



Formation of non-enzymatic protein modifications (nePTMs) on caseins and whey proteins during dry heating and the impact on their *in vitro* infant digestion profile

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ABSTRACT

Thermal treatment during infant formula processing results in the generation of various non-enzymatic protein modifications (nePTMs). In this study, dry heating of a casein or whey protein model system was used to investigate the formation of protein-bound nePTMs after heating from 0 to 72 h. Oxidation, lactosylation, carboxymethylation, 3-deoxyglucosone and hexose modification were the predominant nePTMs, which were found to be protein- and site-specific. Lower solubility of glycosylated caseins resulted in lower *in vitro* digestibility in the gastric phase compared to glycosylated whey protein. After intestinal digestion, the level of carboxymethylation and furosine in the soluble phase increased, especially for 72 h glycosylated casein, which also showed the highest soluble nitrogen level. This study illustrated that the formation of thermal-induced nePTMs of caseins and whey proteins were heating time dependent and linked to digestion efficiency, thus providing novel insights to further understand the effect of nePTMs on proteins' digestion profiles.

1. Introduction

To ensure the microbiological safety and long shelf life of infant formula (IF), multiple thermal treatments are included in its processing. It is widely known that heating processes can induce milk protein denaturation, aggregation and nonenzymatic protein modifications (nePTMs) (Gong et al., 2020; Guo & Huan, 2020). These nePTMs can e.g. change the structure of amino acid side chains and affect functional domains thereby modulating biological and nutritional protein functions, although the modification levels are often low in commercial dairy products (Meltretter et al., 2013). The nePTMs formed in IF include a broad spectrum of chemical modifications which are mainly derived from oxidation, glycation, and glycoxidation of amino acid side chains, due to the presence of lactose and milk proteins, while other mechanisms such as condensation, elimination and deamidation can also occur (Aasmul-Olsen et al., 2024; Lund et al., 2022). Caseins and whey proteins are the two main protein fractions present in milk-based IF. The substantial transformation of these two protein fractions through

heat-induced glycation is structure-dependent. After dry heating, α -lactalbumin was found to be slightly more reactive than β -lactoglobulin for all protein-saccharide combinations, and β -casein showed on average 45 % lower reactivity than the whey proteins (Cardoso et al., 2018). Notably, lysine reactivity to glycation was, as expected, found to increase with increased lysine content but did not possess identical reactivity (Cardoso et al., 2018). Additionally, both κ -casein and α_{s2} -casein contain free cysteine residues, which enable it to form disulphide bonds during heating (Livney & Dalgleish, 2004; Zenker, Teodorowicz, et al., 2020). Therefore, different nePTMs might not be generated uniformly and exhibited protein- and/or site-specific features.

Initially, glycation, also known as the Maillard reaction, causes a reaction between mainly the lysine (Lys) residues of milk proteins with the aldehyde group of reducing sugars, such as glucose or lactose, forming the early glycation product lactulosyllysine and fructose-lysine (FL) (Q. Zhang et al., 2020). At this stage, around 5–20 % of Lys residues can be blocked by Amadori products (AP) in milk-based IF. This blocking of the ϵ -amino groups of Lys residues hinders tryptic digestion as well as

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the action of other digestive enzymes, thereby impairing protein digestibility and decreasing nutritional value by the lowered bioavailability of essential amino acids (Hellwig et al., 2014; Rérat et al., 2002; Wada & Lönnnerdal, 2015). During prolonged heating, the AP degrade to reactive dicarbonyls forming low-molecular heterocyclic compounds, subsequently leading to the formation of advanced glycation end-products (AGEs). Next to glycation, protein oxidation can also be induced during IF production, leading to the formation of methionine sulfoxide, amino adipic semialdehyde, or dityrosine (Baxter et al., 2007; Fenaille et al., 2006; Meltretter et al., 2007, 2008). In the past decade, more than 40 AGEs have been characterized and defined into two forms, one being free-form AGEs on free amino acids, and the other being protein-bound AGEs on amino acids within peptides or intact proteins (Gu et al., 2024). Moreover, several AGE marker compounds have been widely determined for milk products. N ϵ -(carboxymethyl)lysine (CML), N ϵ -(carboxyethyl)lysine (CEL) and pyrroline were found as representative non-crosslinking AGEs while pentosidine and lysine dimers are regarded as important cross-linking AGEs in processed milk (Hegele et al., 2008; Henle, 2005; Q. Zhang et al., 2020).

Up to now, nePTMs in bovine milk protein have mostly been investigated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), or Western blotting combined with various MS techniques (Arená et al., 2010; Mauser et al., 2024; Meltretter et al., 2007, 2013). Further, the combination with enzymatic digestion prior to MS analysis has allowed the identification of different modification types of the proteins and site-specific relative quantification of the glycation products. For example, lactulosyllysine and methionine sulfoxide were found as the most prevalent nePTMs of α -lactalbumin and β -lactoglobulin incubated with lactose in heated model solutions, followed by the cyclization of N-terminal glutamic acid to a pyrrolidone, and the oxidation of Cys and Trp (Meltretter et al., 2007, 2008; Meyer et al., 2011). Recently, untargeted proteomic profiling was applied to identify the novel and unknown modifications of Trp, Cys, His, and Met, combined with lactosylation, carboxymethylation, formylation, glycation of Arg, oxidation, oxidative deamination of N-terminus, and deamidation of Asn and Gln of milk proteins in processed milk (Meltretter et al., 2020). Although site-specific glycation and oxidation modifications were mapped among different milk products, including raw, pasteurized, and UHT milk and infant formula, most studies regarding the effect of MR-modified milk proteins on infants' digestion efficiency are mainly limited to blocked lysine levels. Therefore, a detailed characterization of protein-specific nePTMs of caseins and whey proteins separately is pivotal to understand the changed behaviors of individual milk protein affected by heating and link the effects of various modifications at different sites to digestion efficiency, especially in light of the nePTMs differing with dry heating treatments.

Therefore, the aim of this study was to provide an overview of protein-specific nePTM formed during the heating of caseins and whey proteins, as well as their effects on digestion efficiency. Dry heating was applied to mimic the industrial thermal process of caseins and whey proteins in powdered products. The total CML and furosine were quantified to assess the progress of thermal processing. Then, we characterized nePTMs at both protein and digesta peptide levels. Meanwhile, the detailed effect of heat treatment on digestion efficiency was evaluated by quantifying the release of CML and furosine into the digestive fluid during infant *in vitro* digestion. The newly acquired information may be helpful to understand protein-specific and thermal processing-dependent nePTMs of caseins and whey proteins and their effects on digestion. Our initial hypothesis was that the formation of protein-bound nePTMs is highly heating time dependent and the release of CML and furosine from whole protein to soluble digesta phase after digestion also plays an important role in understanding the digestibility of glycated milk proteins.

2. Materials and methods

2.1. Chemicals

Standard d4-CML, d4-furosine and d4-CEL were purchased from Iris Biotech (Marktredwitz, Germany). Oasis HLB 1 cc Vac Cartridge (10 mg Sorbent per Cartridge, 30 μ m, 100/pk) was obtained from Waters (Massachusetts, USA). Endoproteinase Glu-C was purchased from Sigma-Aldrich (Missouri, USA). ProteaseMAXTM Surfactant, Trypsin Enhancer was obtained from Promega (Wisconsin, USA). PierceTM Quantitative Peptide Assays & Standards was purchased from Thermo Fisher Scientific (Massachusetts, USA). The 3.5 kDa dialysis membrane was purchased from Thermo Fisher Scientific (Massachusetts, USA). All other chemicals were obtained from Sigma Aldrich (Missouri, USA).

2.2. Sample preparation

Fresh bovine milk was collected from Holstein-Friesian cows which were on the campus farm of Wageningen University and Research (Wageningen, The Netherlands). Fat was removed by highspeed centrifuge and excess lactose was removed using 3.5 kDa dialysis membrane tubing, at 4 $^{\circ}$ C, overnight. A tangential flow filtration (TFF) system assembled with pellicon[®] 2 mini filter module (0.1 μ m pore size) was used to separate native casein and whey protein as follow: 1) concentrating process: 450 mL raw skim milk was concentrated 2 times, then 150 mL (1 diafiltrate volume (DV)) retentate was collected as concentrated milk while 300 mL filtrate (2 DV) was collected as whey protein fraction; 2) dialysis process: 1 DV SMUF (simulated milk ultra filtrate) solution was added after collecting each 1 DV of filtrate, which was repeated 4 times until 150 mL concentrated native casein was collected. All the filtrate was regarded as whey protein fraction. Retentate and filtrate fractions were named as casein and whey protein fraction, respectively. Protein and lactose content of the protein fractions was determined by Milkoscan, to calculate the protein-to-lactose ratio. After being lyophilized, the two protein fractions were mixed with lactose, to change the protein-to-lactose ratio to 1:4 (w/w), after which they were evenly grinded by Cryo-miller. Then the mixture was put into a desiccator at a_w 0.60 (saturated KI solution) and incubated at 60 $^{\circ}$ C for 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, and 72 h.

