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Cocoa melanoidins reduce the formation of dietary advanced glycation end-products in dairy mimicking system

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ABSTRACT

The control of Maillard reaction in foods is important to preserve protein nutritional quality. In this study, we investigated the effects of melanoidins obtained from different roasted cocoa beans toward the formation of dietary advanced glycation end-products (d-AGEs) in aqueous solution of whey protein (WP) and glucose, glyoxal and methylglyoxal at 35 °C and pH 7.0. Cocoa melanoidins (4 mg/mL) were more effective to inhibit glyoxal-derived d-AGEs than methylglyoxal-derived d-AGEs, with 74.4% and 48% reduction of *N*-ε-carboxymethyllysine and methylglyoxal-hydroimidazolone formation in WP/glyoxal and WP/methylglyoxal system, respectively. Furthermore, protein-bound *N*-ε-fructosyllysine, measured through furosine, decreased down to 57.2% in presence of cocoa melanoidins in WP/glucose model system suggesting an effective control of the Maillard reaction in an early stage. These findings highlighted that cocoa melanoidins are functional ingredients able to mitigate protein glycation in dairy products during storage.

1. Introduction

Advanced glycation end-products (AGEs) are a group of heterogeneous compounds formed during the Maillard reaction (Poulsen et al., 2013). Caramelization and Maillard reaction induce the formation of α-dicarbonyl compounds (DCs) including 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO), which can readily react with amino terminus of proteins and peptides, lysine ε-amino group, guanidino side of arginine or the thiol group of cysteine residues leading to a cascade of products like *N*-ε-carboxymethyllysine (CML), *N*-ε-carboxyethyllysine (CEL), methylglyoxal-hydroimidazolone (MG-H1) and *S*-(carboxymethyl)cysteine (Hellwig & Henle, 2014). Glycated proteins are formed during food processing and under prolonged storage; the consequent formation of dietary AGEs (d-AGEs) leads to a reduction of protein digestibility and decreases the bioavailability of essential amino acids (Zenker, van Lieshout, van Gool, Bragt, & Hettinga, 2020; Zhao et al., 2017). AGEs are also formed *in vivo* under physiological conditions ranging from free glycated amino acids to large glycated proteins. In pathophysiological conditions, such as diabetes and neurodegenerative disorders, pro-oxidant and inflammatory environment can further promote glycation and oxidation reactions resulting in high accumulation of AGEs particularly for proteins with a low turnover (Rabbani & Thornalley, 2020). How d-AGEs contribute to the pool of endogenous AGE is unclear and different outcomes were found for the various d-AGEs (Delgado-Andrade & Fogliano, 2018). The recommendation to reduce the formation of d-AGE during food processing and storage was suggested by several studies together with the need of careful discrimination between free and protein-bound d-AGEs (van Boekel et al., 2010; Zhao et al., 2019).

Dairy based foods are widely consumed all over the world including flavored milk (Bisig & Kelly, 2017). The industrial production of dairy products requires heat treatment to ensure safe and long shelf life foods. As a consequence, Maillard reaction readily occurs in dairy-based foods because of a combination of prolonged storage and complex composition (Mehta & Deeth, 2016). Zhang et al. (2019) recently reported that CML, CEL and MG-H1/H3 could be considered as markers for evaluation of the extent of the Maillard reaction in conventional and lactose-hydrolyzed ultrahigh temperature milk during storage. Among milk proteins, whey proteins (WP) are largely used as food ingredient and they undergo chemical changes during thermal processing and storage, showing higher concentration of d-AGEs compared to caseins (Pischetsrieder & Henle, 2012). In the frame of the Maillard reaction

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control strategies, the ability of polyphenols to counteract protein glycation has been screened and examples in dairy products include epicatechin (Zhu, Poojary, Andersen, & Lund, 2020), genistein (Kong, Li, Zheng, & Lv, 2015), beetroot juice (Račkauskienė et al., 2015) and secoiridoids derivatives in olive mill wastewater (Troise et al., 2014).

Along with the addition of antioxidant extracts, the possibility to use complex polysaccharide- and protein-based macromolecules formed during food processing, such as melanoidins can be an interesting alternative to improve the 'food naturalness' preventing the addition of artificial additives and preservatives (Román, Sánchez-Siles, & Siegrist, 2017). Melanoidins are the brown heterogeneous high molecular weight polymers formed in foods upon severe thermal treatments like roasting of coffee and cocoa beans. Previous studies proposed that melanoidins can exert functional and health properties acting as antioxidant dietary fiber in food system (Mesías & Delgado-Andrade, 2017; Morales, Somoza, & Fogliano, 2012) and influence gut-brain response and energy intake (Walker et al., 2020).

The ability of melanoidins in scavenging dicarbonyls has been recently pointed out by our group: polyphenols-rich melanoidins like coffee melanoidins can efficiently scavenge DCs under physiological conditions (Zhang, Zhang, Troise, & Fogliano, 2019). Roasting parameters of cocoa beans (time/temperature combination) can influence this activity through changing chemical compositions of melanoidins especially the amount of melanoidins-bound polyphenols. Oracz, Nebesny, and Żyżelewicz (2019) recently reported that the total contents of non-covalently and covalently bound phenolic compounds in cocoa melanoidins ranged from 12.56 to 33.24 mg/100 g dry weight and 407.16 to 649.02 mg/100 g dry weight, respectively, depending on roasting temperature and time. However, the influence of roasting parameters on the ability of melanoidins to inhibit protein glycation and the possible inhibitory mechanism are unclear.

As cocoa-flavored milk is one of the most popular beverages, especially among children, in this study we investigated the inhibitory effect of cocoa melanoidins on WP glycation in WP/glucose or WP/dicarbonyls model under the simulated storage conditions for up to 21 days. The ambient temperature (35 °C) was used to mimic the extreme storage conditions typical of transport across continents, where the temperature exceeds 35 °C (Deeth & Lewis, 2017; Karlsson et al., 2019). In addition, the possible mechanisms underlying such effect were also elucidated.

