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## Glycated bovine serum albumin for use in feeding trials with animal models – In vitro methodology and characterization of a glycated substrate for modifying feed pellets

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1 **Glycated bovine serum albumin for use in feeding trials with animal models – in vitro**  
2 **methodology and characterization of a glycated substrate for modifying feed pellets**

3  
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19  
20 **Abstract**

21 This study investigated different methods to produce N<sup>ε</sup>-carboxymethyl-lysine (CML)-  
22 enriched bovine serum albumin (BSA) as alternatives to the classical approach using  
23 glyoxylic acid (GA) and sodium cyanoborohydride (NaBH<sub>3</sub>CN) which results in toxic  
24 hydrogen cyanide (HCN). The reaction of GA (6 mmol/L) and NaBH<sub>3</sub>CN (21 mmol/L) to  
25 produce CML remained the most effective with CML yields of 24-35%, followed by 13-  
26 24% using 300 mmol/L glyoxal (GO). GA promoted specific modification of lysine to  
27 CML, and fewer structural modifications of the BSA molecule compared with GO, as  
28 evidenced by fluorescence and proteomic analyses. GO promoted greater arginine  
29 modification compared with GA (76 vs 23%). Despite structural changes to BSA with  
30 GO, murine fecal clearance of CML was similar to literature values. Hence, BSA  
31 glycation with 300 mmol/L glyoxal is a suitable alternative to GA and NaBH<sub>3</sub>CN for  
32 generating CML-enriched protein free of HCN, but a CML-only fortification model  
33 remains to be described.

34 **Keywords:** carboxymethyllysine; glycation; BSA; glyoxal

35  
36 **1. Introduction**

37 Advanced Glycation End-Products (AGEs) are a heterogeneous group of compounds  
38 resulting from the non-enzymatic condensation between reducing sugars and amino  
39 groups, primarily lysine and arginine moieties (Perrone et al., 2020). The *in vivo*  
40 glycation of proteins is part of normal metabolism, but pathological implications, as well  
41 as molecular aging, have also been associated with it (Briceno Noriega et al., 2022). In

42 addition to the putative health effects of endogenously formed AGEs, their presence in  
43 food is a matter of concern. In industrial or household cooking processes, the  
44 development of appealing aromas, flavors, and colors goes hand-in-hand with the  
45 formation of dietary AGEs (dAGEs) (Lund & Ray, 2017). It is well-documented that  
46 thermally-processed foods are a key dietary source of these neo-formed compounds.  
47 Endogenous AGEs and dAGEs are thought to exert their pro-inflammatory effect at least  
48 in part through binding with the Receptor for Advanced Glycation End-products (RAGE)  
49 (Nogueira Silva Lima et al., 2021).

50 In rodents, studies of increased exposure to glycated proteins in food have employed  
51 protocols including heating of food (*e.g.* autoclaving, baking) (van Dongen et al., 2021),  
52 supplementing food with free AGEs (Helou et al., 2022), or administering a diet enriched  
53 in protein-bound AGEs (*e.g.* casein, bovine serum albumin) (Grossin et al., 2015). Given  
54 the heterogeneous nature of dAGEs resulting from these different methods, it is difficult  
55 to decipher the effect of a single compound and the precise implications of dAGEs for  
56 health remain elusive. This is partly because the different protocols are intended to  
57 address different research questions. In animal experiments, heating food has the  
58 advantage of representing a process similar to industrial processing or household  
59 cooking and enables the study of a representative cocktail of dAGEs. However, the  
60 disadvantage is that certain micronutrients are degraded, potentially biasing the effects  
61 of dAGEs (Luevano-Contreras & Chapman-Novakofski, 2010). Conversely, the addition  
62 of free AGEs to food has enabled the study of specific glycation products, but this  
63 approach does not represent the main route of exposure to dAGEs as they exist  
64 primarily in the protein-bound form in food. Lastly, administering diets supplemented with  
65 synthetic, well-characterized, protein-bound dAGEs enables the study of a predominant  
66 dAGE and reduces nutritional confounders associated with the heating of foods.  
67 However, the synthesis of protein-bound dAGEs often lacks an extensive assessment of  
68 the precise modifications of glycated proteins.

69 Protein-bound N<sup>ε</sup>-carboxymethyl-lysine (CML) is the most studied dAGE due to its  
70 chemical stability and abundance in food (Scheijen et al., 2016). Dietary CML (dCML)  
71 has been shown to accumulate in organs such as the gut and kidneys (Tessier et al.,

72 2016). Peptides including lysine moieties modified into CML exhibited 7-times greater  
73 affinity for RAGE compared with non-glycated fragments (Xue et al., 2011). Protein-  
74 bound dCML is formed preferentially compared with free CML in thermally processed  
75 foods (van Dongen et al., 2021), making the specific effects of protein-bound dCML on  
76 health a *bona fide* research objective.

77 Many strategies have been used to synthesize protein-bound CML. While no CML-  
78 exclusive synthesis method has been described the most selective approach, to our  
79 knowledge, uses glyoxylic acid and sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ) for the  
80 generation of dCML in proteins including casein, ovalbumin, or albumin (Faist et al.,  
81 2001). Classic reductive amination of glyoxylic acid in the presence of  $\text{NaBH}_3\text{CN}$  has  
82 been reported to convert 16%-44% of the lysine moieties into CML (Andersen et al.,  
83 2012; Ikeda et al., 1996). Despite the high CML yields of this approach, the toxic  
84 hydrogen cyanide (HCN) gas produced is potentially harmful to laboratory personnel and  
85 an inherent contaminant of animal food preparations. Chemically safer alternative  
86 methods have been proposed using sodium borohydride ( $\text{NaBH}_4$ ), sodium  
87 triacetoxyborohydride ( $\text{NaBH}(\text{OAc})_3$ ) reducing agents, and ribose- or glyoxal-driven  
88 reactions (Andersen et al., 2012; Faist et al., 2001; Glomb & Pfahler, 2001). These  
89 methods do not result in the production of HCN, but they may be expensive or require  
90 inflammable or toxic solvents (*e.g.* dichloroethane).

91 Here we present a broad, comparative characterization of classical and alternative  
92 methods of converting lysine into CML, investigating other glycation hotspots such as  
93 arginine residues as well as more complex forms of glycation products as crosslinks.  
94 This work presents, for the first time, not only an alternative to the use of  $\text{NaBH}_3\text{CN}$ , but  
95 also an effective way of producing CML without the nutritional loss associated with heat  
96 treatment. Our goal here was to compare the efficiency of different reducing agents and  
97 carbonyl compounds for dCML fortification of bovine serum albumin (BSA) using an  
98 extensive suite of quantitative and qualitative analytical methods. We employed isotope-  
99 dilution, high-performance liquid chromatography coupled with tandem mass  
100 spectrometric detection (HPLC-MS/MS), western-blot, fluorescence, and proteomic  
101 analyses, using matrix-assisted laser desorption ionization with tandem time-of-flight MS

102 (MALDI-TOF/TOF) and nanoLC with tandem, high-resolution MS (LC-HRMS/MS), to  
103 provide an extensive characterization of the possible modifications of BSA promoted by  
104 different carbonyl compounds. We further studied the efficiency of the incorporation of  
105 protein-bound dCML into mouse feed pellets, their stability over different storage  
106 periods, and finally the clearance of dCML in mouse feces.

## 107 **2. Material and methods**

### 108 **2.1. Chemicals and reagents**

109 Bovine serum albumin (BSA) Fraction V was from Euromedex (Souffelweyersheim,  
110 France) and ribose from TCI Chemicals (Zwijndrecht, Belgium). Ammonium formate  
111 (>99%) and ultra-pure HPLC water were from VWR (Fontenay-sous-Bois, France),  
112 sodium triacetoxyborohydride ( $\text{NaBH}(\text{OAc})_3$ ) from ACROS Organics (Geel, Belgium),  
113 Nonfluoropentanoic acid (NFPA) 97%, formic acid (>95%), acetonitrile (ACN),  
114  $\text{NaBH}_3\text{CN}$ , glyoxylic acid and glyoxal (40% w/v), hydrochloric acid (HCl) 37%, sodium  
115 borohydride ( $\text{NaBH}_4$ ), boric acid ( $\text{H}_3\text{BO}_3$ ), sodium hydroxide (NaOH), lysine (Lys) and  
116 arginine (Arg), as well as all chemicals for proteomics assays were obtained from  
117 Sigma–Aldrich (Saint-Quentin-Fallavier, France). Furosine (Fur), Fur-d<sub>4</sub>, CML, and  
118 CML-d<sub>2</sub> were from Polypeptide Group Laboratories (Strasbourg, France). Labeled  
119 lysine (Lys-<sup>15</sup>N<sub>2</sub>) was from CortecNet (Voisins-le-Bretonneux, France) and Arg-<sup>13</sup>C from  
120 Carl Roth (Karlsruhe, Germany).