2.3. Protein quantification

For solid samples, 15 mg was weighed for DUMAS analysis, while for liquid samples 200 μ L was weighed in tin cups and dried in an incubator at 60 $^{\circ}$ C for at least 6 h. In both cases, samples were prepared duplicate. Then each tin cup was formed into a ball and protein content was detected by DUMAS Flash EA 1112 Protein analyser (Thermo Fisher Scientific, Massachusetts, USA). Cellulose was taken as blank and 6.38 was taken as nitrogen conversion factor as generally used for milk protein (Atallah et al., 2023).

2.4. Protein dissolution

The glycated whey protein and casein were dissolved in SMUF (Jenness & Koops, 1962) at a ratio of 1:12 (w/v) and left shaking for 1 h at room temperature. The soluble phase (supernatant) was collected after the samples were centrifuged at 14,000 RCF, 20 $^{\circ}$ C for 15 mins. Afterward, the supernatant was filtered through a 0.45 μ m cellulose acetate syringe filter, while the insoluble phase (pellet) was washed with SMUF for 3 times. Both the pellet and supernatant were lyophilized and kept at -20 $^{\circ}$ C for further use.

2.5. *In vitro* infant digestion

The 0 h, 6 h and 72 h glycated casein and whey protein samples were chosen for infant *in vitro* digestion (Ménard et al., 2018). The meals

consisted of 1.2 % (w/v) protein and were preheated for 10 min at 37 °C prior to simulated gastric and intestinal digestion. Briefly, in the gastric phase (GP), pH was adjusted to 5.3 while the pepsin activity was set to 268 U/mL digest. The meals were incubated for 60 mins at 37 °C under continuous shaking (25 rpm) in an incubator (Venti-Line, VWR, Radnor, Pennsylvania, USA). In the intestinal phase (IP), the pH was adjusted to 6.6 and trypsin activity in the final digest was adjusted to 16 U/mL. The meals were incubated for 60 mins under the same conditions as in the GP. Samples were taken after 0, 10, and 60 mins in the GP, and 10 and 60 mins in the IP for further analysis. Enzyme activity was stopped by increasing the pH to 6.6 (1 M NaOH) at the end of the GP and by adding 50 µL (0.1 M) pefabloc per mL sample at the end of the IP. Each digestion experiment was performed in duplicate. Enzyme controls were taken containing all substrates of the chosen time point during GP and IP, but samples were replaced by an equal amount of water.

2.6. SDS gel electrophoresis (SDS-PAGE) of undigested and digested samples

As for undigested samples, each sample was diluted to achieve 11.9 µg protein concentration in each gel lane by diluting to a ratio of 4/5/2/10 (v/v/v/v) of the sample, NuPAGE® LDS sample buffer 4× concentrated, NuPAGE® Sample Reducing Agent, and MilliQ. Buffer consisted of 10 mM PBS (pH 7.4), 1 % SDS and 6 M urea, which was used to dissolve the lyophilized insoluble part, while the lyophilized soluble part of glycosylated casein and whey protein was dissolved in SMUF. As for the digesta samples, around 61–66 mg pellet of digests taken after 0, 10, and 60 mins in the GP, and 10 and 60 mins in the IP were dissolved in 25 µL NuPAGE LDS sample buffer (4× concentrated, Invitrogen, USA), 10 µL NuPAGE sample reducing agent (10× concentrated, Invitrogen, USA), and 65 µL Milli-Q water for SDS-PAGE analysis. A Mark 12™ unstained molecular weight (MW) marker was used as standard. Ten microliters of each sample were loaded on a 12 % bis-tris precast polyacrylamide NuPAGE, 1.0 mm, 12-well precast protein gel (Invitrogen, Carlsbad, USA). NuPAGE MOPS running buffer was used to separate the proteins based on their molecular weights at 180 V (constant mode). Gels were stained with Coomassie Brilliant Blue R-250 staining solution and washed with 10 % (v/v) ethanol and 7.5 % (v/v) acetic acid washing solution. The images of each gel were obtained using Image Lab 4.1 software (Biorad, Hercules, USA).

2.7. Degree of hydrolysis

To determine the degree of hydrolysis, 0.5 mL supernatant of digesta was added to 0.4 mL cooled 10 % trichloroacetic acid (TCA) (w/v), vortexed and centrifuged at 10,000 g for 30 mins. No dilutions were needed for supernatants of TCA precipitated undigested meals as well as digested meals in the GP, while meals in the IP were diluted 3 times by MilliQ. Then, 10 µL of the supernatant with 200 µL OPA working agent was incubated in the dark for 15 mins and measured at a wavelength of 340 nm by a microplate reader (Tecan, Meannedorf, Switzerland) according to Mulet-Cabero, Rigby, Brodtkorb, & Mackie (2017). OPA working solution was prepared according to: 3.81 g sodium tetraborate dissolved in approximately 80 mL water. Once dissolved, 0.088 g dithiothreitol (DTT) and 0.1 g sodium dodecyl sulphate (SDS) were added. Then, 0.080 g OPA dissolved in 3 mL ethanol was placed in the solution which was finally made up to 100 mL with HPLC grade water. L-serine (dissolve in 10 mM PBS) was used to obtain a calibration curve in the concentration range between 0.4 and 4 mM. The digestibility was calculated by the formula:

Degree of hydrolysis =

$$\frac{\text{NH}_2(\text{final}) \times \text{Volume}(\text{final}) - \text{NH}_2(\text{initial}) \times \text{Volume}(\text{initial})}{\text{NH}_2(\text{acid}) \times \text{Volume}(\text{acid}) - \text{NH}_2(\text{initial}) \times \text{Volume}(\text{initial})} \times 100 \%$$

where NH₂ (final) is the concentration of free amino groups in digesta sample, NH₂ (initial) is the concentration of free amino groups in the undigested sample (at digestion time 0), and NH₂ (acid) is the total content of free amino

groups in the sample (0.06 g protein) after acid hydrolysis in 6 M HCl for 24 h at 110 °C (Zenker et al., 2020). Each sample was measured in technical triplicates.