2. Materials and methods

2.1. Chemicals

Glyoxal aqueous solution (GO, 40%), methylglyoxal aqueous solution (MGO, 40%), quinoxaline, 2-methylquinoxaline, diethylenetriaminepentaacetic acid (DETAPAC), *o*-phenylenediamine (OPD), *o*-phthaldialdehyde, aminoguanidine and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid (37%), disodium hydrogen phosphate dihydrate, D-(+)-glucose monohydrate, sodium dihydrogen phosphate dihydrate and acetic acid were obtained from Merck (Darmstadt, Germany). Analytical standards *N*-ε-carboxymethyllysine (CML), *N*-ε-carboxymethyllysine-d_2 (CML-d_2), furosine and furosine-d_4 were bought from Polypeptide laboratories (Strasbourg, France). *N*-ε-carboxyethyllysine (CEL) and *N*-ε-carboxyethyllysine-d_4 (CEL-d_4) were purchased from TRC-Chemicals (Toronto, Canada), and methylglyoxal-hydroimidazolone trifluoroacetic acid salt (MG-H1) was obtained from Iris Biotech GmbH (Marktredwitz, Germany).

2.2. Cocoa samples

Dried cocoa beans (Forastero) were provided by CACEP (Villahermosa, Mexico). The raw samples were hand-peeled and roasted in batches of 200 g in a laboratory convection oven (HBG76S651E, Bosch, Munich, Germany). Roasting processes were performed in triplicate at four different roasting parameters as summarized in Table 1 to give four roasted cocoa beans: low roasted cocoa beans (LR-CO); intermediate roasted cocoa-1 beans (IR-CO-1); intermediate roasted cocoa-2 beans (IR-CO-2); dark roasted cocoa beans (DR-CO). At the end of roasting, cocoa beans were cooled for 20 min to room temperature in fume hood for further treatment.

Table 1
Roasting parameters and water content of thermally treated cocoa beans. Low roasted (LR), intermediate roasted (IR) and dark roasted cocoa beans (DR-CO).

	LR-CO	IR-CO-1	IR-CO-2	DR-CO
Temperature (°C)	130	130	150	150
Time (min)	30	120	120	210
Water content (%)	4.30 ± 0.37	3.21 ± 0.30	1.90 ± 0.14	0.54 ± 0.14

2.3. Preparation of high molecular weight cocoa melanoidins

High molecular weight cocoa melanoidins was obtained from four different roasting process according to the methods described by Summa et al. (2008) to yield low roasted (LR-COM), two intermediate roasted (IR-COM-1 and IR-COM-2) and dark roasted cocoa melanoidins (DR-COM). In brief, 200 g of roasted cocoa beans was ground to a fine powder and defatted with petroleum ether. After air-dried at room temperature overnight, the defatted cocoa powder was extracted with water (1:10, w/v) at 80 °C for 20 min. The suspension was centrifuged at 5000 × g for 10 min, and the supernatant was filtered through a Whatman 595 filter paper to remove the insoluble materials. Then, filtrate (400 mL) was dialyzed using a dialysis membrane (MW cutoff > 12.4 kDa, Sigma-Aldrich) for 3 days against 4 L of water at 4 °C with constant stirring. The water was changed 10 times until conductivity reached a value lower than 2.0 μS/cm detected by a conductivity meter (WTW InoLabTM Cond 7110, Fisher Scientific, Sweden). After dialysis, the retentate was frozen at −20 °C and lyophilized. All obtained cocoa melanoidins were then stored at −20 °C until used.

2.4. The quantification of free amino group and dicarbonyl compounds

o-Phthaldialdehyde (OPA) assay was used to determine the free amino groups of the melanoidins before and after incubation with DCs to estimate the possible reaction between amino groups and DCs. Briefly, GO (6.4 mM), MGO (6.4 mM) and four melanoidin solutions (10 mg/mL) were prepared in phosphate buffer (100 mM, pH 7.0). GO/MGO (240 μL) or phosphate buffer (negative control) was mixed with 240 μL of different melanoidin solutions and 720 μL of phosphate buffer before incubation at 35 °C for 2, 5 and 7 days. After incubation, the remaining DCs was measured by mixing 1 mL of samples with 200 μL of 0.2% OPD solution containing DETAPAC (9.6 mM) according to the method described by Zhang et al. (2019). The mixture was kept in the dark at room temperature for 3 h and filtered using a 0.22 μm polyvinylidene fluoride (PVDF) filter before UHPLC-MS/MS analysis as reported in paragraph 2.7. The remaining samples (200 μL) was subjected to OPA assay based on the previous method (Park, Albright, Cai, & Pariza, 2001). Freshly prepared OPA reagent (1500 μL) was added to the samples and the absorbance at 340 nm was measured using an UV–vis spectrophotometer (Cary 50 Varian, Palo Alto, CA) after 120 s of incubation at room temperature. The calibration curve was obtained by using L-serine standard solutions (in the range of 20–150 mg/L) with the same incubation conditions used for the melanoidins samples.

2.5. Effect of cocoa melanoidins on protein glycation in the WP/GO, WP/MGO or WP/glucose reaction model

The concentrations of WP and glucose were based on the chocolate milk (5%–9% carbohydrate and 3.2–3.6% protein) (Roy, 2008) and a concentration of cocoa melanoidins fluctuating between 1.37 and 39 mg/mL in chocolate milk was assayed according to the amount of

defatted cocoa powder added (1%–15%) (Bisig & Kelly, 2017) and the extraction yield of melanoidins from defatted cocoa powder (13.7%–26.0%) (Quiroz-Reyes & Fogliano, 2018). Briefly, WP (35 mg/mL), GO (6.4 mM), MGO (6.4 mM), glucose (1 M), aminoguanidine (6.4 mM) and four melanoidin solutions (10 mg/mL) were prepared in phosphate buffer (100 mM, pH 7.0). WP (250 μL, containing 0.2% sodium azide) was mixed with 500 μL of GO, MGO or glucose solution, and 500 μL of phosphate buffer (negative control, NC), aminoguanidine (positive control) or melanoidins solutions listed above. WP/GO and WP/MGO model systems were incubated at 35 °C for 2, 5, 7 and 14 days, while WP/glucose model system was incubated at 35 °C for 7, 14 and 21 days. After incubation, 0.5 mL of incubated sample was used to analyze the loss of DCs in WP/GO and WP/MGO models as described above and the remaining samples were subjected to acidic hydrolysis and HILIC tandem mass spectrometry for the determination of CML, CEL, MG-H1 and furosine (see below).