121

### 122 **2.2. N<sup>ε</sup>-carboxymethyl-lysine synthesis in different BSA model systems**

123 We compared three approaches for preparing CML-fortified BSA, all representative of  
124 common practice in the literature, and present the molar ratios in Table 1 (Andersen et  
125 al., 2012). The first examined the efficiency of the reducing agents  $\text{NaBH}_4$ ,  $\text{NaBH}(\text{OAc})_3$ ,  
126 and  $\text{NaBH}_3\text{CN}$  in combination with glyoxylic acid (GA-A to GA-F): BSA and glyoxylic  
127 acid were allowed to react for 2h at 37°C to form imine precursors before addition of the  
128 reducing agents. A second approach employed glyoxal at 40% w/v and ribose (R-A to R-  
129 GO-B), while a third employed glyoxal only (GO-A to GO-E). Reactions GA-A to -F and  
130 R-A to R-GO-B, were prepared in a phosphate buffer (0.2 M, pH 8.0) and incubated for  
131 16h at 37°C; reactions GO-A to -E were incubated for 4 days at 37°C under sterile

132 conditions. All samples were then dialyzed for 24h (4°C) using a Spectra/Por 4 MWCO  
 133 14-14 kDa membrane (Spectrum Chemicals) with two water bath changes (1:23 v:v),  
 134 before western-blot and LC-MS/MS analysis. Having thus identified the two best  
 135 approaches for forming protein-bound CML in BSA, three replicates of GA-E (GA-E.1, 2  
 136 and 3) and GO-E (GO-E.1, 2 and 3) were prepared and characterized using western-  
 137 blot, LC-MS/MS, fluorescence spectroscopy and proteomic analyses to assess inter-day  
 138 variability of these reactions (Table 1).

139  
 140 **Table 1.** Model systems for the production of protein-bound CML by modification of BSA  
 141 (Lys - lysine; GA - glyoxylic acid; GO - glyoxal).

Sample	Molar ratio	Final concentration (mmol/L)						
		Lys	GA	GO	Ribose	NaBH <sub>4</sub>	NaBH(OAc) <sub>3</sub>	NaBH <sub>3</sub> CN
Native BSA (Control)	-	175						
GA-A	1 (Lys) : 1.2 (GA) : 4.9 (NaBH <sub>4</sub> )	5	6			22		
GA-B	1 (Lys) : 2.4 (GA) : 3.0 (NaBH(OAc) <sub>3</sub> )	3	7				9	
GA-C	1 (Lys) : 2.4 (GA) : 9.8 (NaBH(OAc) <sub>3</sub> )	3	7				30	
GA-D	1 (Lys) : 0.5 (GA) : 4.9 (NaBH <sub>3</sub> CN)	5	3					21
GA-E	1 (Lys) : 1.2 (GA) : 4.9 (NaBH <sub>3</sub> CN)	5	6					21
GA-F	1 (Lys) : 2.4 (GA) : 9.8 (NaBH <sub>3</sub> CN)	5	12					42
R-A	1 (Lys) : 1.5 (R)	175			300			
R-B	1 (Lys) : 3.0 (R)	175			600			
R-GO-A	1 (Lys) : 0.1 (GO) : 1.5 (R)	175		20	300			
R-GO-B	1 (Lys) : 0.2 (GO) : 3.0 (R)	175		40	600			
GO-A	1 (Lys) : 0.1 (GO)	175		20				
GO-B	1 (Lys) : 0.2 (GO)	175		40				
GO-C	1 (Lys) : 0.6 (GO)	175		100				
GO-D	1 (Lys) : 1.2 (GO)	175		200				
GO-E	1 (Lys) : 1.8 (GO)	175		300				
GA-E.1*								
GA-E.2*	1 (Lys) : 1.2 (GA) : 4.9 (NaBH <sub>3</sub> CN)	5	6					21
GA-E.3*								
GO-E.1**								
GO-E.2**	1 (Lys) : 1.8 (GO)	175		300				
GO-E.3**								

142 Inter-day replicates of (\*) GA-E reactions, and (\*\*) GO-E reactions.

143

### 144 2.3. Western blot analysis of native and glycosylated BSA

145 Protein concentration was determined by the Pierce™ BCA Protein Assay Kit  
146 (ThermoFisher, Courtaboeuf, France). A total of 200 ng of protein was separated in 8%  
147 Bis-Tris (ThermoFisher) gels. Nonspecific binding sites were blocked for 1h at 20°C with  
148 3% milk in tris-buffered saline added to 0.05% Tween 20 (TBS-T); PVDF membranes  
149 (BioRad, France) were incubated overnight at 4°C with rabbit polyclonal anti-CML  
150 primary antibody (Abcam 27684, 1/5000, Cambridge, England), followed by TBS-T  
151 washing and incubation for 2h with anti-rabbit, alkaline phosphatase-conjugated  
152 secondary antibody (Abcam 97048, 1/5000). Membrane revelation was performed with  
153 1-Step™ NBT/BCIP (ThermoFisher).

154

#### 155 **2.4. Fluorescence spectroscopy of native and glycosylated BSA solutions**

156 Fluorescence spectra and intensity were measured in the native BSA and the triplicate  
157 reactions of GA-E (GA-E.1 to 3) and GO-E (GO-E.1 to 3) (Table 1) using a Cary Eclipse  
158 Fluorescence Spectrometer (Agilent, Les Ulis, France) in solutions at concentrations of 1  
159 mg/mL, at pH 8.0.

#### 160 **2.5. Mass spectrometry characterization of native and glycosylated BSA**

##### 161 **2.5.1. LC-MS/MS quantification of lysine, arginine, and CML after acid hydrolysis**

162 Analyses of native and glycosylated BSA (all reactions) were performed in triplicate using  
163 positive electrospray ionization (ESI+) on a Quattro Premier XE triple-quadrupole mass  
164 spectrometer (Waters, Saint-Quentin-en-Yvelines, France) using a Hypercarb column  
165 (100 × 2.1 mm, 5 µm; same-phase guard column 10 × 2.1 mm, 5 µm; column at 10°C;  
166 ThermoFisher). The binary mobile phase was aqueous 12.5 mmol/L NFA (solvent A)  
167 and ACN (solvent B) with the gradient pumped at 200 µL/min over 23 mins as follows:  
168 %B: 0–10 mins, 0–22.5%; 10–15 mins, 60%; 15–23 mins, 0%.

169 Five hundred microliter aliquots of each BSA preparation were lyophilized, and samples  
170 reduced for 2h at room temperature with 500 µL 1M NaBH<sub>4</sub> in sodium borate buffer (pH  
171 9.2). Five hundred microliters of 12M HCl were added, and samples hydrolyzed at  
172 110°C for 21 h. Aliquots of 100 µL hydrolysate were concentrated in a Speed-Vac  
173 (ThermoFisher) and re-solubilized in 1 mL aqueous 12.5 mmol/L NFA. Samples were  
174 filtered (0.45 µm) before preparing x10 dilutions with 12.5 mmol/L aqueous NFA

175 solutions of Lys-<sup>15</sup>N<sub>2</sub> and Arg-<sup>13</sup>C (10 µg/mL) for analysis of Lys and Arg, x200 dilutions  
176 with CML-d<sub>2</sub> (0.2 µg/mL) in NFPA for the analysis of CML.

177 The current study is part of a larger, ongoing collaborative project. An in-house  
178 reference material (lyophilized infant milk formula) was included in each analytical batch  
179 in both LC-MS/MS laboratories (*viz.* the UMR 1167 and ULR 7519 partners (Section  
180 2.6.2.): briefly, one *ca.*50 mg sample of lyophilized milk powder was reduced with NaBH<sub>4</sub>  
181 prior to hydrolysis in 6M HCl (110°C for 21h). This reference material is analyzed  
182 frequently, and results for all compounds were consistently within one standard deviation  
183 of the mean values obtained at both sites (Data not shown).

