Cysteine residues are responsible for the sulfurous off-flavor formed in heated whey protein solutions

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1. Introduction

Whey is the primary by-product of cheese manufacturing and the whey protein fraction is a versatile food ingredient with a high content of essential amino acids (Wijayanti, Bansal, & Deeth, 2014). Bovine whey is composed of about 50% beta-lactoglobulin (β-LG), about 20% alpha-lactalbumin (α-LA) and other low-abundance proteins (Fox, Uniacke-Lowe, McSweeney, & O’Mahony, 2015; McSweeney & Fox, 2013; Walstra, Wouters, & Geurts, 2005). Soluble whey protein aggregates are produced for many food applications by using thermal treatments to achieve desired functionalities such as heat stability for protein beverages (Ryan et al., 2012; Ryan & Foegeding, 2015). However, these thermal treatments also induce unpleasant (e.g., eggy, cabbage-like and sulfurous) flavors, which may be carried over or formed directly in the food product with a negative effect on consumer perception (Jansson, Nielsen, Petersen, & Lund, 2020; Wright, Carunchia Whetstone, Miracle, & Drake, 2006).

The unpleasant flavors are mainly attributed to the formation of volatile sulfur compounds. Wright et al. (2006) suggested that dimethyl trisulfide (DMTS) was associated with the cabbage flavor, while eggy and sulfurous flavors are strongly correlated with the concentration of hydrogen sulfide (H₂S) and carbon disulfide (CS₂) (Jo, Carter, Barbano, & Drake, 2019; Vazquez-Landaverde, Torres, & Qian, 2006). Sulfur-derived off-flavor was recognized by a sensory panel in reconstituted whey protein isolate (WPI, 3% w/v) after 10 min of heating at temperatures of around 60–70 °C and above (Jansson et al., 2020). The significantly higher volatility of H₂S (boiling point of ~60 °C) compared to other sulfur volatiles potentially makes H₂S an important aroma compound in heated whey protein systems.

The formation of H₂S has been found to increase with higher concentrations of free thiol groups (–SH) adjusted by adding β-LG before heating the milk systems (Gaafar, 1987). β-LG is the major source of free thiol groups in WPI and milk and has therefore been suggested to be the primary H₂S precursor (Hutton & Patton, 1952). Strecker degradation of free amino acid cysteine (Cys) in the presence of an α-dicarbonyl compound and thermal degradation of thiamine (vitamin B₁) have been proposed to be pathways for H₂S formation in heated milk by other authors (Al-Attabi, D’Arcy, & Deeth, 2009; Hutton & Patton, 1952; Jo, Benoist, Barbano, & Drake, 2018). However, a commercial WPI with negligible amounts of free amino acids and thiamine generated significant sulfurous off-flavors during heating (Jansson et al., 2020), indicating that free amino acids and thiamine were unlikely to be
responsible for the formation of H$_2$S in heat-treated WPI.

As an alternative pathway to the Strecker degradation of Cys and thiamine degradation, Walstra et al. (2005) suggested a more likely pathway for heat-induced H$_2$S formation in milk via β-elimination of protein-bound free Cys residues (referred to from now on as free Cys residues). In this pathway, low molecular weight thiol-containing compounds, such as free amino acids, are not required for the release of H$_2$S. As a result of this Cys residue desulfurization reaction, a reactive electrophilic by-product (a dehydroalanine (DHA) residue) is also generated (Fig. 1a), which may undergo Michael addition with nucleophilic groups to form stable reaction products. For example, lanthionine (LAN) and lysinoalanine (LAL) are formed from reactions of the unsaturated double bond in DHA with the nucleophilic side chains of Cys and Lys, respectively (shown for LAN in Fig. 1b). These protein cross-links have been identified in various heat-treated food systems (Belitz, Grosch, & Schieberle, 2009; Fennema, Damodaran, & Parkin, 1999; Lagrain et al., 2010).

The present study aimed to identify the origin and study the pathway of H$_2$S formation in heated WPI. Thus, the generation of H$_2$S was investigated in samples of WPI, a whey model system (mixture of α-LA and β-LG), α-LA alone, β-LG alone and sulfur-containing amino acids (methionine [Met] and Cys) after different heat treatments using a gas chromatography-flame photometric detector (GC-FPD). The role of β-elimination of Cys residues in H$_2$S formation was evaluated by comparing the level of DHA residues and its downstream stable products, e.g. LAN and LAL, by site-specific LC-MS/MS-based proteomic analysis. In addition, the role of disulfide bonds on the formation of H$_2$S in whey protein samples was also investigated, since Cys and cystine residues are known to rearrange due to thiol-disulfide exchange reactions, especially during heat treatments (Li, Engholm-Keller, & Lund, 2022).