2.6. Effect of cocoa melanoidins on formation of GO and MGO

Five hundred microliters of glucose (1 M in phosphate buffer, pH 7.0, 0.04% sodium azide) was mixed with 750 μL of phosphate buffer or 250 μL of phosphate buffer and 500 μL of IR-COM-1 solution (10 mg/mL). The mixture was incubated at 35 °C for 7, 14 and 21 days and the formation of GO and MGO was analyzed as described above.

2.7. Determination of quinoxaline derivatives

Determination of quinoxaline derivatives was conducted on a Thermo Ultimate 3000 UHPLC system coupled with a TSQ triple quadrupole mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as detector. The separation of quinoxaline derivatives was achieved on a Kinetex EVO C18 column (150 mm × 2.1 mm, 2.6 μm, Phenomenex, Torrance, CA) with a C-18 precolumn using the following mobile phase: (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. Compounds were eluted at 0.4 mL/min through the following gradient (t in [min]/[%B]): (0/2), (2/2), (8/25), (10/50), (13/95). Positive electrospray ionization was used with the following settings: spray voltage 4.0 kV; capillary temperature 350 °C; dwell time 100 ms; sheath gas and aux gas were set to 10.0 and 5.0 AU (arbitrary unit). The quinoxaline derivatives of GO and MGO were identified by selected reaction monitoring (SRM) mode using the following conditions respectively (in parenthesis collision energy, CE): *m/z* 131 → 77 (CE: 25 V) and *m/z* 145 → 77 (CE: 28 V). The concentrations of quinoxaline and 2-methylquinoxaline were calculated by using external standard calibration curves in the range of 0.02–20.0 mg/L (R^2 greater than 0.999 according to the intraday and interday assays). Trapping capacity was expressed by the percentage of decrease in each dicarbonyl compound (DC) using the following equation (Eq. (1)):

$$\text{Trapping capacity } (\%) = \frac{\text{amount of DC in blank} - \text{amount of DC in samples}}{\text{amount of DC in blank}} \times 100\% \quad (1)$$

2.8. Determination of d-AGEs and protein-bound lysine Amadori compound by UHPLC-MS/MS

CML, CEL, MG-H1 and furosine were determined according to Troise, Fiore, Wiltafsky, and Fogliano (2015) with some modifications and the furosine content was multiplied by a factor of 3.1 to calculate the amount of *N*-ε-fructosyllysine (Krause, Knoll, & Henle, 2003). Briefly, an aliquot of the WP/glucose, WP/GO and WP/MGO model systems (0.75 mL) was mixed with 3.25 mL of HCl (7.4 M) and then heated at 110 °C for 20 h. The hydrolysate was filtered using a PVDF filter (0.22 μm) and 0.2 mL of filtrate was dried under nitrogen flow. The dried samples were reconstituted in 500 μL of 50% aqueous acetonitrile, and 190 μL of sample was mixed with 10 μL of mixed internal standards to obtain a final concentration of 200 μg/L in each sample for all of internal standards before being injected onto a RS 3000 U-HPLC system (Thermo) coupled to a TSQ triple quadrupole. The samples (5 μL) were injected on a thermostated (30 °C) Kinetex HILIC column (2.6 μm, 2.1 mm × 100 mm, Phenomenex) and the flow rate was 0.4 mL/min. The mobile phase consisted of 0.1% formic acid (solvent A), acetonitrile with 0.1% formic acid (solvent B) and 50 mmol/L ammonium formiate (solvent C) with the following gradient program (t in [min]/[%B]/[%C]): (0/80/10), (0.8/80/10), (3.5/40/10), (6.5/40/10). Positive electrospray ionization was used and the source parameters were set as follows: spray voltage 3.0 kV; capillary temperature 310 °C; dwell time 100 ms; sheath gas and aux gas were set to 30 and 25 arbitrary units. SRM mode was used to detect selected AGEs in following transitions, in parenthesis collision energy: CML, *m/z* 205 → 130 (CE: 13 V) and 84 (CE: 26 V); CML-d_2, *m/z* 207 → 130 (CE: 13 V) and 84 (CE: 26 V); CEL, *m/z* 219 → 130 (CE: 14 V) and 84 (CE: 24 V); CEL-d_4, *m/z* 223 → 134 (CE: 14 V) and 88 (CE: 24 V); furosine, *m/z* 255 → 130 (CE: 14 V) and 84 (CE: 30 V); furosine-d_4, *m/z* 259 → 134 (CE: 14 V) and 88 (CE: 30 V) and MG-H1, *m/z* 229 → 166 (CE: 16 V) and 70 (CE: 29 V). MG-H1 was quantified by external calibration curve and other analytes were quantified through the internal standard technique. Limit of detection (LOD), limit of quantitation (LOQ), linearity, coefficient of correlation (r^2) and recovery are shown in Table S1. Data were processed using Xcalibur version 4.0 (Thermo Fisher).

2.9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

WP (7 mg/mL), MGO (2.56 mM) and IR-COM-1 solution (4 mg/mL) were incubated together at 35 °C for 2, 5 and 7 days. After incubation, SDS-PAGE was performed according to Krämer and Davies (2019). In brief, LDS (lithium dodecyl sulfate) sample buffer (4×, Thermo Fisher Scientific) was added to the diluted samples (7 times) and heated at 70 °C for 10 min. WP (10 μg per lane) was then loaded on to 12% Bis-Tris gels (Thermo Fisher Scientific). Five microliters of molecular weight markers (10–250 kDa, Bio-Rad Laboratories) were also used on each gel. SDS-PAGE was conducted at 120 V for 120 min with 4-morpholinepropanesulfonic acid SDS running buffer (20×, Thermo Fisher Scientific). Gels were stained with Coomassie Brilliant Blue R-250, followed by destaining in a 10% ethanol and 7.5% acetic acid aqueous solution until a desired color density level.