2.8. LC-MS analysis of AGEs

Lysine, furosine, CEL and CML levels were determined using liquid chromatography coupled to triple quadrupole mass spectrometer (LC-MS/MS, series: LCMS-8050, Shimadzu Corporation, Kyoto, Japan) by using the method with some modifications (Troise et al., 2016). Briefly, samples consisting of 15 mg protein (based on DUMAS results) were mixed with 4 mL 6 M HCl and heated at 110 °C for 22 h in a heating block for acid hydrolysis with nitrogen saturated in the headspace to avoid interference from oxidation. After acid hydrolysis, 40 µL of the hydrolysates were filtrated by 0.22 µm filter and 40 µL filtrate was dried under nitrogen and reconstituted in 1 mL ultrapure water. Subsequently, solid phase extraction was applied to extract the target compounds. Afterwards, extracted samples were dried under nitrogen and reconstituted by 1 mL 50 % (v/v) acetonitrile. After being diluted below 1 ppm (0.01 ppm to 1 ppm), 190 µL sample was mixed with 10 µL 10 ppm internal standard. The internal standard was 10 ppm of mixed d4-lysine, d4-furosine, d4-CEL and d4-CML. Calibration standards were 190 µL 0.001–1 ppm of mixed lysine, furosine, CEL and CML, mixed with 10 µL internal standard. For analysis, the UPLC unit consisted of a SIL-30 AC autosampler, a LC-20ADXR solvent delivery module, DGU-20ASR degassing unit, a CTO-20 AC column oven and a FCV-20AH2 valve unit. The samples (5 µL) were injected on an Atlantis™ Premier BEH Z-HILIC 1.7 µm, 2.1 mm * 100 mm (Waters Chromatography B.V., Etten-Leur, the Netherlands) connected to an Atlantis™ Premier BEH Z-HILIC 1.7 µm, VanGuard™ fit cartridge 2.1 mm * 5 mm (Waters Chromatography B.V., Etten-Leur, the Netherlands). The flow rate was set at 0.4 mL/min and the column temperature at 30 °C. The mobile phases consisted of 0.1 % formic acid (solvent A) and acetonitrile with 0.1 % formic acid (solvent B) with the following elution profile (t in [min]/[%B]): (0/90), (1.10/90), (3.5/40), (6.5/40), (8.0/80) and (13.0/80). MS data was collected for 13 mins. Positive ionization mode was used for all MS analyses. The voltage of the turbo ion-spray ionization was 4.0 kV. The temperature of electrospray ionization probe, desolvation line, and heat block were set at 300 °C, 250 °C, and 400 °C, respectively. The pressure of the collision-induced dissociation gas was 4 kPa whereas the flow rates of the drying gas, nebulizer gas, and heating gas were set at 10 mL/min, 3 mL/min, and 10 mL/min, respectively. The electrode voltage of Q1 pre bias (collision cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre bias (collision cell energy exit potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were optimized using the flow injection analysis of standard compounds (20 ppm) in water:ACN (1:1 v:v) using the support software (Shimadzu Corporation, Kyoto, Japan). The most abundant fragment ion was selected for quantitation and the second and third abundant fragments in ion yield were selected as structural confirmation based on the optimized single reaction monitoring transition (for details, see Supplementary Table 4). Calibration curves were linearly regressed as peak area ratio of standards/internal standard against corresponding standards' concentration. Lysine, furosine, CEL and CML were quantified according to the calibration curves and their peak area ratio (analytes/internal standards). The final content was shown as µg/g protein for lysine, furosine, CEL and CML.

2.9. Analysis of protein-bound nePTMs

2.9.1. Endoproteinase hydrolysis

For the analysis of peptide-bound nePTMs all glycosylated samples (from 0 to 72 h) were first dialyzed by 3.5 kDa dialysis membrane for 24 h to remove buffer salts and lactose (Mauser et al., 2024). Then, the retentates were lyophilized and dissolved in 0.1 % ProteaseMAX-solution at 5 µg/µL. Later, 1 µL of 0.2 M DTT and 2 µL iodoacetamide-solution were

added for reduction and alkylation of cysteines. Furthermore, the protein samples (40 μL each) were transferred to Eppendorf tubes and hydrolyzed by the addition of 20 μL of endoproteinase GluC (0.2 $\mu\text{g}/\mu\text{L}$) in 100 mM Tris-HCl-buffer (pH 7.8). The tubes were shaken in an Eppendorf thermo mixer for 16 h at 37 $^{\circ}\text{C}$. Following 30 min of incubation with 2 μL 100 % formic acid at room temperature for stopping the enzymatic hydrolysis, 12,000 rpm centrifugation was applied to separate the Protease-MAX by-products. Under these conditions, 95–100 % of the cleavage sites in β -lactoglobulins were hydrolyzed, which was monitored by targeted analysis of peptides with missed cleavages in full scan chromatograms.

2.10. Targeted MicroLC-IM-QTOF analysis

All measurements were performed on a Dionex Ultimate 3000 RS UHPLC system (Thermo Fisher Scientific, Dreieich, Germany) coupled online to an electrospray ionization (ESI) source and a trapped ion mobility time-of-flight mass spectrometer (timsTOF Pro, Bruker Daltonics, Bremen, Germany) (Mausser et al., 2024). A reversed phase C18 capillary column (YMC-Triart C18, 500 $\mu\text{m} \times 100 \text{ mm}$, 3.0 μm particle size, 12 nm pore size, YMC Europe, Dinslaken, Germany) was applied with an injection volume of 5 μL for Glu C digestion samples and 2 μL for *in vitro* digesta samples. A flow rate of 30 $\mu\text{L}/\text{min}$ was used with the following gradient: 15 min 2 % B, 5 min 2 % B, 65 min 45 % B, 65.5 min 95 % B, and 75 min 95 % B, 75.5 min 3 % B, and 90 min 2 % B. Eluent A was 0.1 % formic acid in ultrapure water (18.2 M Ω , Milli-Q Synergy, Merck, Darmstadt, Germany) and eluent B was acetonitrile with 0.1 % formic acid (Ultra-LC-MS grade, Carl Roth). The first 2 min after injection were diverted into the waste. The column oven was kept at 35 $^{\circ}\text{C}$. All MS experiments were carried out in positive mode with the following source parameters: end plate offset 500 V, capillary voltage 4500 V, nebulizer gas pressure 0.5 bar, dry gas flow was 5.0 L/min, and dry gas temperature 190 $^{\circ}\text{C}$. In the Funnel 1, the Deflection Delta was set to 70.0 V and RF to 350 Vpp. In Funnel 2 and in the Multipole the corresponding RF were set to 200 Vpp. In the collision cell, collision energy was set to 10 eV and Collision RF was 1500 Vpp. The ion energy in the quadrupole was set to 5.0 eV. Ions were detected in PASEF mode. The resulting data were processed applying label free quantification with the software-assisted database search tool PEAKS Online (Bioinformatics Solutions Inc., Waterloo, Canada). A targeted nePTMs database (53 nePTMs) was also included in the DB algorithm searches. Peptides that featured only carbamidomethylation of cysteine were considered as unmodified. The database was first chosen as “Uniprot-SwissProt” with the taxon “*Bos taurus* (cow)” and all the signal peptides in databases were removed. A contaminant database was applied as published elsewhere and was manually adapted to account for Glu-C autoproteolytic peptides. (Frankenfield et al., 2022).

2.11. Statistical analysis

GraphPad Prism 10.1.2 (GraphPad Software, LaJolla, CA) was used for statistical analysis unless otherwise indicated. For peptide-bound nePTMs analysis, data processing and graphs creation were performed by Python 3.12.4 (<https://www.python.org>). In addition, R package of ALASCA was used for further multivariate data analysis, which is available from (<https://github.com/andjar/ALASCA>). Data manipulation of nePTMs analysis by proteomics: The peptides with only one of the triplicates showing the area data will be taken as missing data and be excluded. The sum of the area of peptides detected for a specific protein in each injection is taken as 100 %. Firstly, LFQ was applied to quantify the peptides (modified and unmodified) common to all groups. Secondly, the abundance of a specific modification was calculated by dividing the sum of the peak areas of peptides containing the specified modification by the sum of all peptides contained in the specific protein. Finally, the percentage of each nePTMs were used to do statistics analysis. Raw data was normalized by recalculating into a percentage to

make sure the difference in abundance of each protein between each group can be narrowed down since the samples were present in a matrix without standardizing the abundance of all specific proteins.

3. Results and discussions

3.1. The impact of heat treatment on milk protein properties

Dry heating of casein and whey protein in the presence of lactose led to changes in the protein structure that affected their solubility. It was obvious that the color of both glycosylated casein and whey protein gradually turned to dark brown with heating times increasing from 0 to 72 h (Supplementary Fig. 1). Moreover, the color unevenly distributed between the supernatant and pellet of the samples. The brown color was predominantly observed in the pellet of glycosylated casein while in the case of glycosylated whey protein, it was mainly observed in the supernatant. This phenomenon might be due to the protein-dependent solubility of brown nitrogenous polymers and copolymers after dry heating in the presence of lactose. To test this, the nitrogen level of soluble and insoluble fractions was quantified as shown in Supplementary Fig. 1. Glycosylated casein samples had 90 % insoluble nitrogen, which was much higher than for glycosylated whey protein (around 50 %). This indicates that the solubility of glycosylated protein is highly protein-structure-dependent. Whey proteins generally have higher solubility because of the large number of surface hydrophilic residues (Mavropoulou & Kosikowski, 1973). In contrast, the glycosylated caseins present in the pellet of this sample may have low solubility due to the difficulty of casein micelles in general to resolubilize, as has earlier been shown (Zenker, Raupbach, et al., 2020). In addition, during prolonged heating, the soluble nitrogen content of glycosylated casein increased a little, whereas the soluble nitrogen content in glycosylated whey protein samples decreased with heating time. Both changes might be due to the Maillard reaction (MR)-induced molecular interactions. Whey proteins could react with lactose leading to the formation of high molecular weight polymers, resulting in increased insolubility. In addition, the low temperature at 60 $^{\circ}\text{C}$ combined with the low a_w at 0.6 used in this study favored MR over protein unfolding. On the other hand, the solubility of caseins might be promoted by connecting hydrophilic sugar groups to free amino groups (Kato et al., 1992). To further understand the effect of heating on the protein compositions of the soluble and insoluble protein fractions, SDS-PAGE was applied, as shown in Fig. 1.