### 184 **2.5.2. Proteomic analyses**

185 Proteomic analyses were performed on the native BSA and the triplicate reactions of  
186 GA-E (GA-E.1 to 3) and GO-E (GO-E.1 to 3) (Table 1). Matrix-assisted laser  
187 desorption/ionization with tandem time-of-flight spectrometric detection (Sciex 4800+  
188 MALDI-TOF/TOF, Sciex, Framingham, MA, USA) (Pien et al., 2022) was used to  
189 determine the molecular weight of the native and glycosylated BSA (GA-E and GO-E).

190 The native BSA and triplicate GA-E and GO-E glycosylated BSA samples were then  
191 subjected to protein digestion (100 µg) performed with an eFASP method (enhanced  
192 Filter Aided Sample Preparation) (Erde et al., 2014; Helle et al., 2018) using  
193 chymotrypsin at a protein:enzyme ratio of 1:50 (w/w). After digestion, the peptide  
194 concentration was determined with Denovix 11 + (DS-11+, Denovix, Wilmington, USA),  
195 and the concentration was adjusted to 1 µg/µL by dilution with ultrapure water containing  
196 0.1% formic acid (Sigma-Aldrich). NanoLC-MS/MS was performed on an Orbitrap Q  
197 Exactive plus mass spectrometer via a U3000 RSLC Microfluidic HPLC System  
198 (ThermoFisher Scientific, Massachusetts, USA) (Helle et al 2018). One microliter of the  
199 peptide mixture at a concentration of 1 µg/µL was injected with solvent A (5%  
200 acetonitrile and 0.1% formic acid v/v) for 3 min at a flow rate of 10 µl/min on an Acclaim  
201 PepMap100 C18 pre-column (5 µm, 300 µm i.d. × 5 mm). The peptides were separated  
202 on a C18 Acclaim PepMap100 C18 reversed phase column (3 µm, 75 mm i.d. × 500  
203 mm), using a linear gradient (5-40%) of solution B (75% acetonitrile and 0.1% formic  
204 acid) at a rate of 250 nL.min<sup>-1</sup>. The column was washed with 100% solution B for 5



205 minutes and then re-equilibrated with solvent A. The column and pre-column were  
206 maintained at 40°C. The analysis duration was 185mins. The LC runs were acquired in  
207 positive ion mode with MS scans from m/z 350-1,500 in the Orbitrap at a resolution of  
208 70,000 at m/z 400. The automatic gain was set at  $1e10^6$ . Sequential MS/MS scans were  
209 acquired at 35,000 resolution at m/z 400 in the high-energy collision/dissociation cell for  
210 the 15 most-intense ions detected by full-MS survey scans. Automatic gain control was  
211 set at  $5e10^5$ , and the collision energy was 28 eV. Dynamic exclusion was set at 90s and  
212 ions with 1 and more than 8 charges were excluded.

213 Proteomics data were processed with PEAKS X+ (Bioinformatics software, Waterloo,  
214 Canada) against a homemade database containing BSA sequences (Swissprot  
215 accession number, P02769). Precursor ion mass tolerance was 10 ppm and fragment  
216 ion mass tolerance 0.05 Da. Three missed cleavages were allowed. Cysteine  
217 carbamidomethylation (C) (+57.02 Da) was set as a fixed modification. Deamidation  
218 (NQ) (+0.98 Da), Oxidation or Hydroxylation (MRYPNKD) (+15.99 Da), Carboxymethyl  
219 (KR) (+58.005 Da), Glyoxal-derived hydroimidazolones (R) (+39.994 Da), N<sup>ε</sup>-acetyl-  
220 lysine (K) (+42.010 Da), N<sup>ε</sup>-formyl-lysine (K) (+27.994 Da) were selected as variable  
221 modifications. Six variable PTMs (post-translational modifications) were allowed per  
222 peptide. PEAKS PTM and SPIDER algorithms were run with similar parameters to  
223 search for 313 PTMs, and peptide mutations and homology. The false detection rate  
224 (FDR) was set to 1%, and minimal ion intensity for modification was set to 2%.

225 Raw files from eFASP were analyzed using Mass Spec Studio v 2.1.2.3107 (Sarpe et  
226 al., 2016). Thus, GOLA (2S)-amino-6-(2-((5S)-amino-5-  
227 carboxypentylamino)acetamido)hexanoic acid hydrochloride salt (+39.994 Da), GODIC  
228 (2S)-2-amino-6-[[2-[[[(4S)-4-amino-4-carboxybutyl]amino]-1,4-dihydroimidazol-5-  
229 ylidene]amino]hexanoic acid (+21.984 Da) and GOLD (6-{1-[(5S)-5-ammonio-6-oxido-6-  
230 oxohexyl]imidazolium-3-yl}norleucinate) lysine – lysine crosslink (+34.992 Da) were  
231 selected as variable modifications, and carbamidomethyl cysteine and methionine  
232 oxidation were set as a dynamic modifications. Trypsin was selected as an enzyme with  
233 3 missed cleavages. The error of MS and MS/MS precursors was set to 10 ppm, while  
234 the minimum charge of peptides was 3 and the maximum 8. Peptide cross-links with a

235 score higher than 18 were considered for further analysis. The percentages presented  
236 correspond to the proportion, relative to the control BSA, of a given modification on a  
237 specific peptide modified by the GA or GO reactions. These percentages were  
238 calculated for each of the triplicate proteomics analyses, enabling an average value to  
239 be calculated for a given modification of a given peptide (Pien et al., 2022). The MS  
240 proteomics data have been deposited on the ProteomeXchange Consortium  
241 (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository  
242 (Vizcaíno, et al., 2012) with the data set identifier PXD041212.

## 243 **2.6. Stability of rodent feed pellets enriched with native or glycated BSA**

244 Rodent feed pellets (A04) were prepared by SAFE Custom Diets (Augy, France) and  
245 enriched with native or glycated BSA (GO) by extrusion. A total of 7.5 g/kg<sub>pellets</sub> of  
246 glycated BSA was incorporated into pellets to give a dCML diet with 200 µg<sub>CML</sub>/g<sub>pellets</sub>.  
247 Similarly, 7.5 g/kg<sub>pellets</sub> of non-glycated, native BSA was incorporated into pellets as a  
248 control diet (STD). A 50 kg batch of the STD diet and a 40 kg batch of the dCML diet  
249 were prepared. Each preparation was divided into vacuum-sealed plastic bags  
250 containing 10 kg each, submitted to gamma radiation (25 kGy), then stored at 20°C for  
251 up to 80 days. Individual bags were opened for use approximately every 20 days and  
252 the assays described below were performed.

253

### 254 **2.6.1. Quantitation of total protein content**

255 Total protein in feed pellets was determined in triplicate using the Dumas method in a  
256 LECO FP528 nitrogen analyzer (Garges les Gonesse, France). One hundred milligrams  
257 of powdered pellets were analyzed according to AOAC method 990.03 (AOAC, 1995).  
258 The classical nitrogen-to-protein conversion factor of 6.25 was used to calculate protein  
259 content.

### 260 **2.6.2. LC-MS/MS quantification of lysine, CML, and furosine after acid hydrolysis**

261 Duplicate analyses of feed pellets were performed in ESI+ mode on a TSQ Quantum  
262 Ultra MS/MS (ThermoFisher) using a Raptor PolarX column (100 x 2.1 mm, 2.7 µm;  
263 same-phase guard column 5 x 2.1 mm, 2.7 µm; column at 40°C; Restek, Lisses,  
264 France). The binary mobile phase, pumped at 500 µL/min over 13 mins, consisted of

265 10% 200 mmol/L ammonium formate in ACN (solvent A) and aqueous 0.5% formic acid  
266 (solvent B), with the following gradient: %B: 0–3.5 min, 12%; 3.5–8 min, 12–70%; 8–13  
267 min, 12%.

268 Thirty-milligram sub-samples of crushed, homogenized pellets were accurately weighed,  
269 and samples were either reduced with NaBH<sub>4</sub> prior to hydrolysis (quantification of Lys  
270 and CML) or hydrolyzed in 6M HCl without prior reduction (110°C for 21h - quantification  
271 of Fur). The analysis procedure was similar to that described in section 2.5.1., except  
272 re-solubilization and dilutions were in aqueous 0.01 M HCl (x20 for Lys, x2 for CML, x10  
273 for Fur).