2.10. Statistical analysis

All experiments were performed in three independent replicates unless otherwise stated. Significant differences ($p < 0.05$) between means were analyzed by Tukey's HSD test using the SPSS statistics (v. 23.0, IBM, Armonk, NY). The error bars in all figures correspond to the standard deviation (SD).

3. Results and discussion

3.1. Time course of GO and MGO trapping capacity and free amino group contents of cocoa melanoidins

Previous findings demonstrated that dicarbonyl scavenging ability of food melanoidins mainly comes from the melanoidins-bound polyphenols fraction (Zhang et al., 2019) and time course investigation was conducted to ascertain the role of free amino groups in melanoidins during carbonyl scavenging process. Fig. 1 presents that IR-COM-1 and LR-COM reduced GO down to 67.8% and 45.3% within 2 days respectively, displaying the highest and the lowest GO scavenging capacity among four cocoa melanoidins. The higher polyphenols content in IR-COM-1 can explain its higher trapping activity than LR-COM. According to Oracz and coworkers, the well-known carbonyl scavengers

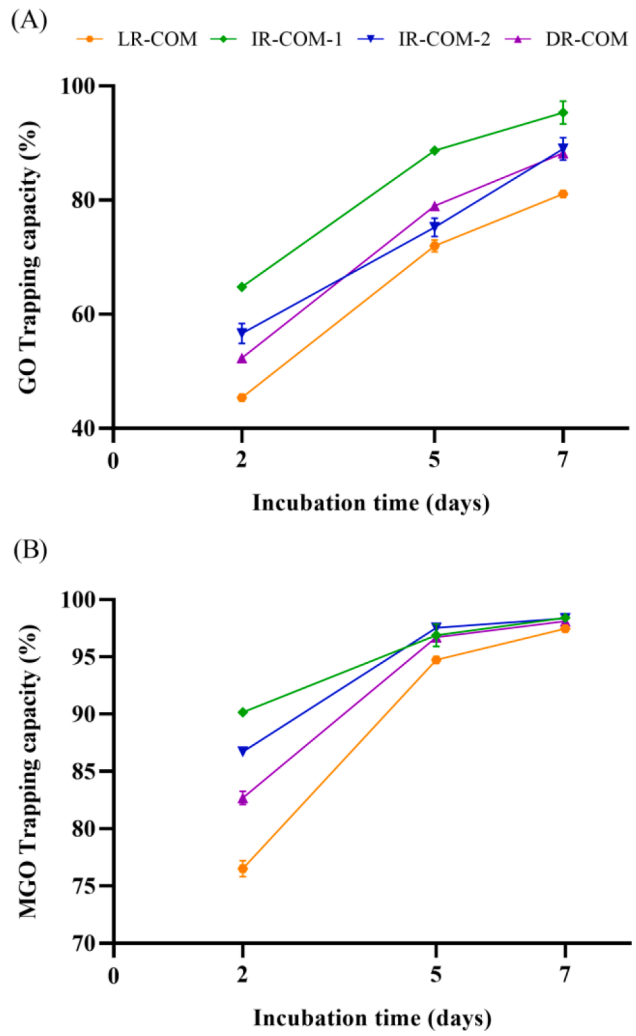

Fig. 1. Time-course of glyoxal (A, 1.28 mM) and methylglyoxal (B, 1.28 mM) trapping capacity of cocoa melanoidins (2 mg/mL). Results are expressed as mean ± SD for n = 3. Low roasted (LR), intermediate roasted (IR) and dark roasted-cocoa melanoidins (DR-COM).

catechin and its derivatives are the predominant polyphenols in cocoa melanoidins, and the intermediate roasting conditions lead to the highest total phenolic content and antioxidant activity of high molecular weight cocoa melanoidins (Oracz & Zyzelewicz, 2019; Oracz et al., 2019), which is in line with our results in Fig. S1 and parallels the dicarbonyl scavenging assay (Fig. 1). More than 90% of MGO was reduced by each cocoa melanoidins within 5 days, while trapping efficacy of cocoa melanoidins in GO system was lower than that for MGO, indicating that the methyl group of methylglyoxal do not exert any steric hindrance. These results highlighted the fundamental role of roasting parameters in determining the functional properties of cocoa melanoidins in line with the observation on coffee and other foods (Mesías & Delgado-Andrade, 2017). Quiroz-Reyes and Fogliano (2018) previously reported that both roasting temperature and time significantly influenced the yield, polyphenols content and antioxidant activity of cocoa melanoidins as a result of modifications of chemical composition and molecular rearrangement in cocoa beans.

The free amino group content in melanoidin samples is reported in Table 2: DR-COM sample (46.8 μmol/g) was characterized by a lower content of free amino groups compared to melanoidins extracted from low roasted cocoa samples. These results indicated that severe roasting conditions promoted the reaction between amino groups of melanoidin-bound proteins and other components including polyphenols and sugar,

Table 2
Content of free amino group (μmol/g of melanoidins) in cocoa melanoidins after 2, 5 and 7 days of incubation with or without glyoxal (GO) and methylglyoxal (MGO).

	Control	GO	MGO
LR-COM			
0 days	73.0 ± 2.1 a		
2 days	72.8 ± 1.1 a	65.8 ± 3.9 ab	57.2 ± 3.3 cd
5 days	68.1 ± 4.1 a	63.7 ± 3.1 ab	52.6 ± 2.7 cd
7 days	66.9.1 ± 2.8 ab	62.7 ± 3.7 bc	51.3 ± 2.8 d
IR-COM-1			
0 days	69.1 ± 1.9 a		
2 days	67.6 ± 2.0 a	64.6 ± 3.9 a	50.9 ± 3.3 bc
5 days	64.8 ± 2.4 a	61.8 ± 4.0 a	48.8 ± 2.7c
7 days	63.1 ± 2.7 a	60.5 ± 1.3 ab	47.8 ± 2.2c
IR-COM-2			
0 days	53.7 ± 3.1 a		
2 days	51.3 ± 1.7 a	48.6 ± 3.3 ab	31.0 ± 2.8c
5 days	49.5 ± 3.1 ab	47.2 ± 2.2 ab	30.1 ± 2.2c
7 days	47.3 ± 2.9 ab	45.9 ± 1.3b	29.4 ± 2.4c
DR-COM			
0 days	46.8 ± 2.4 a		
2 days	45.7 ± 1.9 a	44.3 ± 1.7 a	33.8 ± 3.3 bc
5 days	44.3 ± 2.8 a	40.5 ± 1.8 a	29.4 ± 2.2c
7 days	44.1 ± 2.0 a	40.2 ± 3.1 ab	28.8 ± 5.0c