2. Materials and methods

2.1. Chemicals and reagents

Powdered whey protein isolate (WPI) (Lacprodan® DI-9224, containing 89% protein and < 0.2% fat and lactose, based on dry matter) was obtained from Arla Foods Ingredients (Videbæk, Denmark). α-Lactalbumin (α-LA) from bovine milk (calcium-depleted, Lot # SLBT2518, 96%, product # L6010), β-lactoglobulin (β-LG) from bovine milk variant A/B (Lot # SLBS6536, 99%, product # L0130), L-Cys (97%), L-Met (99%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetic acid, tris(2-}

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**Fig. 1.** Cys β-Elimination of Protein-Bound Cys Residue (a), Michael Addition between Dehydroalanine (DHA) and Cys Residues (b), and β-Elimination of Protein-Bound Cystine and the Conversion of Persulfide into Cys Residue (c). Adapted with Modifications from (Nashef et al., 1977; Whitaker & Feeney, 1983).
carboxymethylphosphate (TCEP), iodoacetamide (IAA), sodium sulfide and TrpZean® bovine recombiant trypsin, sodium hydroxide, urea, sodium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium acetate, endopeptidase Lys-C, and LiChroSolv LC-MS grade acetonitrile were obtained from Merck (Søborg, Denmark). Staphylococcus aureus endopeptidase-Glu-C was obtained from Worthington (Lakewood, NJ, USA). HiPerSolv LC/MS grade formic acid (99 %) was purchased from VWR (Søborg, Denmark). Double-deionized water was produced using a Milli-Q system (Millipore, Bedford, MA, USA). All reagents were of analytical grade.

2.2. Preparation of protein and amino acid samples

α-LA (2.45 mg/mL), β-LG (15.88 mg/mL), a whey model (2.45 mg/mL α-LA and 15.88 mg/mL β-LG), Cys (0.69 mg/mL), Met (0.54 mg/mL), sulfur amino acid mixture (0.69 mg/mL Cys and 0.54 mg/mL Met) and 3 % WPI (w/w) solutions were prepared in 10 mM phosphate buffer (pH 7.0) for LC-MS/MS analysis. All protein samples were also prepared in 55 mM HEPES buffer (pH 7.0) for proteomic and thiol analyses. Protein and amino acid concentrations in the prepared solutions were chosen to reflect their concentrations in a 3 % WPI (w/w) solution (Li, Nielsen, & Lund, 2022).

2.3. Heat treatments for proteomic analysis

Samples (200 µL) in HEPES buffer were heated at 60, 70, 80, 90 °C for 10 min or 90 °C for 120 min in polymerase chain reaction (PCR) tubes (Molecular BioProducts Inc., San Diego, CA, USA) in a thermal cycler (Applied Biosystems 2720, version 2.09, Thermo Fisher Scientific Inc., Singapore) as previously described (Li, Engholm-Keller, & Lund, 2022). Heating at 90 °C for 10 min was representative for a typical industrial heat treatment used for producing functional protein aggregates, 90 °C for 120 min was considered as an extreme heat treatment. Another heat treatment at 160 °C for 160 s was chosen to mimic an ultra-high-temperature (UHT) treatment as preliminary data revealed formation of off-flavor levels corresponding to the levels produced in pilot-scale equipment for UHT treatment (data not shown), thus allowing more closely comparison to industry-relevant process conditions. In this UHT-like treatment, each 1 mL of sample solution in glass vials (item no. ML 33003 V, Mikrolab Aarhus A/S, Aarhus, Denmark) were sealed with silicone/polytetrafluoroethylene (PTFE) septa and crimp caps (item no. ML 33034L, Mikrolab Aarhus A/S, Aarhus, Denmark) and heated for 160 s in a pre-heated aluminum block (catalog no. 460-0008, VWR, Søborg, Denmark) at 160 °C. The samples were first cooled at room temperature for 1 min and then immersed in ice-water.