According to Fig. 1A, representing glycosylated caseins, two dominant bands corresponding to α -lactalbumin (~14 kDa) and β -lactoglobulin (~18 kDa) were visible. This might be due to small amounts of whey protein that cannot be completely removed by the TFF filtration method. With longer heating, the bands of κ -casein (22 kDa), α_{s1} -casein and β -casein (both ~26 kDa), and α_{s2} -casein (29 kDa) gradually appeared in the soluble fraction, with their intensity increasing. This result was coherent with the increasing soluble nitrogen level for longer heated glycosylated casein samples (Supplementary Fig. 1). However, the intensity of the bands of monomeric α_{s1} -casein and β -casein, α_{s2} -casein, and κ -casein (22 kDa), as well as insoluble aggregates (> 93 kDa) in the insoluble fraction of glycosylated caseins was higher compared to those in soluble fraction, as shown in Fig. 1C. This result agrees with the high insoluble nitrogen level. In both soluble and insoluble fractions of glycosylated whey proteins, the band intensity of α -lactalbumin and β -lactoglobulin decreased while the aggregates, which probably consist of α -lactalbumin and β -lactoglobulin and/or their aggregates, gradually increased until 72 h (Fig. 1B and D). The trapping of material in the SDS-PAGE wells occurred in both glycosylated caseins and whey proteins, especially after 24 h, indicating that higher levels of glycosylation led to increased aggregation. The potential molecular interactions involved in the aggregation during glycosylation are highly heat treatment dependent. Besides, hydrophobic interactions and hydrogen bonds might also lead to aggregation, causing a loss of solubility during the heat treatment, while the covalent interactions caused by MR-induced cross-linking

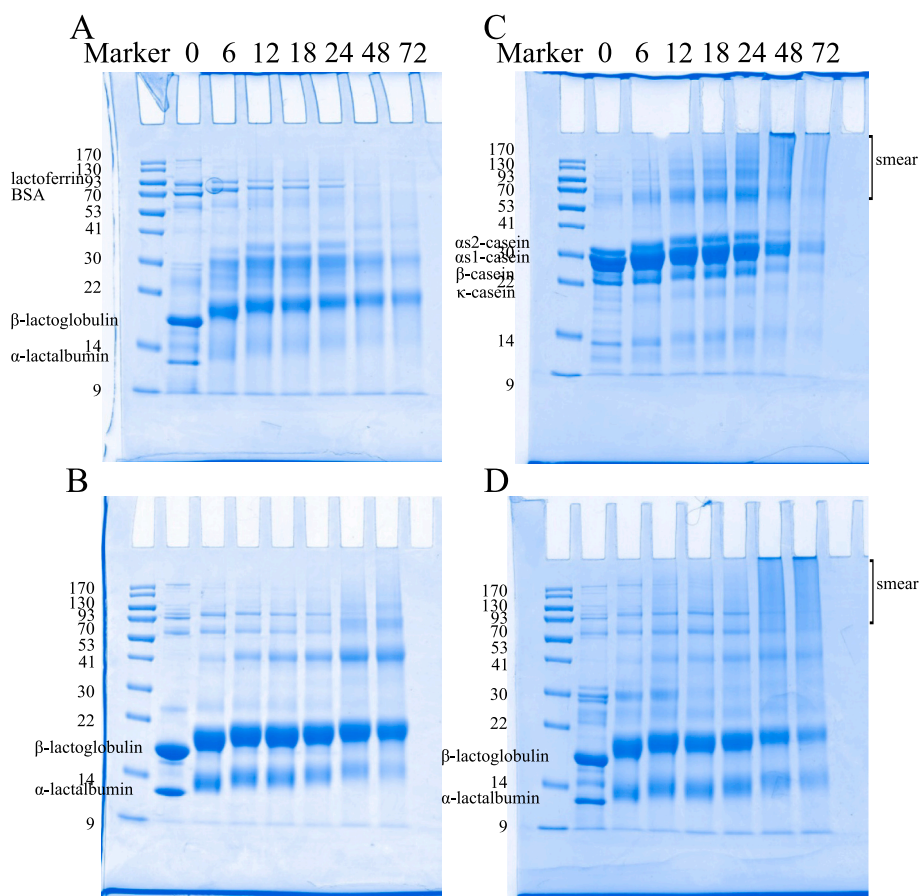


Fig. 1. The change of soluble (A) and insoluble (C) protein compositions of glycosylated casein as well as soluble (B) and insoluble (D) protein compositions of glycosylated whey protein after dry heating.

might lead to insolubility during prolonged heating (Fan et al., 2018; Zenker, Raupbach, et al., 2020). Overall, the contribution of glycation to the decreasing solubility appeared to be heat treatment dependent, which was in line with previous findings suggesting that the glycation-induced crosslinking, but also disulphide bond formation, hydrogen bonds and hydrophobic interactions can all contribute to loss of solubility in glycosylated samples (Zenker, Raupbach, et al., 2020). Therefore, several representative markers were quantified to determine the effects of the heating process.

3.2. Furosine, lysine, CML and CEL levels in insoluble and total fractions

To determine the MR levels in the insoluble and total fractions, the furosine, Lys, CML and CEL levels were quantified. Fig. 2 illustrates the progress of glycation by showing the total abundance of these markers of glycation. The extremely low level of CEL in both casein and whey proteins indicates that CEL is not the primary AGE formed in milk proteins during thermal processing. In the case of caseins, the Lys level decreased with a concomitant increase in CML and furosine, especially during the first 12 h (Fig. 2A-D). The furosine level exhibited a significant increase during the first 6 h and reached up to 951 $\mu\text{g/g}$ protein while the CML level increased significantly from 24 h and reached up to 308 $\mu\text{g/g}$ protein ($p < 0.05$). The same pattern could be seen in glycosylated whey proteins (Fig. 2E-H). Notably, CML and furosine levels increased significantly as Lys levels decreased during the first 6 h ($p < 0.05$), occurring earlier than the increase observed in caseins, where CML level significantly increased after 24 h. This might be due to the higher reactivity of whey protein towards dry heating than casein, as it has higher Lys levels. However, the maximum level of CML and furosine of glycosylated whey protein, 113 and 1018 $\mu\text{g/g}$ protein, respectively, were

lower than those of glycosylated casein, possibly due to the higher levels of lysine in casein. The CML and furosine levels in the insoluble fractions displayed a similar tendency, but at far lower levels than those seen in the whole sample. It could be concluded that CML and furosine exist mostly in the soluble fraction, in a soluble protein-bound form. To better understand the impact of dry heating on glycosylated casein and whey protein samples, more in-depth identification of protein-bound nePTMs was performed, as described in the next section.

3.3. Analysis of protein-bound nePTMs

Heating causes various kinds of chemical changes in proteins, such as peptide bond cleavage, dephosphorylation, racemization, oxidation, β -elimination, disulfide exchange, Maillard reactions in the presence of sugars, and last but not least deamidation of the amino acids Gln and Asn (Metwalli & Van Boekel, 1998). To better understand the relationship between nePTMs and extent of dry heating at the protein level, endo-proteinase GluC was applied to specifically cleave proteins C-terminally to glutamic acid and aspartic acid. In total, there were 17 nePTMs identified in glycosylated caseins while 14 nePTMs found in glycosylated whey proteins. Here, we only focused on glycation-related nePTMs detected in α_{S1} -, α_{S2} -, β - and κ -casein as well as α -lactalbumin and β -lactoglobulin for further data analysis.