Data are presented as the mean ± SD for n = 3. Different letters in the same melanoidins group indicate significant difference according to Tukey's HSD test at $p < 0.05$. Low roasted (LR), intermediate roasted (IR) and dark roasted-cocoa melanoidins (DR-COM).

which led to the decline in free amino groups (Wang, Qian, & Yao, 2011). Upon incubation with DCs, the concentration of free amino groups was reduced faster in the presence of MGO than GO, especially in the first 2 days of incubation. This data suggested that amino residues of melanoidin-bound proteins contributed to the dicarbonyl scavenging ability of cocoa melanoidins and is in line with the high reactivity of MGO toward amino groups (Meade, Miller, & Gerrard, 2003). Considering the composition of model system, the content of available amino groups in cocoa melanoidins was 0.11–0.18 μmol, which was much lower than the content of GO/MGO (1.54 μmol) highlighting the predominant role of melanoidin-bound polyphenols in scavenging DCs.

3.2. *Effects of cocoa melanoidins on the formation of CML, CEL and MG-H1 in the WP/GO and WP/MGO model systems*

The ability of cocoa melanoidins to control CML formation was evaluated in WP-GO model system. Fig. 2A shows that GO concentration decreased over the incubation time in all of reaction models, with a more marked decline in cocoa melanoidins groups compared with control group. After 7 days of incubation, the scavenging of GO was close to 100% in the presence of melanoidins and IR-COM-1 decreased the GO content more efficiently than other three melanoidins during the first 5 days of incubation (Table S2). However, after 14 days of incubation, 7.2% of initial GO was still detectable in control group, indicating the effective scavenging for GO in the presence of cocoa melanoidins. Fig. 2B highlighted that 12.2% of GO reacted with lysine to form CML in control group after 14 days of incubation based on the initial concentration of GO (2.56 μM) in model system. Focusing on model systems with cocoa melanoidins, the content of CML was significantly reduced by all four melanoidins. Furthermore, the inhibition of CML formation by IR-COM-1 reached up to 74.4% after 14 days of incubation, followed by IR-COM-2, DR-COM and LR-COM, which can be related to the higher dicarbonyl scavenging activity of IR-COM-1 than that of other cocoa melanoidins (Fig. 1). CML is a well-characterized and extensively studied AGE, which is used as a marker of Maillard reaction in foods (Delgado-Andrade, 2016), particularly in dairy products that contain higher CML concentration than other food categories (Assar, Moloney, Lima,