## 274 **2.7. Total CML quantification in mouse feces**

275 Mouse feces from animals fed with either the STD or dCML diets were collected from  
276 two independent cages housing three animals each. A total of 3 g of feces were  
277 collected (weekly collections from the same cages over 3 weeks), and the results  
278 presented here represent the averaged CML concentrations in feces over this period.  
279 The LC-MS/MS analyses of mouse feces were performed in triplicate (500 mg  
280 subsamples) (section 2.5.1). The animals from which feces were collected were not  
281 sacrificed at this stage since they were part of another study (Project approval number:  
282 23208-2019120215543800 v4; they were later euthanized by intraperitoneal injection of  
283 ketamine (150 mg/kg) and xylazine (10 mg/kg) followed by cervical dislocation for  
284 collection of biological fluids and organs).

## 285 **2.8. Statistical analyses**

286 Data are represented as means ± standard deviation. One way ANOVA test followed by  
287 Tukey's posthoc test was performed for multiple comparisons (BSA-GA vs. Native BSA  
288 (control) vs. BSA-GO) of the significance of differences between the control group and  
289 the other samples. Analyses were performed using GraphPad Prism 9.0 (San Diego,  
290 CA, USA) at  $\alpha = 0.05$ .

291

## 292 **3. Results and discussion**

### 293 **3.1. Quantification of CML, lysine, and arginine in BSA model systems using** 294 **glyoxylic acid or ribose with or without glyoxal**

295 We explored different approaches to establish an efficient method for CML  
 296 fortification of BSA which avoided heating or the use of NaBH<sub>3</sub>CN. In a first set of  
 297 experiments, five reactions (GA-A to -F) with equimolar quantities of lysine were  
 298 incubated with different molar ratios of GA and NaBH<sub>4</sub>, NaBH(OAc)<sub>3</sub>, or NaBH<sub>3</sub>CN  
 299 (Table 1). The formation of CML ranged between 13.0 ±1.3 and 235.0 ±23.5 μmol/g  
 300 BSA, corresponding to a minimum of 1% of lysine transformed into CML using  
 301 NaBH(OAc)<sub>3</sub> (GA-B and -C), and a maximum of 24% of CML using NaBH<sub>3</sub>CN (GA-E)  
 302 (Table 2). No linear increase in CML yield was observed with increasing molar ratios of  
 303 glyoxylic acid and NaBH<sub>3</sub>CN to lysine (GA-F), but increasing GA and NaBH<sub>3</sub>CN resulted  
 304 in a higher loss of lysine, indicating that modifications other than CML took place under  
 305 these more reactive conditions. The CML yields (%) are presented in Table 2 with  
 306 corresponding percentage losses of lysine.

307  
 308 **Table 2.** CML yield (% lysine converted) and lysine loss (%) in native BSA and in BSA  
 309 glycated with glyoxylic acid (GA) using reductive amination with NaBH<sub>4</sub>, NaBH(OAc)<sub>3</sub>, or  
 310 NaBH<sub>3</sub>CN, or from BSA glycated with ribose (R) +/- glyoxal (GO; see also Table 1).

Sample	CML Yield		Lysine loss
	μmol/g BSA	% of lysine converted into CML	% of initial lysine
Native BSA (Control)	0.5 ± 0.05	0.05 ± 0.005	0
GA-A	29.0 ± 2.9	3.0 ± 0.3	3.0 ± 0.3
GA-B	13.0 ± 1.3	1.0 ± 0.1	1.0 ± 0.1
GA-C	13.0 ± 1.3	1.0 ± 0.1	1.0 ± 0.1
GA-D	104.0 ± 10.4	11.0 ± 1.1	12.0 ± 1.2
GA-E	235.0 ± 23.5	24.0 ± 2.4	24.0 ± 2.4
GA-F	182.0 ± 18.2	20.0 ± 2.0	29.0 ± 2.9
R-A	19.0 ± 2.3	2.1 ± 0.2	37.5 ± 2.0
R-B	15.5 ± 1.0	1.7 ± 0.1	58.5 ± 5.0
R-GO-A	20.2 ± 2.5	2.2 ± 0.2	41.2 ± 2.7
R-GO-B	32.0 ± 3.2	3.5 ± 0.1	51.7 ± 5.0

311  
 312 Different boron hydrides have been extensively used as reducing agents in the synthesis  
 313 of protein-bound CML. Overall, we observed that NaBH<sub>4</sub> (GA-A) and NaBH(OAc)<sub>3</sub> (GA-B  
 314 and -C) were relatively ineffective compared with NaBH<sub>3</sub>CN (GA-D to -F). Others have

315 reported that  $\text{NaBH}_4$  is a less selective reagent, requiring a pre-incubation phase for  
316 imine formation (Ranu et al., 1998), while  $\text{NaBH}(\text{OAc})_3$  has a limited reduction activity  
317 due to the stereo effect of the three acetoxy groups (Abdel-Magid et al., 1996). As  
318 anticipated,  $\text{NaBH}_3\text{CN}$  proved to be the most effective reducing agent due to the  
319 preferential reduction of iminium ions formed under oxidative conditions at physiological  
320 pH (Borch et al., 1971). The CML yields presented here were similar to other studies  
321 using comparable synthetic routes. The study of Ikeda et al. (1996), working on CML-  
322 BSA fortification via reductive amination of a BSA-GA system, reported 16% of lysine  
323 was converted to CML. In another protein model (casein), lysine modification to CML  
324 was shown to be 38-55% using  $\text{NaBH}_3\text{CN}$  (Andersen et al., 2012; Faist et al., 2001).  
325 To examine whether other carbonyl compounds could improve CML yield, we tested the  
326 synergy of ribose with glyoxal. Ribose has an unstable cyclic form, rendering it a more  
327 reactive sugar compared with hexoses (Wei et al., 2012), which could favor the glycation  
328 of proteins (Glomb & Monnier, 1995). Here, reactions including ribose (R-A and R-B, R-  
329 GO-A and R-GO-B; Table 1) exhibited no real increase in CML production over the BSA-  
330 GA system, reaching a maximum of 3.5% lysine transformation to CML. However, high  
331 percentage losses of lysine were observed (38%-59%), suggesting the transformation of  
332 lysine residues into glycation products other than CML (Table 2). In line with the CML  
333 yields observed after 16h of reaction between BSA and ribose, the glycation of BSA by  
334 ribose alone was shown to produce 0.1 and 10.3% of CML after 5h and 170h incubation  
335 at 37°C, respectively (Glomb & Monnier, 1995).

### 336 337 **3.2. Quantification of CML, lysine, and arginine in a new BSA model system** 338 **using only glyoxal**

339 We then examined a glycation system using glyoxal as the only carbonyl compound  
340 (GO-A to -E, Table 1). Under these conditions, CML was formed proportionally with the  
341 glyoxal concentration: 0.5, 1, 4, 9 & 13% of lysine modification with 20, 40, 100, 200,  
342 and 300 mmol/L of glyoxal, respectively (Figures 1a and 1b). Exceeding 300 mmol/L of  
343 glyoxal did not generate more CML (data not shown). Nevertheless, the increase in  
344 glyoxal concentrations promoted a concomitant loss of lysine (3, 6, 10, 22, and 31%),  
345 accompanied by the emergence of high-molecular-weight BSA complexes visible on

346 western blot membranes (Figure 1b). The glycation process likely changed the intrinsic  
347 characteristics of BSA. This phenomenon has been demonstrated recently in a BSA-  
348 glucose model where glycation-induced conformational changes were reported, such as  
349 the loss of the secondary  $\alpha$ -helix structures of BSA (Peng et al., 2023). In addition,  
350 glyoxal is a well-known protein crosslinking agent, resulting in the formation of GOLD,  
351 GOLLA, and GODIC (Glomb & Pfahler, 2001; Lopez-Clavijo et al., 2014), which may  
352 explain the occurrence of a higher proportion of high molecular weight (>130 kDa)  
353 protein with increasing glyoxal concentrations (Figure 1b). We discuss this in more detail  
354 later in this text.

355 Although serum albumins (bovine or human) have a higher proportion of lysine than  
356 arginine, glyoxal would preferentially promote limited protein modifications of arginine  
357 rather than lysine residues (Henning & Glomb, 2016). While other amino acids such as  
358 tryptophan, histidine and cysteine are also susceptible to modification with glyoxylic acid  
359 and glyoxal (Herraiz et al., 2022), and modifications of cysteine were observed in our  
360 proteomics analyses (data not shown), the focus of our study was the more significant  
361 modifications of lysine and arginine residues of our BSA protein. In a recent investigation  
362 by Henning et al. (2022), less than 4% of the lysine in a BSA-GO (18 mmol/L) model  
363 system was converted to CML, while almost 10% of the total arginine residues were  
364 converted into N<sup>ω</sup>-carboxymethyl-arginine (CMA) (Henning et al., 2022). From our  
365 experience, a gradual loss of Coomassie blue staining was noticed with the increase in  
366 glyoxal concentration (Figures 1b and 1d). This protein staining occurs through the  
367 interaction of the dye with basic (lysine, arginine, and histidine) and hydrophobic  
368 moieties (phenylalanine and tryptophan), and arginine has been indicated as a primary  
369 interaction site with Coomassie anionic species (Georgiou et al., 2008). Hence, the high  
370 arginine loss in GO-E samples (>70%, Figure 1e) may have resulted in reduced  
371 Coomassie blue staining (Figures 1b and 1d). In addition, it is important to highlight that  
372 the overall electrostatic affinity is reduced when glycation takes place. The conversion of  
373 lysine, a basic amino acid, into CML which includes an acidic carboxylate, may also  
374 have reduced the extent of Coomassie blue staining in both GA-E and GO-E samples.