To better understand the data related to the changes of each nePTM, ALASCA was applied. ALASCA is a concept of the extended ANOVA simultaneous component analysis (ASCA+) framework combined with general linear models and principal component analysis (PCA) to decompose and visualize the separate effects of experimental factors (Jarmund et al., 2022; Madssen et al., 2021). The ALASCA analysis of glycosylated caseins is shown in Supplementary Fig. 2, with principle

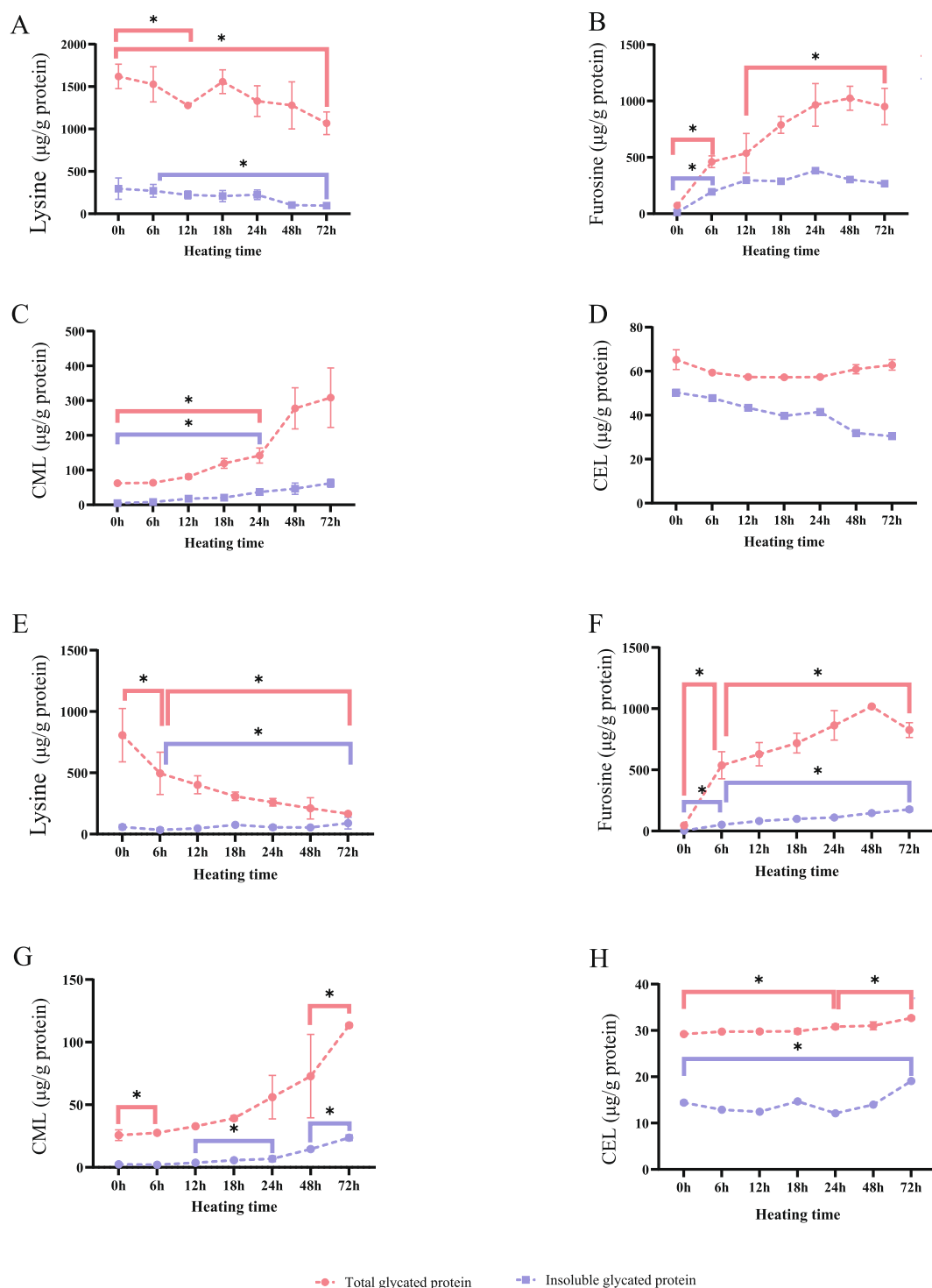


Fig. 2. The changes of lysine, furosine, CML and CEL level of glycated casein (A) to (D) after dry heating; The changes of lysine, furosine, CML and CEL level of glycated whey E) to H) after dry heating.

component 1 (PC1) and 2 (PC2) contributing 69 % and 23 %, respectively. PC1 showed a monotonic increasing trend over time with a rapid increase after 24 h (Supplementary Fig. 2). Notably, protein-bound oxidation, lactosylation, carboxymethylation, 3-deoxyglucosone (3-DG) modification, and hexose modification, showed large positive loadings on the first component (Supplementary Fig. 2). The positive contributions of these nePTMs to PC1 indicate their increase over time. Similarly, the ALASCA analysis of glycated whey proteins is shown in Supplementary Fig. 3, with component 1 and 2 contributing 82 % and

9.8 %, respectively. PC1 showed a continuous increase until 18 h followed by fluctuations until 72 h (Supplementary Fig. 3). This non-linear trend after 18 h was coherent with the data that several nePTMs didn't show continuous changes over time (as showed below). Besides, oxidation, lactosylation, carboxymethylation and hexose modification showed strong positive loadings on PC1 (Supplementary Fig. 3). In light of the loading scores of PC1 from the ALASCA analysis, relevant nePTMs were selected and discussed with regard to their presence on individual proteins in the next section.

3.4. Protein-bound nePTMs of caseins and whey proteins

The changes of nePTMs derived from α_{S1} -, α_{S2} -, β - and κ -casein over heating time can be found in Fig. 3 while for α -lactalbumin and β -lactoglobulin those can be found in Fig. 4. Here, we discuss the changes of specific nePTMs with heating time based on the early stage, middle stage and advanced stage of glycation, including the concomitant effect of oxidation.

3.4.1. The protein-bound nePTMs markers at the early stage

Lactosylation is known as an important indicator of the early stage of milk protein glycation. Here, the lactosylation was found as: N-terminal (Lactosylation N-terminal), Lys and Arg (Lactosylation (KR)), Cys (Lactosylation (C)) and Trp (Lactosylation (W)). The highest abundance was found for lactosylation (KR), followed by N-terminal lactosylation. Lactosylation (KR) in α_{S2} -casein increased steeply, up to 30 %. As for the N-terminal lactosylation, the highest level was observed in β -casein after 12 h, being 4 % (Fig. 3). Lactosylation of Trp occurred mostly in α_{S2} -casein while lactosylation of Cys was mostly found in κ -casein after 18 h (Fig. 3). Differences in lactosylation levels observed among individual caseins might be due to the limitation of number of accessible amino groups. Moreover, the decrease of N-terminal lactosylation might indicate the conversion from lactosylation to further AGEs due to the decomposition of Amadori compounds, reacting with free amino groups via Strecker degradation (Kastrup Dalsgaard et al., 2007). In terms of glycated whey proteins, lactosylation (KR) was mostly present in β -lactoglobulin with a maximum level of around 17.5 % after 12 h while that of α -lactalbumin was only around 1.5 %. In addition, lactosylation on N-terminal, Cys, and Trp were also predominantly found in β -lactoglobulin (Fig. 4). This might not only be due to the lower extent of lactosylation for α -lactalbumin compared to β -lactoglobulin being related to the lower numbers of Lys residues, being 15 for β -lactoglobulin and 12 for α -lactalbumin, but also the Lys accessibility during dry heating (Czerwenka et al., 2006). This result indicated that lactosylation of caseins and whey proteins mostly occurred in Lys, Arg and N-terminal sites. In addition, the degradation and conversion of lactose to hexose during dry heating may occur in parallel and/or after lactosylation (Milkovska-Stamenova & Hoffmann, 2016, 2017). As for caseins, hexosylation was equally found in Asn, Ser and Tyr (Hexose (NSY)) and in Asn, Gln and Leu (Hexose (NQL)). However, in terms of whey proteins, hexose were mostly found on Trp, Lys and Ile (Hexose (WKI)) and reached up to 13 % in β -lactoglobulin, which was much higher than Hexose (NSY) (Fig. 4). Overall, lactosylation and hexose are the main nePTMs in both caseins and whey proteins, indicating their important role as key peptide-bound markers for the early glycation stages.

3.4.2. The intermediate and advanced stage nePTM glycation markers

3-DG, one of the α -dicarbonyls, is a C6 reactive intermediate generated via vinylogous β -elimination at C3 of a glucose unit in both Maillard reaction and sugar degradation (Ashraf et al., 2015; Zhang et al., 2019b). Here, 3-DG was mostly found in α_{S1} -casein and increased continuously over heating time, especially after 24 h (Fig. 3). This suggested that α -dicarbonyl formation might be favored during prolonged heating. Notably, reactive α -dicarbonyl compounds and α -amino groups of free amino acids can react to form Strecker aldehydes or the nucleophilic side chains of amino acids (most importantly lysine), which may have led to different AGEs being formed.