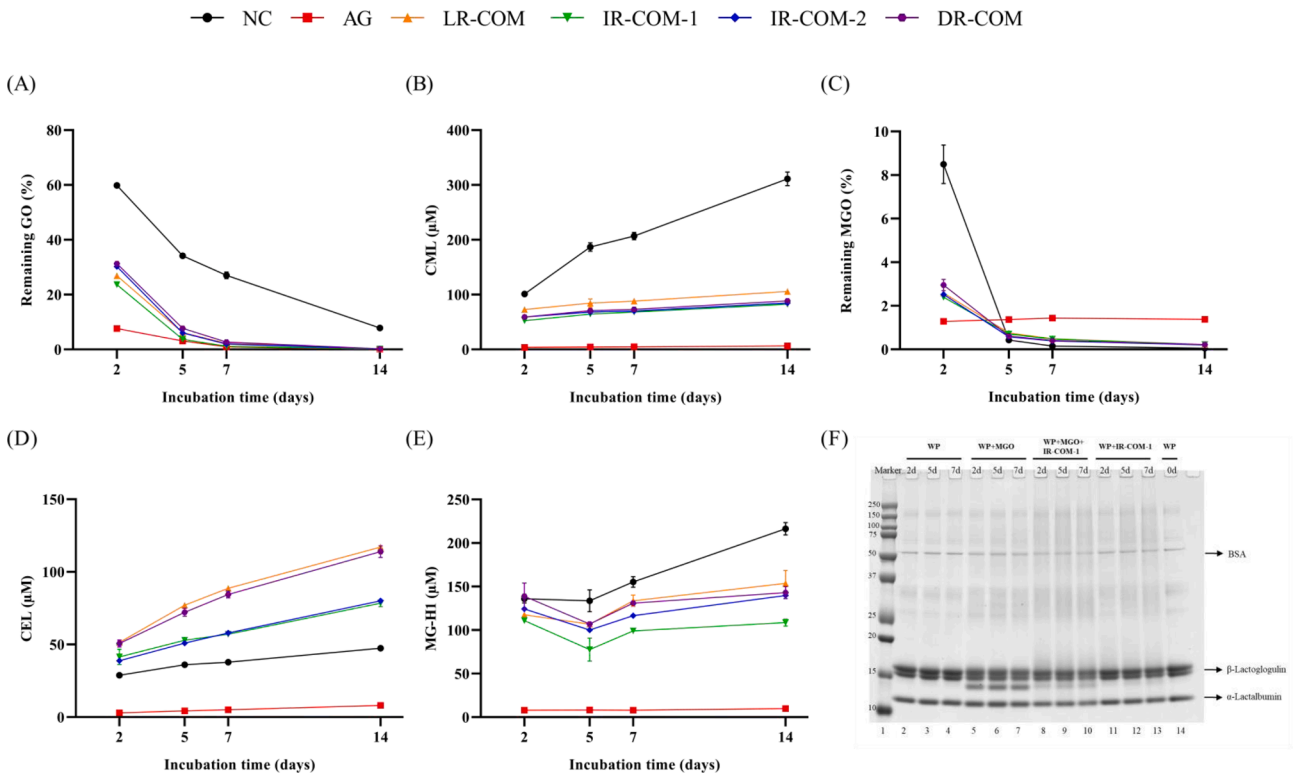

Fig. 2. Effects of four cocoa melanoidins on the percentage of remaining dicarbonyl compounds and the formation of *N*-ε-carboxymethyllysine (CML) in whey protein/glyoxal model (A: glyoxal; B: CML), as well as *N*-ε-carboxyethyllysine (CEL) and methylglyoxal-hydroimidazolone (MG-H1) in whey protein/methylglyoxal model (C: methylglyoxal; D: CEL; E: MG-H1) after 2, 5, 7 and 14 days of incubation. F: SDS-PAGE molecular weight band patterns of whey protein glycation and inhibitory effect of IR-COM-1 at 35 °C after 2, 5 and 7 days of incubation. Negative control (NC), aminoguanidine (AG), low roasted (LR), intermediate roasted (IR) and dark roasted-cocoa melanoidins (DR-COM). Data are expressed as mean ± SD for n = 3.

Magee, & Ames, 2009). Considerable amount of CML was generated from day 5 to day 14 in control group, while the increase of CML was controlled in the presence of melanoidins during the same time window. These results can be explained by the evidence that more than 90% of GO was lost within 5 days in melanoidins groups and supported the hypothesis that cocoa melanoidins inhibits CML formation in whey protein system through scavenging GO.

The percentage of remaining MGO in the presence of cocoa melanoidins in WP/MGO model was showed in Fig. 2C. The remaining MGO in control group was significantly higher than that in the groups containing melanoidins after 2 days of incubation period, indicating that part of MGO in reaction model was scavenged by cocoa melanoidins; the loss of MGO was more than 90% within 2 days in all models. The concentration of MG-H1 was 216.4 μM in control group after 14 days of incubation and upon the addition of cocoa melanoidins, the highest inhibition of MG-H1 formation was found in IR-COM-1 group, reaching up to 48% after 14 days (Fig. 2E). Furthermore, IR-COM-1 still exerted the inhibition on MGO-derived AGEs formation due to the higher formation of MG-H1 than that of CEL. Similar results were observed by Zhang et al. (2019) who found that MG-H1 was the major MGO-derived d-AGEs in lactose-hydrolyzed milk during the storage. Aminoguanidine, as a potent AGE-inhibitor, suppressed more than 80% of CEL and 90% of CML and MG-1 formation. Considering these results and comparing with the data in WP/GO model system, cocoa melanoidins were less effective in inhibiting MGO-derived d-AGEs formation, although they showed higher trapping capacity for MGO than GO. This could be explained by the higher reactivity of MGO than GO toward amino acids in whey proteins. At the same incubation time, amino groups react more efficiently with MGO than with GO, lowering the amount of MGO trapped by cocoa melanoidins. These findings indicated that the reactivity of α-dicarbonyls toward proteins should be considered when correlating dicarbonyl trapping capacity to inhibitory effect on protein glycation.

The effects of cocoa melanoidins on MGO-induced protein modification was further investigated by using SDS-PAGE (Fig. 2F). WP without addition of reactants showed three bands of about 14 kDa, 18 kDa and 66.5 kDa corresponding to *α*-lactalbumin, *β*-lactoglobulin and bovine serum albumin (lane 14), respectively. In the presence of MGO (lane 5–7), the formation of a new protein band below that of *β*-lactoglobulin was clearly detectable in accordance with Krämer et al. (2019) who suggested that this band was due to the formation of a *β*-lactoglobulin derivative induced by MGO. In presence of IR-COM-1 (lane 8–10), the intensity of the MGO-induced band decreased by 72.7%, confirming that cocoa melanoidins can counteract protein damage. In addition, no difference was found within three incubation time-points for WP/MGO and WP/MGO/melanoidins model system, which could be explained by the loss of MGO within 2 days as presented in Fig. 2C.

3.3. *Effects of cocoa melanoidins on the formation of CML, CEL, MG-H1 and N-ε-fructosyllysine in the WP/Glucose reaction model*

Glucose was used as substrate to evaluate the protective effects of cocoa melanoidins against the whey protein glycation, which proceeds faster than lactosylation. All of four cocoa melanoidins significantly inhibited the formation of CML, with IR-COM-1 being the most effective one (88.3% reduction after 21 days, Fig. 3A). This is in line with the results on Fig. 2, indicating that the better performance of IR-COM-1 in inhibiting d-AGEs formation in WP/glucose model can be related to its higher dicarbonyl scavenging activity compared to other three cocoa melanoidins tested. Concerning arginine modifications, IR-COM-1 was the most effective in suppressing the formation of MG-H1 among cocoa melanoidins (55.6% reduction after 21 days). Conversely, the concentration of CEL was higher in the cocoa melanoidins system than control group (Fig. 3B). Considering these results, we hypothesized a negative contribution of melanoidins in the formation of CEL through reacting