For proteomic analysis, protein samples were categorized into two groups (i.e. non-reduced and reduced groups) for investigation of different protein modifications. The pH of samples from the non-reduced group was adjusted to 6.5 with 50 mM sodium acetate (pH 6.0) after heat treatment. Then, free Cys residues were alkylated (via carbamidomethylation) using 15 mM IAA/4 M urea for 30 min in the dark (at 30 °C) before storing the samples at –20 °C. Samples from the reduced group were immediately frozen at –20 °C after heat treatment, while the reduction and alkylation processes were performed after the enzymatic hydrolysis as described in the ‘‘2.5.2 LC-MS/MS’’ section below.

2.4. Gas chromatography-flame photometric detector (GC-FPD) analysis

One milliliter of each untreated protein or amino acid sample in phosphate buffer was transferred to a sealed glass vial as described in the ‘‘2.3 Heat Treatments for Proteomic Analysis’’ section above. Samples were then heated following the abovementioned temperature/time combinations in the preheated aluminum block and stored at 10 °C before analysis on an Agilent GC7890B gas chromatography system equipped with a flame photometric detector (FPD Plus, Agilent Technologies Inc., Santa Clara, CA, USA). After five min incubation at 32 °C in the autosampler (G6501B GC Sampler 80, Agilent Technologies Inc., Santa Clara, CA, USA), 250 µL of headspace was injected through a splitless injection liner at 250 °C (single taper Ultra Inert (UI) with glass wool, 900 µL, Agilent Technologies Inc., Santa Clara, CA, USA). Analytes were separated on a polyethylene glycol (PEG) column (J&W DB-WAX Ultra Inert, 30 m × 0.25 mm, 0.25 µm, Agilent Technologies Inc., Santa Clara, CA, USA) with 1.2 mL/min hydrogen flow. The oven temperature was kept at 32 °C for six min, and increased to 230 °C at 50 °C/min for five min, followed by a five min final holding time. The FPD was operated in sulfur mode at 255 °C.

A HgS stock solution was prepared by dissolving 385 mg of sodium sulfide in 50 g of phosphate buffer (pH 8.5) (Vazquez-Landaverde et al., 2006). The stock solution was diluted into a series of different concentrations in phosphate buffer at pH 7.0 for the preparation of the standard curve. Data of the GC-FPD analysis was analyzed using MassHunter Quantitative Analysis software (ver. 10.1.49, Agilent Corp., Wilmington, DE, USA).

2.5. Quantification of total free thiols

The concentration of total free thiol groups in all protein samples was determined by the DTNB assay (Ellman, 1959). Protein samples were diluted appropriately and transferred (100 µL) to a 96-well-plate (Thermo Fisher Scientific Inc., Rochester, NY, USA), together with a series of freshly prepared Cys standard solutions (in 55 mM HEPES buffer (pH 7.0)) with concentration ranging between 0.1 and 1.0 mM. The working reagent was prepared by mixing 8 M urea in 10 mM phosphate buffer (pH 8.0) with freshly prepared 10 mM DTNB in 100 % ethanol at a 4:1 ratio. Working reagent (120 µL) was added to the wells containing the samples and standards and the plate was incubated at room temperature in the dark for 20 min. The absorbance at 412 nm was measured by a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) at 30 °C. The concentration of total free thiols in the protein samples was calculated based on the calibration curve of Cys with the subtraction of the blank signal, which was the absorbance of the working reagent.

2.6. Proteomic analysis

2.6.1. Specific enzymatic hydrolysis

β-LG samples from the non-reduced group were hydrolyzed by two times sequential addition of 2.5 % Glu-C (enzyme-to-protein ratio (w/w) 2.5:100) followed by 3 h and 15 h incubations, respectively. Other protein samples from the non-reduced group were hydrolyzed with 1 % Lys-C for 3 h, followed by hydrolysis using 5 % trypsin for 15 h. For all samples from the reduced group, pH and urea concentration were adjusted to 6.5 and 4 M, respectively, with an equal volume of 8 M urea/50 mM phosphate buffer (pH 6.0). A triple-protease sequential hydrolysis was then conducted with sequential addition of 1 % Lys-C (3 h), 5 % trypsin (15 h), and two times 2.5 % Glu-C (3 + 15 h). All enzymatic hydrolyses were conducted at pH 6.5 and 30 °C, and the urea concentrations were always diluted to 1 M with 50 mM sodium acetate (pH 6.5) after Lys-C hydrolysis.