375

### 376 **3.3. Fluorescent characterization of native and glycated BSA**

377 While CML is not fluorescent, other AGEs such as argpyrimidine, HA-triosidine,  
378 pentosidine, and vesperlysine are (Perrone et al., 2020). The excitation and emission  
379 wavelength ranges for fluorescent AGEs are around 320-380 nm and 380-440 nm,  
380 respectively. Measuring fluorescence at their classical wavelengths is an easy and  
381 inexpensive way to estimate the formation of AGEs other than CML during the protein-  
382 bound CML fortification, and additionally provides an indication of the specificity of the  
383 chemical reaction.

384 We determined the fluorescence spectra in GA-E and GO-E samples, then compared  
385 their fluorescence using 340 nm and 425 nm as excitation and emission wavelengths,  
386 respectively (Figure 2). Greatest fluorescence intensity was observed in the GO-E  
387 samples, indicating extensive formation of fluorescent glycation adducts or crosslinks  
388 under these conditions. A 15-fold difference in fluorescence intensity was observed  
389 between the GA-modified BSA and the GO-modified BSA ( $p < 0.0001$ ; Figure 2b). None  
390 of the fluorescent AGEs listed above are known to be formed from glyoxylic acid or  
391 glyoxal alone, however, and their formation pathways have been shown to involve  
392 reducing sugars with at least 3-carbons (Tessier et al., 2003). The fluorescent adducts  
393 or crosslinks formed in our BSA-GA or BSA-GO systems therefore remain to be  
394 elucidated.

395

### 396 **3.4. Proteomic characterization of native and glycated BSA**

397 In an attempt to identify the modifications promoted by the glycation of BSA, we  
398 further compared the triplicate GA-E (GA-E.1 to 3) and GO-E reactions (GO-E.1 to 3 –  
399 Table 1) using proteomic approaches. To overcome the decrease in digestion yield by  
400 trypsin following the creation of CML and CMA, which remove the basic sites recognized  
401 by this enzyme (Lapolla et al., 2004), we performed a chymotrypsin digestion which is  
402 not hampered by this change as it cleaves the protein after large, hydrophobic amino  
403 acids. The CML incidence (%) was calculated relative to non-glycated, native BSA. The  
404 average CML yield from the BSA-GA system was  $29 \pm 6\%$ , while the CML yield from the  
405 BSA-GO incubations was similar at  $21 \pm 3\%$  (Figure 1c). Both the CML yields and shifts  
406 in electrophoretic migration of the protein were relatively reproducible among the  
407 triplicates (Figures 1c and 1d).

408           The native BSA protein contains 59 lysine residues, of which 52 exhibited  
409 modifications in both native and glycated BSA (Supplemental material). Differences in  
410 the incidence of lysine modifications between native BSA and the GA- or GO-glycated  
411 BSA were statistically significant for only 34 of these residues ( $p < 0.05$ , Figure 3a).  
412 Among these, GA promoted a higher incidence of CML in residues commonly glycated  
413 by both reagents. This was observed for 17 lysine residues where the intensity of  
414 modifications promoted by GA was significantly higher compared with GO ( $p < 0.05$ ). Six  
415 further residues were exclusively modified in the GA-E samples (K155, K183, K204,  
416 K211, K235, K495). These observations suggest a greater chemical efficacy of the  
417 glycation of lysine residues to CML by GA compared with GO, while the glycation of  
418 lysine residues only accessible to GA might explain the slightly higher CML yield we  
419 observed in our BSA-GA system. Some of the modified residues in Figure 3a accord  
420 with previous studies on the glycation of BSA by fructose. Hinton & Ames (2006)  
421 identified nine lysine residues particularly susceptible to glycation with fructose (K28,  
422 K36, K151, K180, K399, K437, K495, K523, K548), eight of which feature among the  
423 residues in Figure 3a, suggesting that glycation of lysine to CML occurs preferentially at  
424 these specific sites on the BSA protein.

425           A significant incidence of arginine modifications was only observed with glyoxal. A  
426 small proportion of arginine residues R371 and R507 were converted into CMA (Figure  
427 3b), but the main glycation adduct of this amino acid was G-H1, which exceeded 80% of  
428 the R232 and R507 residues in the GO-E samples (Figure 3c). The incidence of  
429 glycation modifications of residues R232, R371, and R507 did not completely explain the  
430 average 76% arginine loss we observed in our BSA-GO system, involving a total of 23  
431 arginine residues (Figure 1e). This implies that low-incidence modifications may affect  
432 these residues, but that their detection was limited by the sensitivity of our proteomics  
433 method.

434           MALDI-TOF analysis comparing native BSA, GA- and GO-modified BSA revealed  
435 a molecular weight change from 66,762 Da (native BSA) to 68,043 Da (GA-E samples)  
436 and 68,830 Da (GO-E samples), a difference of 1281 Da and 2068 Da, respectively. The  
437 percentage incidence of CML on the lysine residues in Figure 3a enabled an estimate of



438 CML-related increases in the molecular weight of glycated BSA, and showed increases  
439 of 1033 Da (GA) and 608 Da (GO). Hence, the CML-related increment in mass (Da)  
440 accounted for 80% of the total mass gain in the GA-E samples, but only 30% of the  
441 mass gain in the GO-E samples. As expected, then, the increases in molecular weight  
442 observed by MALDI-TOF were not explained by the formation of CML alone, but were  
443 likely also due to the formation of other glycation adducts and crosslinks. These results  
444 are also in line with evidence of a greater diversity of glycation products in GO-E  
445 samples seen in the western blot (Figure 1b and 1d) and fluorescence assays (Figure  
446 2).

447 We detected signals for high molecular weight molecules by both western blot  
448 (Figure 1b and 1d) and MALDI-TOF (data not shown) which we hypothesize result from  
449 the formation of BSA dimers and trimers associated with glycation crosslinking. The GO-  
450 E samples exhibited a significant incidence of the lysine-lysine crosslink GOLD  
451 (LSHK<sup>130</sup>DDSPDLPK<sup>138</sup>LK<sup>140</sup>PDPNTLCDEF) between two identical peptides from two  
452 different BSA molecules which was not detected in either the native BSA nor the GA-E  
453 samples (Figure 3d). This may explain the higher proportion of high molecular weight  
454 (>130 kDa) bands clearly observed in western-blot membranes of these samples  
455 (Figures 1b and 1d). Both GA- and GO-BSA exhibited signals for the lysine-lysine,  
456 glyoxal-derived cross-link GOLA (QEAK<sup>346</sup>DAFLGSF and AEDK<sup>336</sup>DVCK<sup>340</sup>NY,  
457 SALTPDETYVPK<sup>523</sup>AF and DEK<sup>528</sup>LF), but this was significant only in the GA-E  
458 samples. The low incidence of these crosslinks accords with the literature, where the  
459 formation of GOLD, GOLA (two lysine-lysine crosslinks), or GODIC (a lysine-arginine  
460 crosslink) are described as being of minor quantitative importance (Henning et al.,  
461 2022). The low signal intensity from the depleted fractions in such crosslinks observed  
462 here could be improved by a pre-concentration of BSA dimers and trimers prior to  
463 proteomic analysis.

464

### 465 **3.5. Stability of rodent feed pellets enriched with native or GO-glycated BSA**

466 The work described so far was intended to provide a more complete  
467 understanding of precisely which glycation products would be added to rodent feed

468 pellets enriched with glycated BSA. The experimental design involved long-term  
469 exposure to high dCML, and so CML and furosine (an acid-hydrolysis derivative of the  
470 Amadori product fructose-lysine) were rigorously quantified by LC-MS/MS; we were also  
471 curious as to the stability of our BSA-enriched pellets over time. Both STD and dCML  
472 diets were respectively supplemented with equal amounts of non-glycated, native BSA  
473 and glyoxal-glycated BSA (equivalent to GO-E samples). The inclusion of native or GO-  
474 modified protein was performed in batches by SAFE Diets and both were stored at 20°C  
475 in 10 kg bags. The concentrations of lysine, total protein, furosine and CML of the initial  
476 batches are presented in Figure 4, which also illustrates their stability over time.