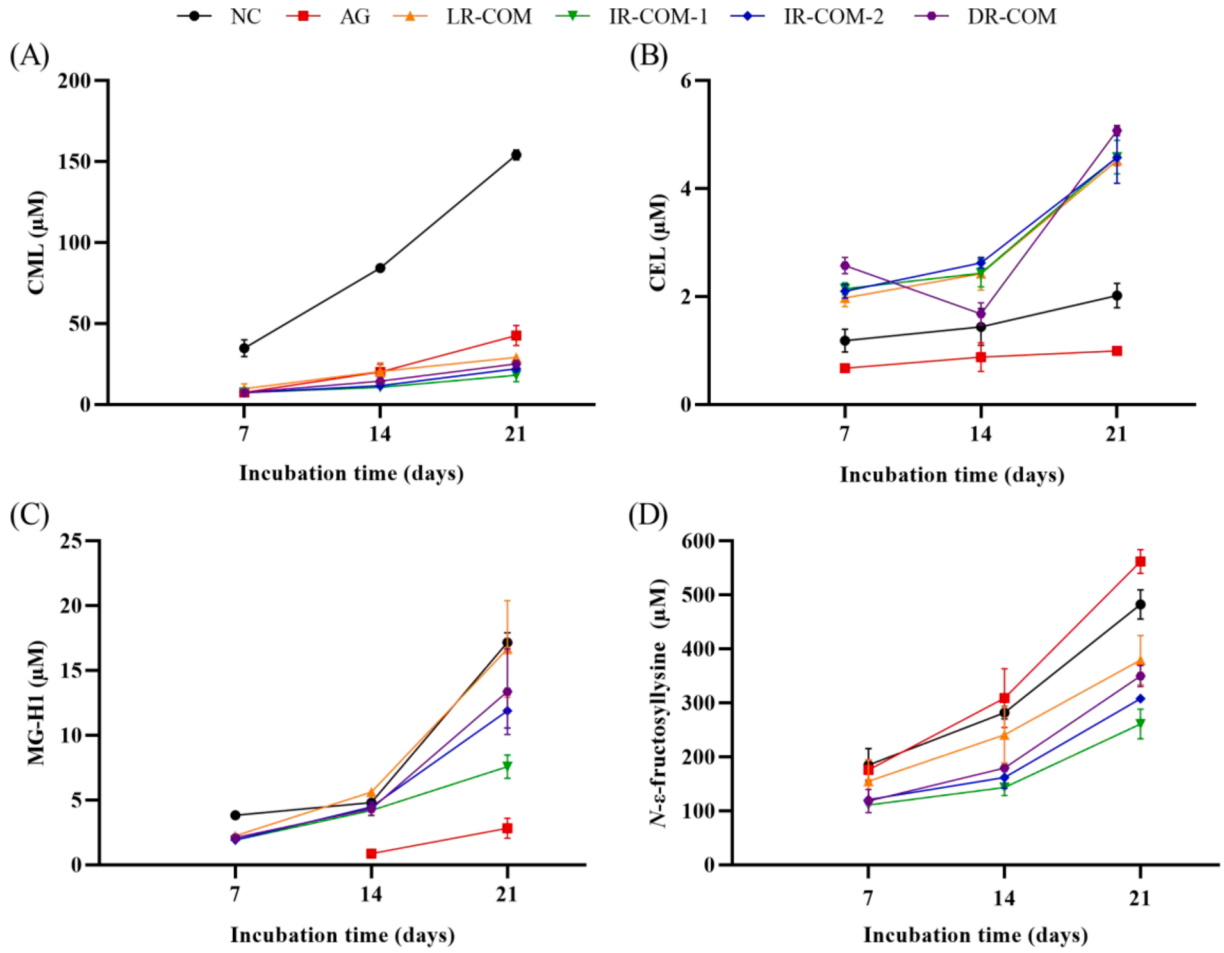

Fig. 3. Concentration of *N*-ε-carboxymethyllysine (CML), *N*-ε-carboxyethyllysine (CEL), methylglyoxal-hydroimidazolone (MG-H1) and *N*-ε-fructosyllysine (measured through furosine) in the whey protein/glucose model with or without cocoa melanoidins after 7, 14 and 21 days of incubation. Negative control (NC), aminoguanidine (AG), low roasted (LR), intermediate roasted (IR) and dark roasted-cocoa melanoidins (DR-COM). Data are expressed as mean ± SD for n = 3.

with WP as observed in Fig. S2. However, the formation of CEL was much lower than those of CML and MG-H1, suggesting cocoa melanoidins were effective in reducing d-AGEs formation in WP/glucose model system.

N-ε-fructosyllysine was analyzed through furosine to gain the insights into the relationship between cocoa melanoidins and related compounds formed during the early stage of protein glycation (Fig. 3D). The presence of cocoa melanoidins caused a significant decrease in *N*-ε-fructosyllysine content, while aminoguanidine showed no effect on *N*-ε-fructosyllysine formation during the incubation. These results emphasized that cocoa melanoidins inhibited the glycation of protein-bound lysine through multiple pathways. During roasting process, the phenolic rings bound to melanoidins skeleton can be oxidized to quinone, which can react with amino groups in WP leading to the loss of available reaction sites for glucose (Guerra & Yaylayan, 2014). The macromolecule formed by melanoidins and WP could minimize the interaction between WP and glucose through steric hindrance and the reduction of *N*-ε-fructosyllysine can be the reason why cocoa melanoidins was less effective than aminoguanidine in inhibiting CML formation in WP/GO model but more effective in WP/glucose model.

Hodge pathway is one of the possible routes for the generation of CML: the formation of ketoamine Amadori products is followed by Amadori rearrangement and subsequent oxidation and degradation leading to the formation of CML as indicated in Fig. 4 (Hodge, 1953). The reduction of *N*-ε-fructosyllysine formation induced by cocoa melanoidins confirmed our hypothesis that cocoa melanoidins can inhibit CML formation through decreasing the generation of lysine Amadori compound (Fig. 4, Route A). Another possible pathway for the formation of d-AGEs involves the nucleophilic attack of guanidino, amino, thiol groups, and amino terminus of peptides and proteins on free DCs formed by degradation of Schiff bases and sugar (Fig. 4, Namiki pathway and Wolff pathway). In presence of aminoguanidine, no effect on the formation of Amadori compound was observed in WP/glucose model, but aminoguanidine inhibited 72.4% of d-AGEs formation (CML, CEL and MG-H1) in total. Aminoguanidine hardly suppressed AGEs formation from Amadori product at *post*-Amadori stage but guanidine derivative is effective in glucose/lysine system through scavenging dicarbonyls (Fig. 4, Route B) (Glomb & Monnier, 1995). We hypothesized that a relevant contribution for d-AGEs formation in WP/glucose model system was provided by Wolff and Namiki pathways.