As mentioned in section 3.2, total CML as a representative Lys-derived AGE is found abundantly in glycated caseins. Similarly, the protein-bound CML, which were found on Lys, Trp and N-terminal (CML-KWX@N-term), were predominantly observed in α_{S1} -casein, α_{S2} -casein, and β -casein, with a maximum around 6 % after 72 h (Fig. 3). Besides, in the case of glycated whey proteins, the increasing level of CML-KWX@N-term was observed in both β -lactoglobulin and α -lactalbumin, which reached up to 10 % (Fig. 4). The CML could be generated from the Hodge pathway (1), where CML is formed by the oxidation of

Amadori products, and the Namiki pathway (2), where CML is formed by the condensation of the nucleophilic sites on proteins and peptides, with free glyoxal formed from degradation of Schiff bases (W. Zhang et al., 2019). Notably, a few of the CML modifications of glycated caseins were also present on Cys and Lys ("Carboxymethyl (CK)") although the maximum levels were below 0.5 % (Fig. 3). Besides, CML modified Ser, Cys and Gln were found in glycated whey proteins, with the maximum only being 1 % in β -lactoglobulin (Fig. 4). This result indicated that Lys, Trp and N-terminal are the most likely sites for the generation of protein-bound CML in both caseins and whey proteins.

3.4.3. The nePTMs markers of protein oxidation

The essential and semi-essential amino acids of milk proteins are very susceptible to oxidation leading to their degradation and formation of undesirable protein modifications, including protein cross-links, leading to loss of nutritional value. The oxidation of Pro to pyroglutamic acid, dioxidation on Met, oxidation of Cys to cysteic acid were all found to be positively correlated with heating time, especially after 24 h. Meanwhile, dehydration was accompanied by the oxidation reaction. It is noteworthy that the oxidation of Met was mostly found in α_{S1} -casein and increased up to 11 % (Fig. 3). In addition, oxidation of proline to pyroglutamic acid was also mostly found in α_{S1} -casein while the oxidation of cysteine to cysteic acid could only be found in α_{S2} -casein. Besides, in terms of whey proteins, the oxidation of Met was found to mostly occur in β -lactoglobulin and reached up to 10 % while the level of oxidation of Cys to cysteic acid in β -lactoglobulin and α -lactalbumin were lower than 0.4 % (Fig. 4). Although the level of oxidation of Cys to cysteic acid is quite low, Cys is typically the most readily oxidized sulfur amino acid and is therefore the primary target of oxidation, generating cysteic acid. The inherently reactive nature of the sulfur-containing amino acids makes them the major oxidation target for both radical and two-electron oxidants, with relatively high reaction rate constants (Li et al., 2022). Moreover, the high abundance of oxidation occurring in His, Trp and Met, indicates that they are the key amino acids sensitive to the heating-induced oxidation. Additionally, the rapid increase of the alpha-ketoamide N-terminal modification indicates the oxidation of the N-terminal amine, which was observed in α_{S1} -casein at 18 h. However, the examined structure has a highly reactive α -dicarbonyl group and, therefore, probably underwent further reactions resulting in its decrease after 72 h. Notably, deamidation is predominantly observed in β -casein after 24 h and α -lactalbumin after 18 h, which indicates that the hydrolysis process occurred mostly after extreme heating. However, the deamidation level varied among individual proteins, which indicates that the hydrolysis process might have occurred disproportionately due to the type of amide and accompanied with other further reactions (Metwalli & Van Boekel, 1998).

Overall, the heat-induced nePTMs occurred in proteins not only located on the N-terminal or Lys residue, but also on the other residues. Different protein-bound nePTMs were found to different extents on the individual milk proteins. The results indicate that the formation of protein-bound nePTMs is protein specific, and Lys and N-terminal sites were most likely modified during glycation, especially for lactosylation and CML. More importantly, the formation of nePTMs is highly sensitive to the stage of the heating progress. To further understand the effect of dry heating on protein digestion, the digestibility of 6 h and 72 h glycated caseins and whey proteins were studied, as discussed in section 3.5.

3.5. Digestibility and the release of AGEs during digestion

Caseins and whey proteins heated for 6 h (early glycation stage) and 72 h (late glycation stage) were selected for *in vitro* infant digestion, while a non-heated sample was taken as control. Firstly, remaining intact proteins of glycated casein and glycated whey protein samples after *in vitro* digestion were visualized by SDS-PAGE, as shown in Fig. 5. Intact caseins obviously remained present during GP, whereas they were

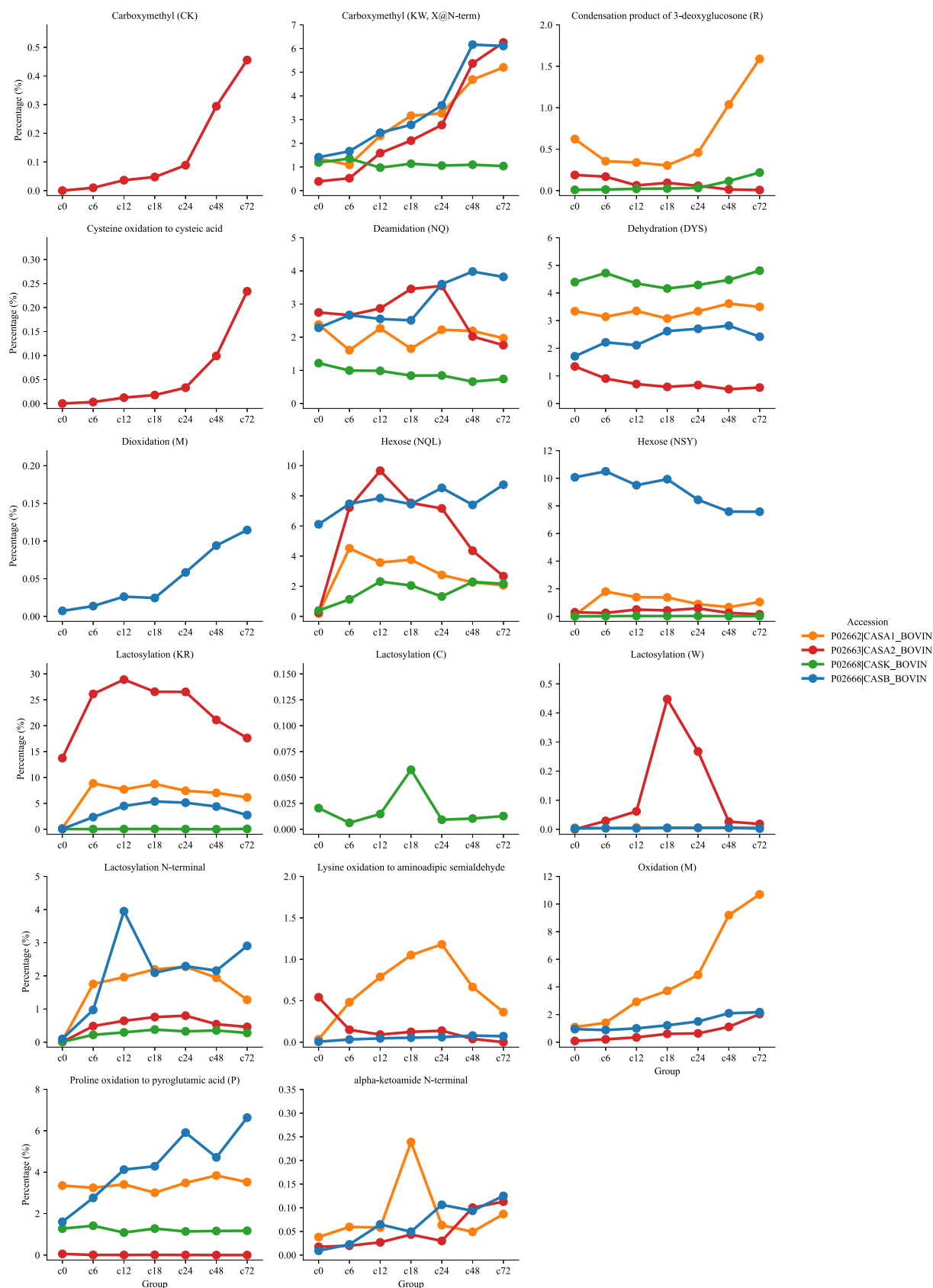


Fig. 3. The changes of glycation-related nePTMs of α 1-, α 2-, β - and κ -casein over heating time.