2.6.2. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

Samples from the reduced group were reduced using 13 mM TCEP and alkylated (via carbamidomethylation) using 30 mM IAA, with both incubations being conducted with shaking for 30 min at 25 °C in the dark. All digested samples were acidified with formic acid to a 5 % final concentration, transferred to a 96-well PCR plate (Axygen PCR-96-FS-C, VWR, Søborg, Denmark) with a seal mat (Axygen AM-96-PCR-RD, VWR, Søborg, Denmark), and stored at 10 °C before analysis. The LC-MS/MS analysis was conducted as previously described (Li, Engholm-Keller, & Lund, 2022) on a UHPLC Ultimate 3000 system equipped with a reversed-phase C18 column (Aeris 1.7 µm PEPTIDE XB-C18 100 Å, 100
× 2.1 mm, Phenomenex, Torrance, CA, USA) coupled with a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (MS) (Thermo Fisher Scientific Inc., Roskilde, Denmark).

2.6.3. Modification database search on linear peptides

Peptide modifications of samples from the non-reduced group were identified by the Proteome Discoverer software (ver. 2.2.0.388, Thermo Fisher Scientific Inc., Roskilde, Denmark) with previously described settings (Li, Nielsen, Engholm-Keller, & Lund, 2022). Cys → DHA (DHA (-34 Da), Cys → persulfide carboxamidomethylation (persulfide) (+89 Da) and Cys carboxamidomethylation (alkylation) (+57 Da) were chosen as variable modifications, while N-term → pyruvic acid (-33 Da) and C-term → amidation (-104 Da) were selected as variable modifications at any peptide N-terminus and C-terminus, respectively (Supporting Information, Figure S1). Database search results (.msf file) were validated in the Skyline software (ver. 20.1.0.76, University of Washington, Seattle, WA, USA) by manually checking retention time, isotopic correlation and fragment ion distribution for each modification (Li, Nielsen, Engholm-Keller, & Lund, 2022; Schilling et al., 2012). Databases containing β-LG (P02754) or α-LA (P00711) (downloaded from Uniprot [http://www.uniprot.org], accessed in September 2019) were selected for samples of β-LG and α-LA, respectively, while their combination was used for searches of the whey model and WPI.

2.6.4. Modification database search for cross-linked peptides

Cross-linked peptides were identified and validated as previously described (Li, Engholm-Keller, & Lund, 2022) using pLink (ver. 2.3.9, Institute of Computing Technology, Chinese Academy of Sciences, Beijing, China) and Skyline (ver. 20.1.0.76, University of Washington, Seattle, WA, USA) software, respectively. Custom-defined linkers “LAN”, between two Cys residues (-34 Da); “LAL”, between Cys and Lys residues (-34 Da) and “histidinoalanine (HAL)” between Cys and His residues (-34 Da) were included in the pLink database search based on corresponding protein databases as mentioned above (Fan, Meng, Lu, Zhang, Yang, Chi, Sun, Dong, & He, 2015). Glu-C and/or trypsin were set as digestion enzyme(s) according to the proteolytic treatment and Cys carboxamidomethylation (alkylation) (+57 Da) was selected as a variable modification. Relative quantification of cross-linked peptides was conducted for samples subjected to the reducing condition.

2.6.5. Relative quantification and data analysis

Relative quantification of modifications and cross-links were performed for selected validated peptides in the Skyline software by integrating the area-under-curve (AUC) of the three first isotopologue ion masses for each validated peptide to calculate the theoretical cystine mass in the standard curve/slope of the standard curve. Statistical analysis was performed by one-way ANOVA with a Tukey-Kramer HSD post-hoc test in JMP Pro 15 (SAS Institute Inc., Cary, North Carolina, USA). All MS. triplicates, unless otherwise mentioned, and results were shown as mean concentration of free thiol in the unheated sample from Equation 1 was replaced by the free thiol concentration measured after the heat treatment ([free thiol]unheated), while calculating the formation extent of H2S from Cys residue(s) to avoid over-estimation (Equation 2).