Therefore, the effect of cocoa melanoidins on the formation of GO and MGO during the incubation of glucose was investigated. After 21 days of incubation, the concentration of formed GO in the absence of cocoa melanoidins was higher than that of MGO, reaching 62.79 and 6.79 μM, respectively (Table S4). When glucose was incubated with cocoa melanoidins, the concentration of GO and MGO was considerably reduced to 5.12 and 0.52 μM, respectively. Even though cocoa melanoidins can directly scavenge GO and MGO, previous studies reported that the sequestration of transition metals is effective to inhibit α-dicarbonyl formation from glucose (Chetyrkin et al., 2011). Thus, the ability of melanoidins to block oxidative pathways via chelating metal ions (Morales et al., 2012) and their antioxidant activity (Fig. S1) can be two factors in keeping under control both Namiki and Wolff pathway as both routes require the presence of oxygen and transition metals, such as

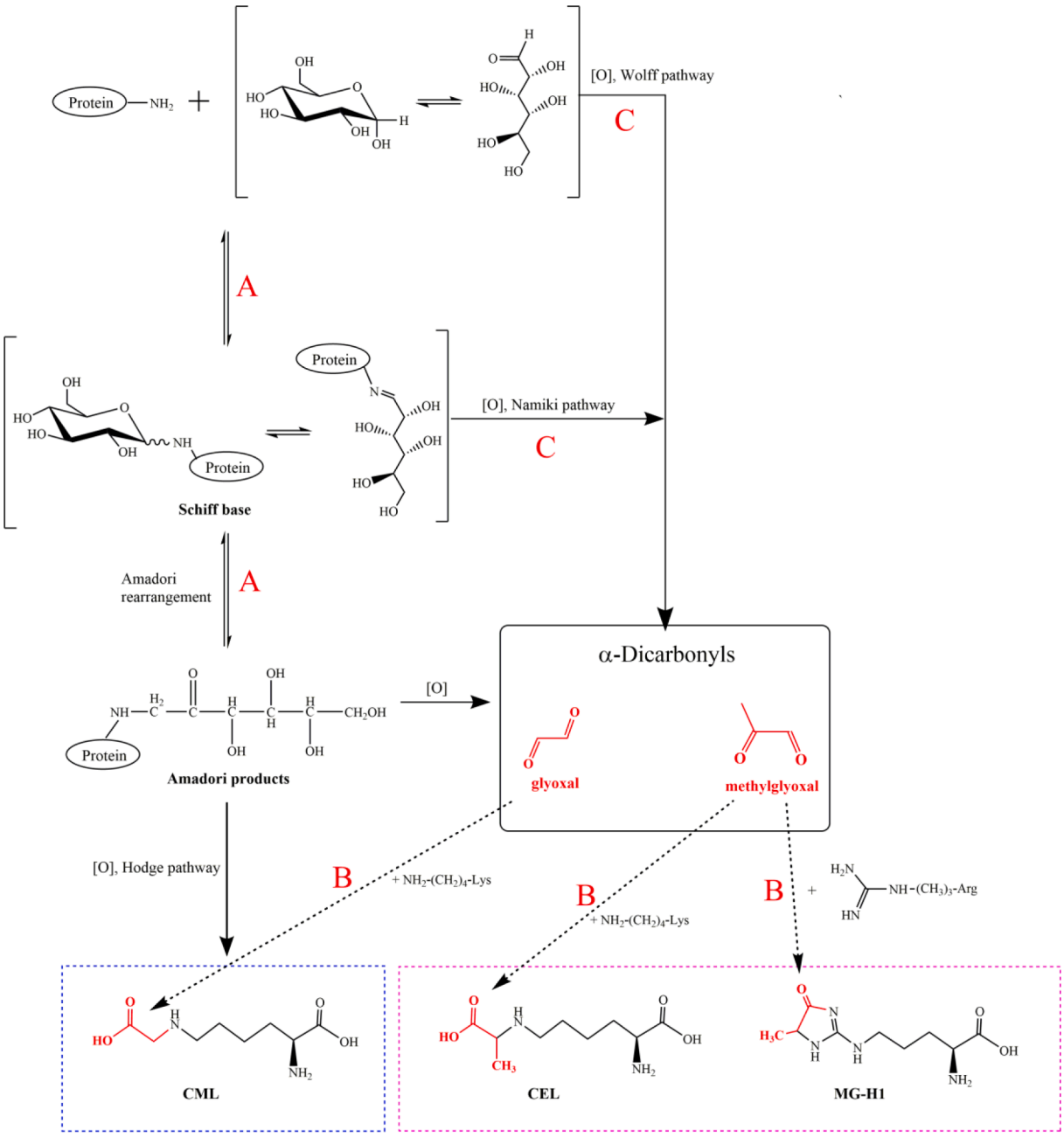

Fig. 4. Proposed mechanisms for inhibition of cocoa melanoidins on the formation of *N*-ε-carboxymethyllysine (CML) and methylglyoxal-hydroimidazolone (MG-H1): A, decrease of protein-bound Amadori compounds; B, dicarbonyl compounds scavenging; C, blockage of oxidative fragmentation of Schiff bases and sugar.

copper and iron. (Fig. 4, Route C). This can be another explanation of higher antiglycation activity of IR-COM-1 compared to that of other cocoa melanoidins.

4. Conclusion

Our study clearly shows that cocoa melanoidins (at a final concentration of 4 mg/mL) successfully inhibited the formation of CML and MG-H1 in milk-model system during incubation at 35 °C for up to 21 days, and their efficacy was influenced by the roasting conditions of cocoa beans. Based on the determination of *N*-ε-fructosyllysine and monitoring glucose fragmentation, the control of Maillard reaction by cocoa melanoidins is mainly due to: (1) reducing the formation of Amadori products; (2) scavenging α-dicarbonyls such as GO and MGO formed from the degradation of glucose and Maillard reaction intermediates, and (3) preventing the oxidation of glucose and Maillard reaction intermediates. While free polyphenols can fast react with α-dicarbonyls, bound polyphenols are a reservoir of bioactive compounds, whose exposure to reactive compounds can be modulated through thermal treatments. Considering multiple reaction routes, we demonstrated that cocoa melanoidins can be a promising tool to control the d-AGE formation in dairy products during storage: this strategy can improve the quality of milk cocoa beverages.

CRediT authorship contribution statement

Hao Zhang: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing - original draft. **Antonio Dario Troise:** Methodology, Formal analysis, Visualization, Supervision, Writing - review & editing. **Hui Zhang:** Project administration,

Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Vincenzo Fogliano:** Methodology, Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128827.

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