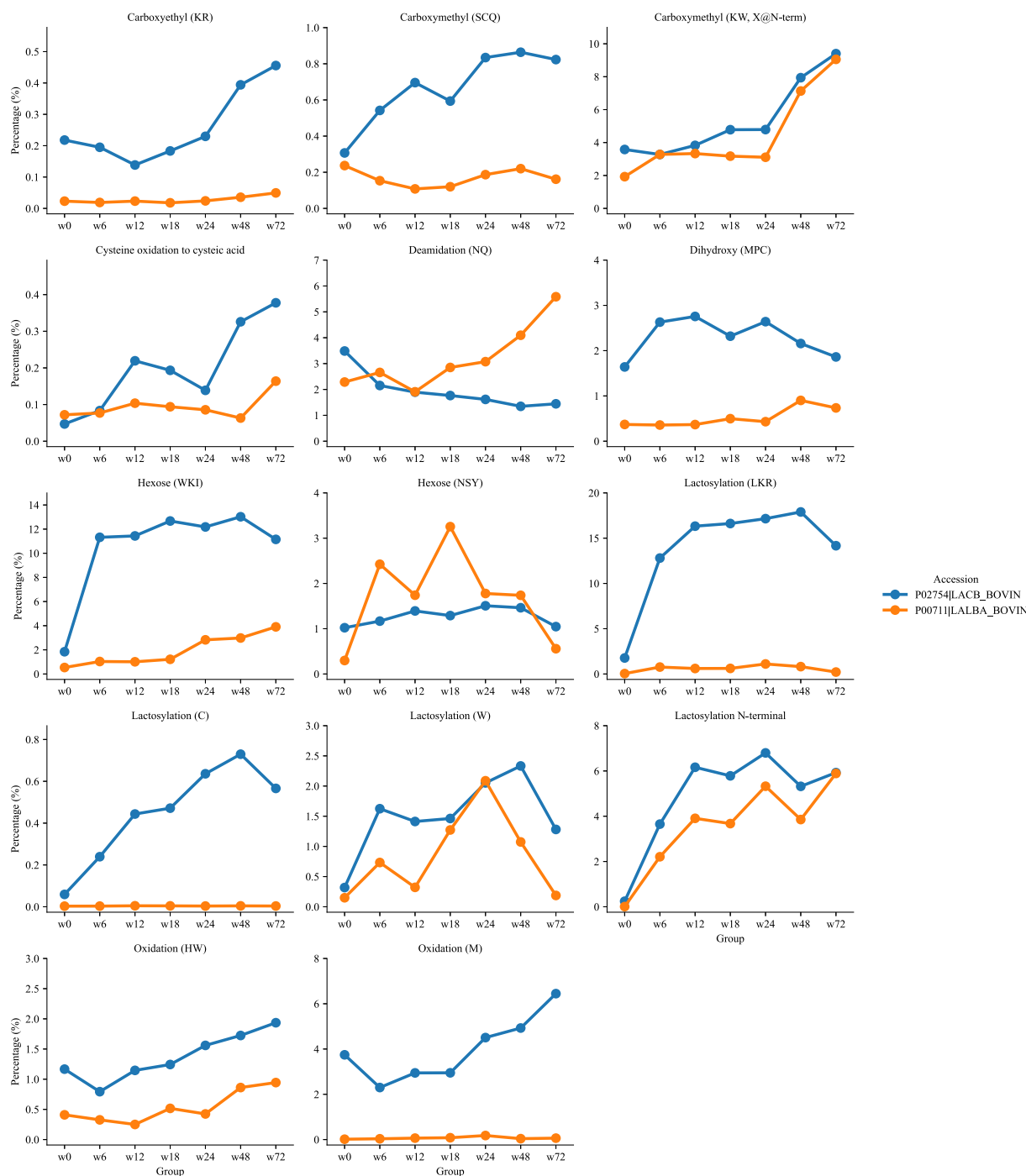


Fig. 4. The changes of glycation-related nePTMs of α -lactalbumin and β -lactoglobulin over heating time.

not visible anymore during IP (Fig. 5A-E). The disappearance of the casein bands was accompanied by the appearance of large peptides of ~ 10 kDa, indicating the formation of large casein-derived peptides during IP. Moreover, a smear of aggregated protein in pellets of 72 h glycated caseins over the whole lane appeared on the gel and more intense protein bands could be found in the pellet of glycated caseins than the control. As for glycated whey proteins, intact β -lactoglobulin and α -lactalbumin remained until GP60, whereas they were not visible anymore during IP (Fig. 5D-F). Meanwhile, bands around 10 kDa, indicating the presence of large whey-derived peptides, remained visible until the end of intestinal digestion. However, there were no intact proteins remaining after IP10, either in the supernatant or the pellet of digesta.

The degree of hydrolysis of all samples increased significantly during the IP, especially the first 10 min (Fig. 6A). At the end of digestion, the highest DH was observed in non-heated casein (50 %), followed by 6 h and 72 h glycated casein (33 %), with a similar pattern seen in the glycated whey protein groups, where the non-heated whey proteins showed the highest DH (88 %) at the end of IP, being significantly higher than that of 72 h glycated whey (40 %) ($p < 0.05$) (Fig. 6B). Notably, during the IP, the soluble nitrogen level of the 72-h glycated casein sample increased more than the other glycated casein samples. However, this wasn't seen in the case of glycated whey proteins. This indicates that the structure of caseins after 72 h heating was mostly damaged by glycation and more accessible to enzymes than the other glycated casein samples, which resulted in the highest level of release of