477 The STD and dCML diets did not differ in initial concentrations of lysine, total  
478 protein or furosine levels ( $p>0.05$ ) (Figure 4a - c). The dCML diet contained  $270.5 \pm 40.6$   
479  $\mu\text{g CML/g D}_{\text{ry}}\text{W}_{\text{eight}}$  of food, approximately 10-fold more CML than the STD diet ( $26.3$   
480  $\pm 0.9 \mu\text{g CML/g D}_{\text{ry}}\text{W}_{\text{eight}}$  of food) (Figure 4d). The different diets were fed to mice during  
481 80 days of experiments and the stability of lysine, total protein, furosine, and CML was  
482 assessed by analyzing a sample at the opening of each 10 kg bag. While small  
483 fluctuations in these concentrations were expected due to the relatively small sample  
484 size taken from each bag, the overall stability of the lysine and total protein contents was  
485 good (Figures 4a and 4b).

486 While there was no difference in initial furosine levels between the two diets,  
487 concentrations rose significantly in both diets over the 80 days of storage ( $p<0.05$ ; this  
488 effect was also verified in other batches, data not shown). Furosine is a product formed  
489 during acid hydrolysis of Amadori products, and is a widely used surrogate measure of  
490 fructose-lysine (Erbersdobler & Somoza, 2007). We observed an increase in furosine  
491 with storage time which we hypothesize results from the presence of reactive sugars in  
492 the raw material used for rodent food preparation. This increase was not affected by the  
493 type of BSA used to fortify the feed pellets (either native or glycated), and the formation  
494 of fructose-lysine during the storage of common foods is widely described. For example,  
495 an increase in furosine of up to 37% has been reported in infant formulas stored at 25°C  
496 (Bosch et al., 2008). To our knowledge, our results present for the first time the evolution  
497 of an early glycation product in animal feed pellets stored at 20°C.

498 The stability of CML in rodent food over 80 days at 20°C is shown in Figure 4d.  
499 The STD diet, supplemented with native BSA, showed small but nonetheless significant  
500 reduction in dCML contents over time ( $p<0.05$ ). The dCML diet, supplemented with GO-  
501 glycated BSA, exhibited a decrease in CML over time from 40 days in stock ( $p<0.05$ ).  
502 Very few studies have assessed the stability of CML in foods during cooking or storage.  
503 This may be linked to the widely held belief that AGEs are, by definition, chemically  
504 stable molecules with low reactivity that simply accumulate over time. However, we have  
505 already observed a degradation of CML during prolonged cooking of meats  
506 (unpublished data), and Cheng et al. (2014) have also shown a decrease in CML over  
507 time in cookies heated from 180 to 230°C. But the decrease in dCML content we  
508 observed was after long-term storage of the feed pellets at room temperature, and the  
509 time/temperature combination is thus totally different from the observation by Cheng et  
510 al. (2014). And since we observe an increase in fructose-lysine during storage, it is  
511 possible that at least part of the CML is lost to the formation of N- $\epsilon$ -carboxymethyl-N- $\epsilon$ -  
512 (1-deoxyfructosyl)-l-lysine (CMFL), which has been recently described in model systems  
513 (Hellwig et al., 2022). This newly reported glycation product confirms the reactivity of the  
514 secondary amino group of CML, and we have already observed the formation of N- $\epsilon$ -di-  
515 carboxymethyl-l-lysine in model systems (unpublished data), but CMFL is the first  
516 heterogeneous, multiple glycation to be described. Future analyses of rodent pellets will  
517 include CMFL, but with regard to the current study we are not aware of any reports of  
518 chemical instability of CML during storage and, while the decrease is small, its apparent  
519 instability in fortified rodent chow requires further consideration.

520

### 521 **3.6. dCML excretion in mouse feces**

522 Mice fed with the STD diet had an average dCML intake approximately 10-fold  
523 less than animals on the dCML diet. The daily exposure to dCML was estimated to be  
524 2.1 mg CML/kg body weight in the STD group and 23 mg CML/kg in the dCML group.  
525 We confirmed that CML fecal excretion was proportional to this disparity in CML  
526 concentrations between the 2 diets. The average CML concentration in fresh feces of  
527 the STD and dCML groups was  $35.00 \pm 2.64 \mu\text{g CML/g}_{\text{feces}}$  in the STD group, and  $299.67$   
528  $\pm 14.74 \mu\text{g CML/g}_{\text{feces}}$ , respectively ( $p<0.05$ ). When CML excretion was expressed as a

529 percentage of ingested CML, we found a small but significant difference between groups  
530 corresponding to 26% (STD) and 33% (dCML), or roughly 1/3 of the ingested CML  
531 ( $p=0.02$ ). Overall, CML fecal excretion was found to be proportional to the daily dietary  
532 intake. Others have reported similar results in mice, rats, and humans. A study which  
533 quantified the fecal elimination of CML by adult rats reported an excretion of about 37%  
534 among animals that consumed extruded proteins (Alamir et al., 2013). Our results are  
535 also in line with a previous study in humans exposed to a low- or high-CML diet (22.5-  
536 31.7% CML excretion) (Delgado-Andrade et al., 2012). The excretion of CML is reported  
537 to be associated with clearance into urine as well as feces, partial retention in organs  
538 and tissues, as well as partial metabolism, essentially by colonic microbiota (Hellwig et  
539 al., 2019; Tessier et al., 2016). However, a complete picture of all the mechanisms that  
540 govern dCML accumulation, metabolism, and excretion remains to be elucidated.

#### 541 **4. Conclusions**

542 In summary, we have shown that an efficient and reproducible synthesis of protein-  
543 bound CML can be achieved with the use of glyoxal in a BSA model system. The  
544 reaction of glyoxylic acid and  $\text{NaBH}_3\text{CN}$  to produce CML remains the most effective  
545 method with respect to the CML yields, resulting in fewer structural modifications of the  
546 BSA molecule compared with glyoxal. We recognize that the glyoxal concentrations  
547 used here may seem extreme, but it was necessary to minimize the volume of reactions  
548 to be dialyzed for the large-scale production of protein-bound CML. Although no food  
549 models perfectly mimic the glycation of BSA, and none of the reactions herein can be  
550 described as representative of glycation as it may occur during industrial or domestic  
551 food preparation, we aimed to add glycated BSA to animal feed pellets in a way that  
552 minimized alterations to the food matrix. The fecal excretion of CML in animals on the  
553 dCML diet, produced from extruded pellets fortified with glyoxal-modified BSA, was very  
554 similar to literature values for CML excretion in both animals and humans. This suggests  
555 that, despite the highly artificial conditions associated with our manufacture of a  
556 synthetic dCML diet, CML absorption/excretion dynamics in our murine model remained  
557 similar to those reported elsewhere.

558 Although using glyoxylic acid with NaBH<sub>3</sub>CN induced more specific glycation of the  
559 BSA's lysine residues, the results of our comparative study underline that even this  
560 method does not generate CML alone. Acid hydrolysis followed by isotope-dilution LC-  
561 MS/MS allowed us to precisely determine the concentrations of CML, lysine and  
562 arginine. A limitation of this approach is of course that it was not possible to quantify  
563 other AGEs more or less sensitive to acid hydrolysis such as CMA and G-H1, but our  
564 proteomic analyses, using a milder enzymatic hydrolysis, enabled us to reveal the  
565 presence of these AGEs. Lysine-lysine dimers GOLD and GOLA occurred at only a low  
566 frequency, indicating a low probability of formation of these AGEs even under the very  
567 intensive conditions of *in vitro* glycation, but which was also close to the limits of  
568 sensitivity for our analytical technique. It may be that further characterization of these  
569 samples, notably the number and type of crosslinks, will raise further research  
570 questions, but this study nevertheless adds significant knowledge to facilitate the  
571 production of glycated proteins for the study of dietary AGEs.

572 **Declaration of interests:** Authors declare no conflict of interest.