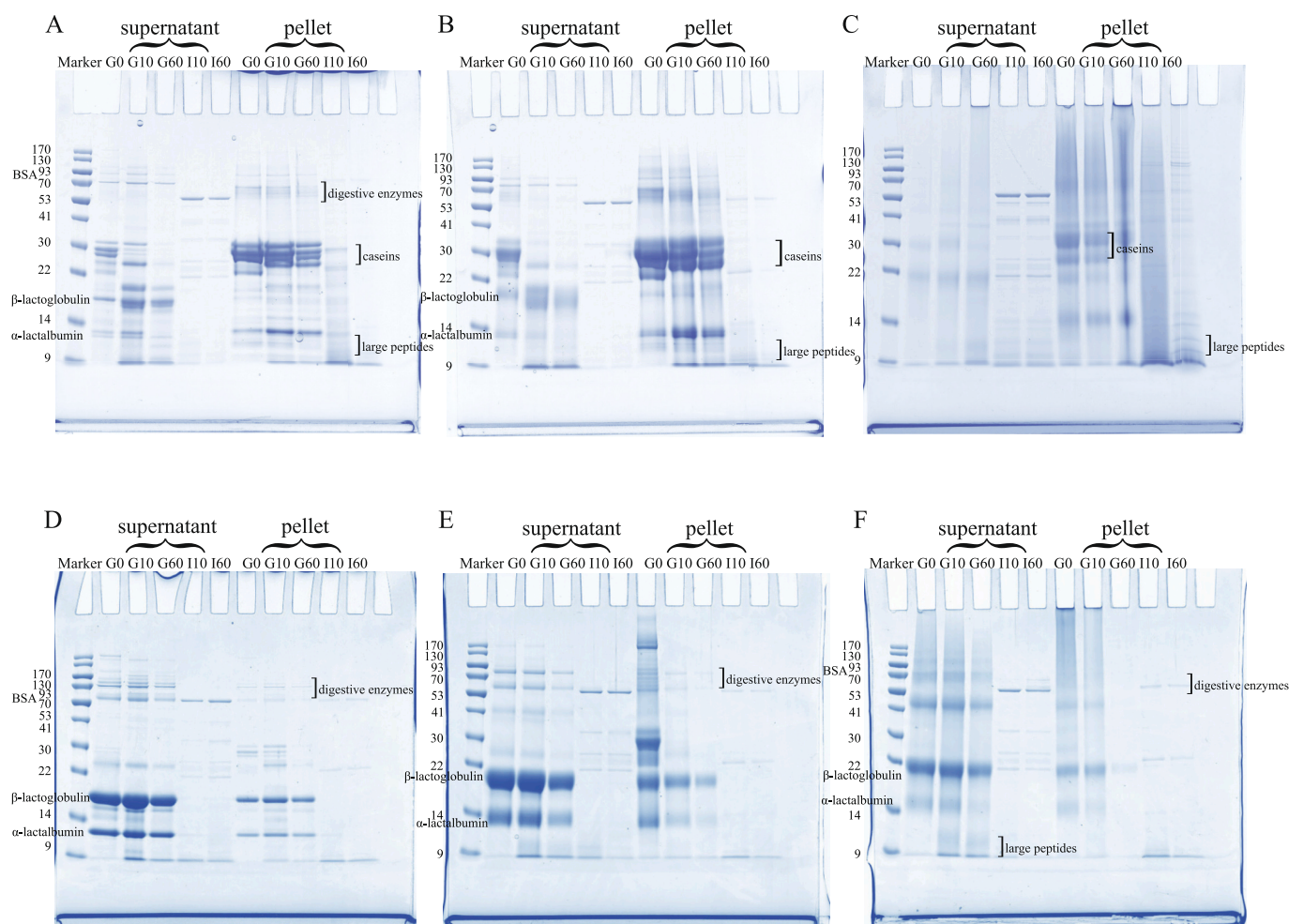


Fig. 5. Digestive protein composition in supernatant and pellet of non-heated (A), 6 h (B) and 72 h (C) glycated casein, and non-heated (D), 6 h (E) and 72 h (F) glycated whey.

the soluble nitrogen from its inner structure. In conclusion, although glycation impaired digestion of both caseins and whey proteins, whey proteins were digested to a larger extent than caseins, as shown by the higher soluble nitrogen level and hydrolysis degree at the end of digestion.

Concomitantly, the release of CML and furosine to the digestive supernatant was highly correlated with the transfer of soluble nitrogen (Fig. 6E and F). In GP0, the lower CML and furosine level in the supernatant of the glycated casein samples was correlated with the lower casein solubility (Supplementary Fig. 1). From GP0 to GP60, there are increases of the furosine and CML level in the supernatant of both glycated caseins and whey proteins. Moreover, there was a significant increase from GP60 to IP10 of 72 h glycated caseins ($p < 0.05$), which agrees with the increase in its soluble nitrogen level. This supports our hypothesis that there is a positive correlation between digestibility and release of glycation products, which is linked to the soluble nitrogen level. Moreover, the intestinal digestion phase might result in a large release of glycation products, especially for long heat-treated caseins. To be noticed, protein-bound nePTMs will also highly affect the release of the digestive nePTMs which might be cleaved to their free forms or short peptides-bound forms. Therefore, future research needs to determine the protein-bound PTM levels in the soluble and insoluble phase separately, to determine where the release of CML/furosine during digestion is coming from.

4. Conclusion

In this study, dry heating was applied to mimic the effect of thermal treatment on milk proteins, especially during infant formula processing. This study provided a comprehensive and systematic overview of the nePTMs in dry heating conditions, which also occurs during the production of infant formula. Moreover, oxidation, lactosylation, carboxymethylation, 3-DG and hexose modifications were found as the main protein-bound nePTMs that positively correlated to the heating time. We also found that the changes of nePTMs with heating time were protein-specific, which possibly is related to the level and accessibility of amino acids, indicating the impact of protein selection in IF production. In terms of heating impact on digestion efficiency, the lower protein solubility might result in lower digestibility, especially during the gastric digestion phase, while the release of CML and furosine from the insoluble pellet to the supernatant mainly occurred during intestinal digestion, which was correlated to the soluble nitrogen levels. Moreover, the impaired digestion efficiency caused by specific AGEs provided new knowledge to understand the impact of heating processes on infant digestion and its relation to nePTMs levels. Overall, this study provides knowledge for understanding protein-, site-specific and heating-dependent protein-bound nePTMs on caseins and whey proteins in a dry heating model, as well as their effect on digestion, which may be useful in designing improved heating processes for infant formula with optimal digestion kinetics.

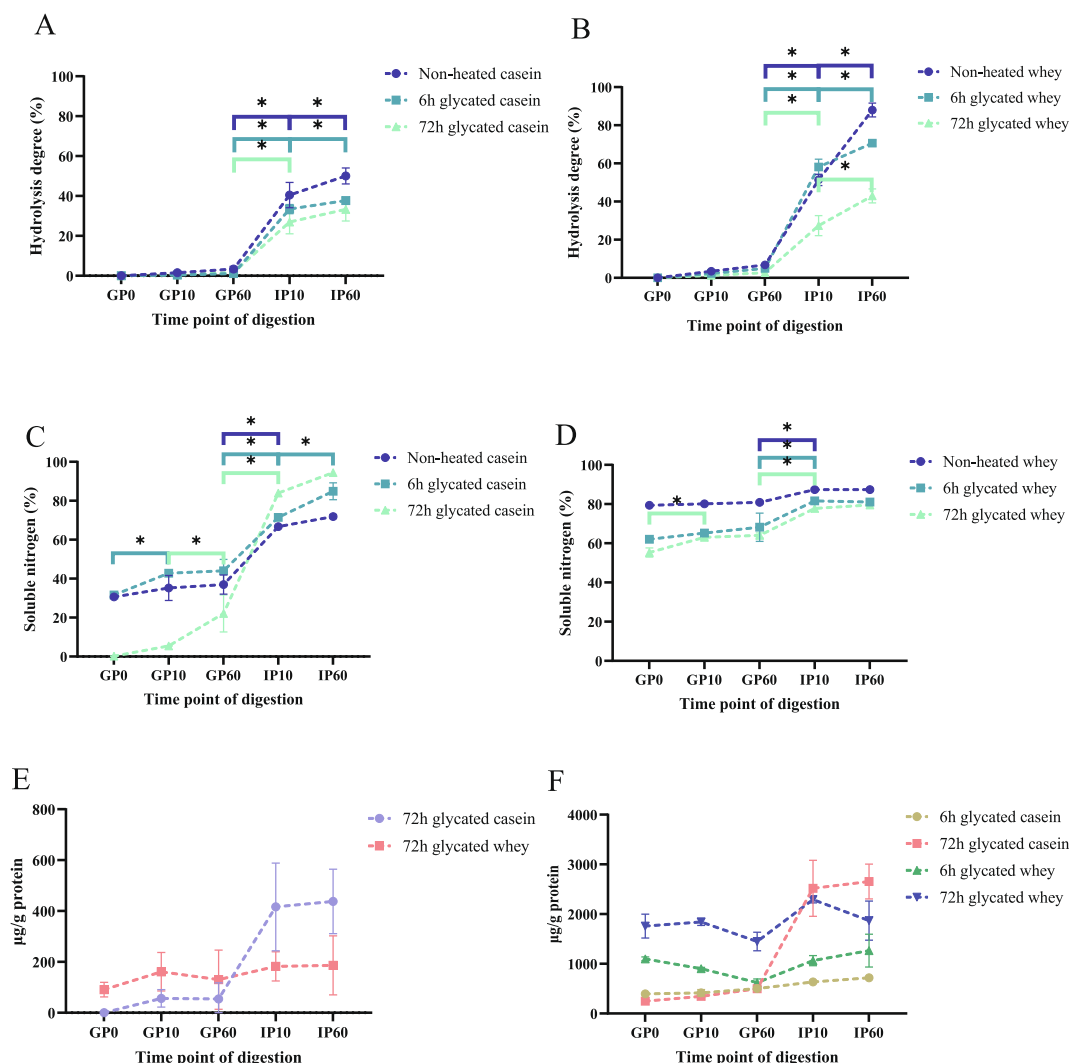


Fig. 6. Digestibility of non-heated protein, 6 h glycated and 72 h glycated protein at each digestion time point. Soluble nitrogen of glycated casein A) and whey B), hydrolysis degree of glycated casein C) and whey D), and the release level of CML E) and furosine F).

CRediT authorship contribution statement

Yifan Liang: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Data curation, Conceptualization. **Hannah Zenker:** Writing – review & editing, Visualization, Validation, Supervision, Software, Methodology, Data curation, Conceptualization. **Andreas Mauser:** Writing – review & editing, Methodology, Investigation. **Monika Pischetsrieder:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Josep Rubert:** Writing – review & editing, Validation, Supervision, Software, Project administration, Formal analysis, Data curation. **Kasper Hettinga:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

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.2025.145896>.

Data availability

Data will be made available on request.

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