573 **Ethical approval:** All experiments carried out in animals were in accordance with the  
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578

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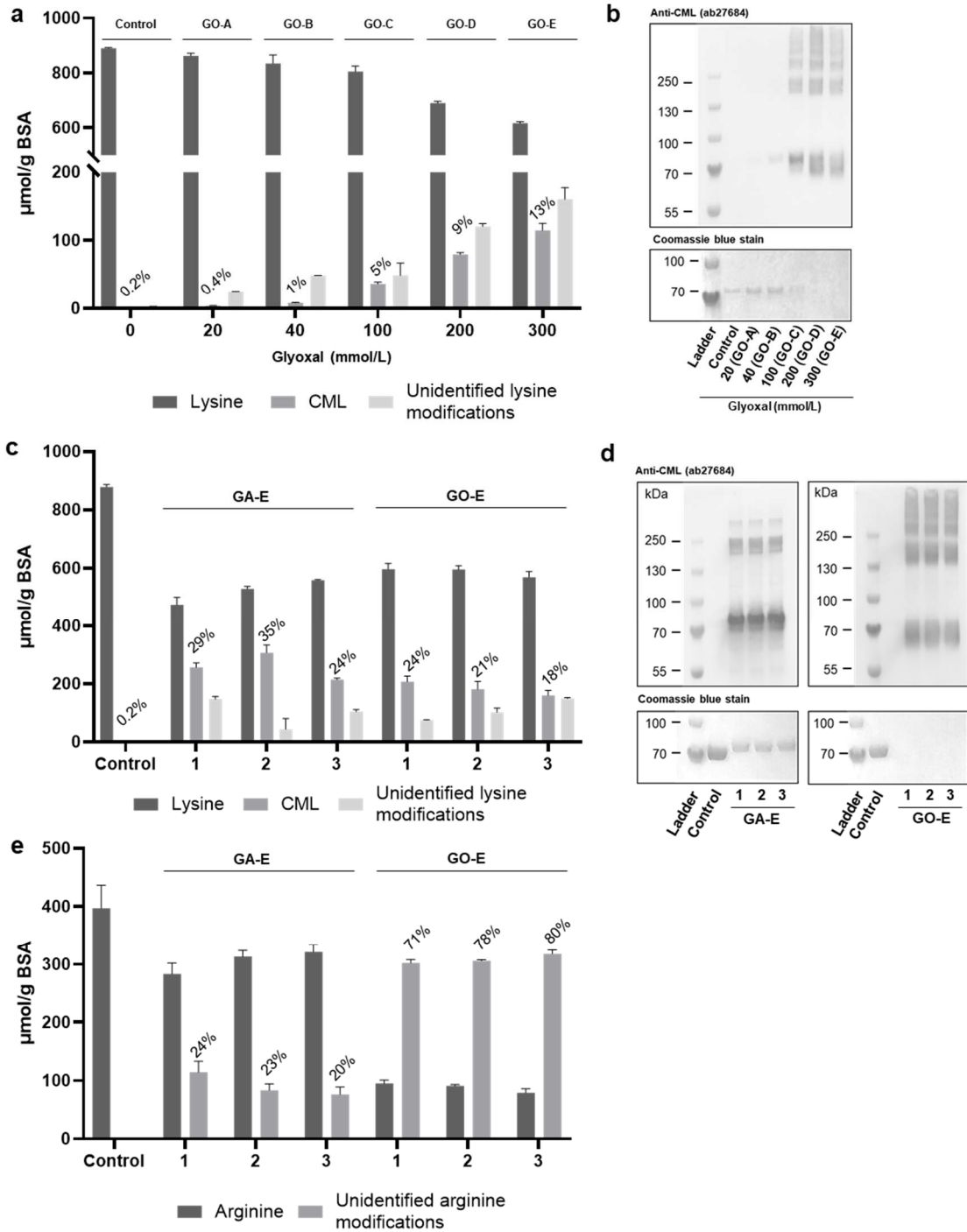
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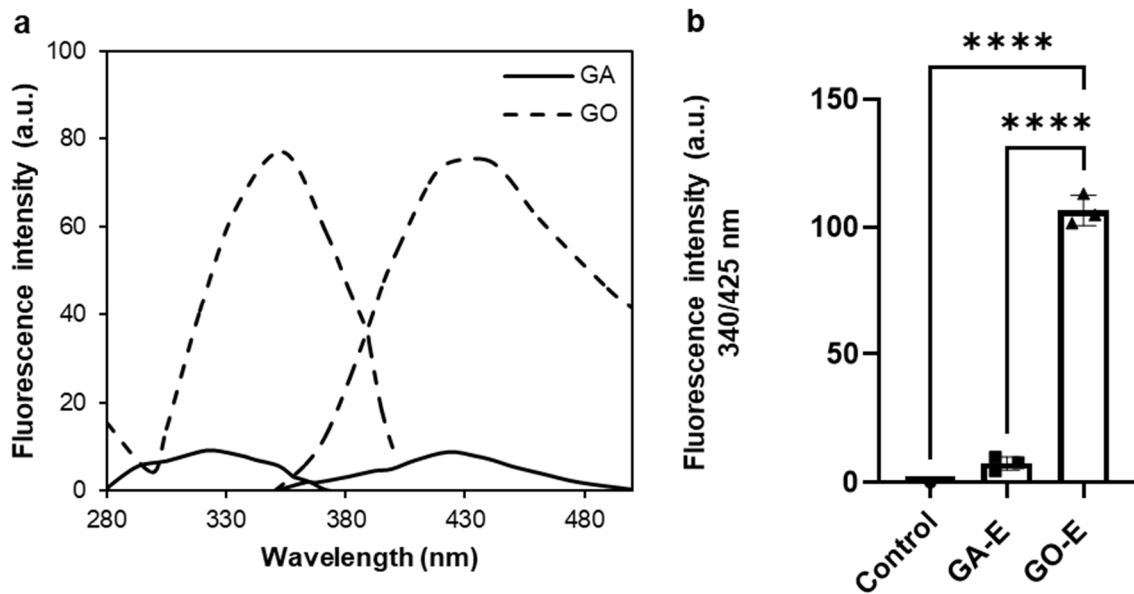


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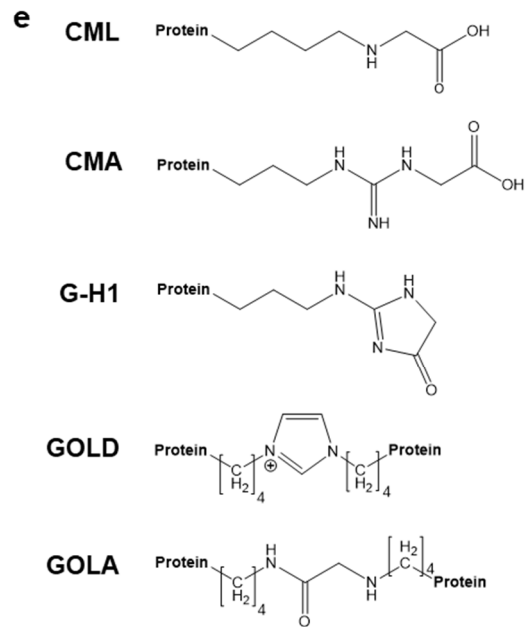
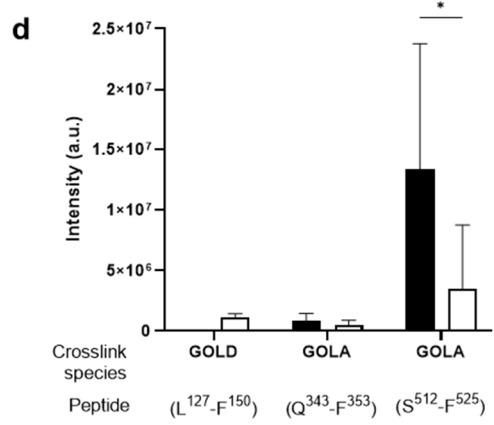
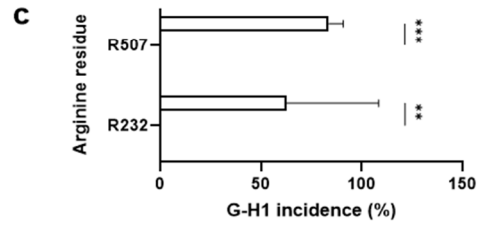
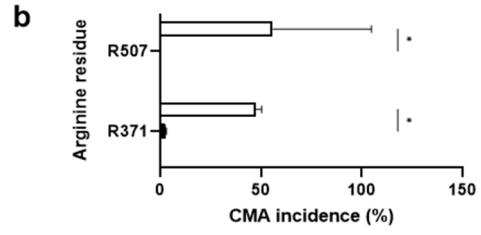
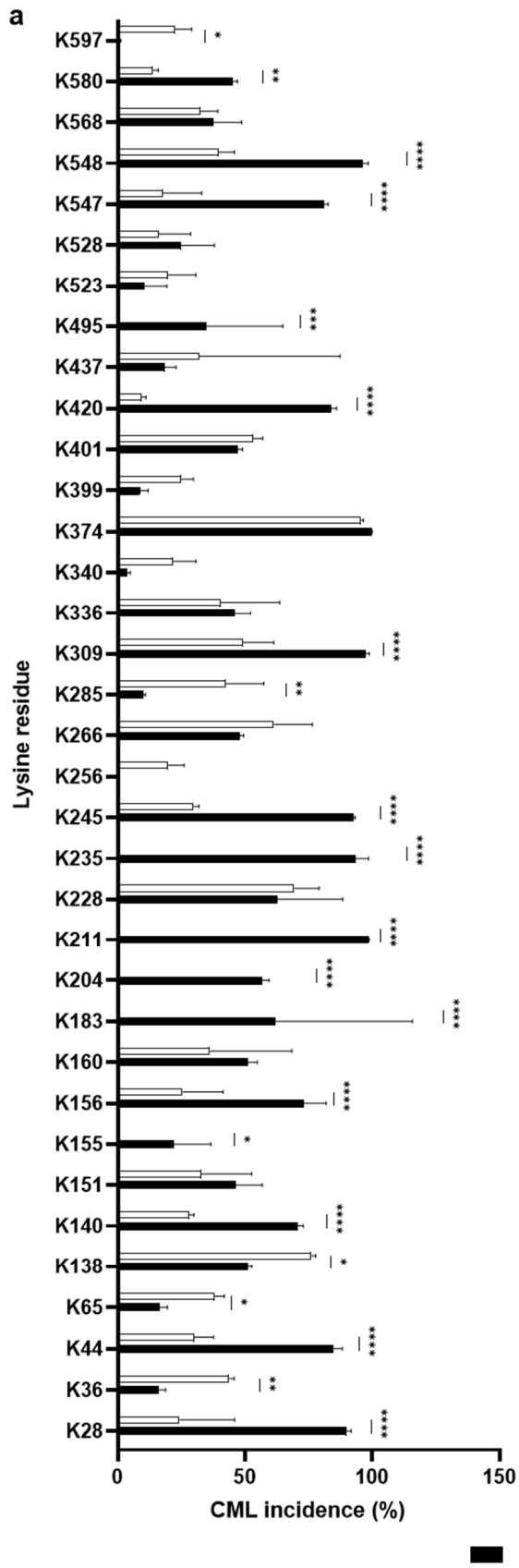


**Figure 1.** CML formation after incubating BSA with glyoxylic acid (GA) or glyoxal (GO). (a) LC-MS/MS quantification of lysine, CML, and the estimation of lysine modifications other than CML in samples GO-A to -E. Percent notations indicate the proportions of lysine residues converted to CML in GA- and GO-BSA glycation systems relative to native BSA. (b) Protein-bound CML detection on a

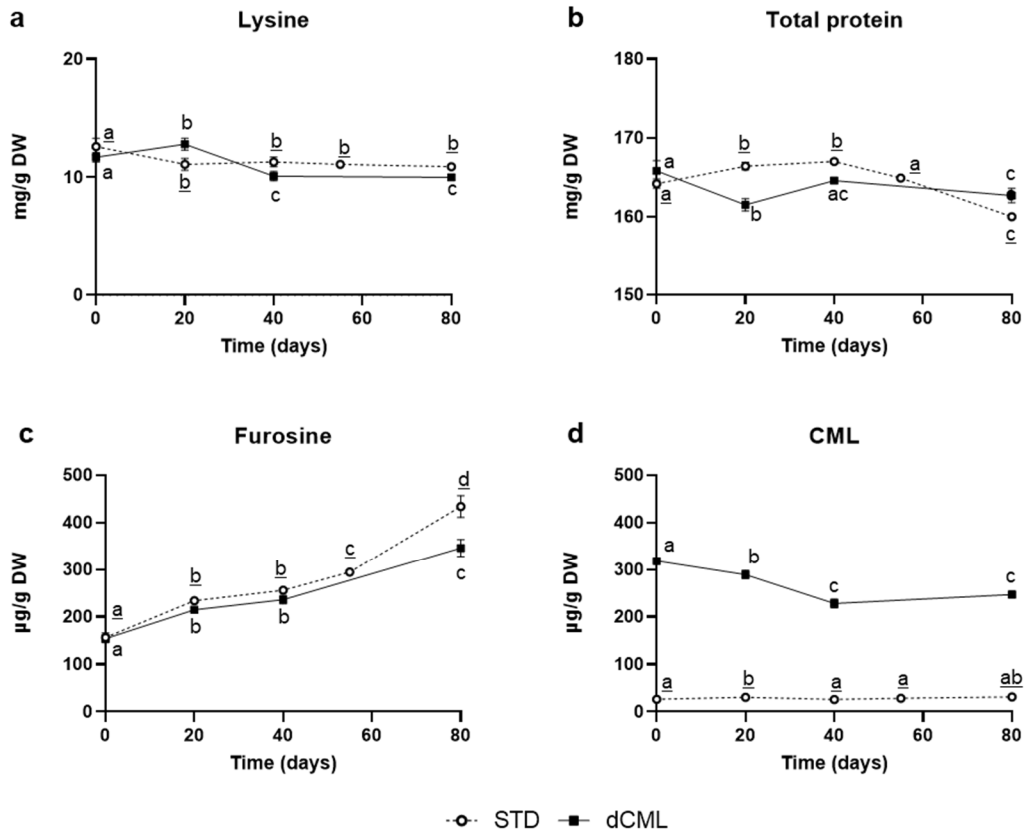
western blot with increasing concentrations of glyoxal. Respective Coomassie blue staining of the acrylamide gel in GO-A to -E samples. (c) LC-MS/MS quantification of lysine, CML, and the estimation of lysine modifications other than CML within GA-E triplicates (GA-E.1, GA-E.2, GA-E.3) and GO-E triplicates (GO-E.1, GO-E.2, GO-E.3). Percent notations indicate the number of lysine residues converted to CML relative to native BSA. (d) Protein-bound CML detection on a western blot on GA-E and GO-E replicates, together with their respective Coomassie blue staining on acrylamide gel. (e) LC-MS/MS quantification of arginine and its modifications on GA-E and GO-E replicates. Percent notations indicate the number of arginine residues converted to unknown arginine modifications, again relative to native BSA.



**Figure 2.** (a) Excitation and emission spectra of glyoxylic acid-modified BSA (GA-E samples) and glyoxal-modified BSA (GO-E samples) and (b) fluorescence intensity of native BSA (control), GA-E and GO-E samples at pH 8. Fluorescence excitation was set to 340 nm for the emission spectra, and emission to 425 nm for the excitation spectra. **Control versus GA groups:  $p= 0.4305$ .** Bars represent fluorescence intensity means  $\pm$  SD of the GA-E (GA-E.1 to 3) and GO-E triplicates (GO-E.1 to 3). \*\*\*\*  $p \leq 0.0001$ .



**Figure 3.** Proteomic analysis of glyoxylic acid (GA) and glyoxal (GO) modified BSA. Only the residues that were significantly modified relative to the original amino acid in non-glycated, native BSA have their *p* value indicated. (a) Average CML incidence (%) in GA- and GO-modified BSA molecules. Average CMA (b) and G-H1 (c) incidence (%) in GA- and GO-modified BSA. (d) Identification of potential crosslink sites on GA- and GO-modified BSA. Bars represent mean  $\pm$  SD values (n=3). (e) Chemical structures of the main glycation products identified by proteomic analyses. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$  for comparisons between the control (non-glycated, native BSA), GA-BSA, and GO-BSA. Bars corresponding to non-glycated BSA are all equal to zero.



**Figure 4.** Temporal LC-MS/MS quantification of (a) lysine, (b) total protein, (c) furosine, and (d) CML in extruded mouse food pellets enriched with native BSA (STD) or GO-BSA to mimic a CML-rich diet (dCML), over 80 days of storage at 20 °C. Values are expressed as a function of the dry weight (DW) of rodent food. Different letters represent significant difference within diets. Underlined letters correspond to comparisons within STD diet, while non-underlined letters show comparisons within dCML at  $\alpha=0.05$ .