Thus, when [free thiol]heated > [free thiol]unheated

The extent of formation of H2S from Cys residues [%] = \frac{[H_2S]_{\text{heated}}}{[\text{free thiol}]_{\text{unheated}} + [H_2S]_{\text{heated}}} \times 100\% \tag{2}

The extent of disulfide cleavage [%] = \frac{[\text{free thiol}]_{\text{heated}} - [\text{free thiol}]_{\text{unheated}}}{2 \times \text{[theoretical cystine]}} \times 100\% \tag{3}

2.7. Quantification of lanthionine (LAN) after acidic hydrolysis

Quantification of LAN was performed by using a previously validated and published method (Akilhoglu & Lund, 2022). Briefly, aliquots (200 µL of protein samples (1 mL aliquots were used for α-LA samples) were mixed with 6 M HCl (final concentration) and hydrolyzed in a microwave system (Biotage Initiator, Uppsala, Sweden) in 2 mL microwave reaction vials. The hydrolysate was evaporated to dryness using a centrifugal vacuum evaporator (Savant® SPD131DDA SpeedVac Concentrator, Thermo Fisher Scientific Inc., Roskilde, Denmark). The residue was dissolved in Milli-Q water and filtered through a 0.20 µm regenerated cellulose syringe filter (Phenomenex, Torrance, CA, USA). A stable isotopically labeled internal standard, lysine-d4, (Iris-Biotech, Markredwitz, Germany) was added to each hydrolyzed sample, followed by mixing with an equal volume of acetonitrile. Five microliter of each sample was injected into the UHPLC-MS system, which was equipped with a Synchroscan HILIC column (100 × 2.1 mm, 1.7 µm, Thermo Fischer Scientific Inc., Roskilde, Denmark), and analyzed following the chromatographic conditions and mass spectrometric details previously described by Akilhoglu and Lund (2022). The concentration of LAN in acid-hydrolyzed protein samples was calculated based on the linear calibration curve of external standards, i.e. 10–4,000 ng/mL LAN (Iris-Biotech, Markredwitz, Germany), with normalization based on the signal of internal standard in each sample.

2.8. Statistical analysis

All heating (or control) experiments were performed as independent triplicates, unless otherwise mentioned, and results were shown as means ± standard deviation (SD). Limits of detection (LODs) were calculated according to the formula: LOD = 3.3 × (standard deviation of the standard curve/slope of the standard curve). Statistical analysis was performed by one-way ANOVA with a Tukey-Kramer HSD post-hoc test in JMP Pro 15 (SAS Institute Inc., Cary, North Carolina, USA). All raw data in the present study was the same as in our previously published papers (Li, Engholm-Keller, & Lund, 2022; Li, Nielsen, Engholm-Keller, & Lund, 2022), where the extent of disulfide rearrangement and protein oxidation was investigated. The modification search results are available via MassIVE (https://massive.ucsd.edu) with identifier MSV000088811.

3. Results & discussion

3.1. β-LG and Cys are the key precursors of heat-induced H2S in WPI

Heat-induced formation of volatile sulfur compounds was investigated in WPI, whey proteins (α-LA and β-LG) and sulfurous amino acids (Cys and Met) by specific sulfur detection using GC-FPD. Among all...
tested protein and amino acid samples, H$_2$S was the only detected volatile sulfur compound after all employed heat treatments. The H$_2$S concentrations detected in the present study were above its reported sensory threshold (Table 1), which is ca. 0.3 μM in water (Rychlik, Schieberle, & Grosch, 1998), and were significantly (>3,000 times) higher than other volatile sulfur compounds (e.g. DMDS) detected in WPI after heat treatments (at up to 90 °C, 10 min) in a previous study (Jansson et al., 2020). These results demonstrate that H$_2$S has a significant role in off-flavor formation in heated WPI solutions.

In WPI, H$_2$S was detected after heat treatment at 90 °C for 10 min at a concentration of 4.0 ± 0.1 μM, which significantly increased to 23.3 ± 0.4 and 38.1 ± 8.6 μM after heating at 90 °C for 120 min and the UHT-like treatment, respectively. The detected H$_2$S concentrations in the present study were also much higher than the values found in most heated milk products as summarized by Al-Attiabi et al. (2009). The gas phase of the samples in the present study was injected directly into the GC-FPD system, which minimized the loss of volatile compounds that could potentially occur during the additional extraction step used in previous studies for H$_2$S quantification. The limited amount (below the limit of detection of the method [LOD = 1.3 μM]) of H$_2$S in 80 °C (10 min) heated WPI samples observed in the present study may partially explain the significantly different levels of aromas that were perceived by smell between 80 and 90 °C (10 min) heated WPI in the study of Jansson et al. (2020). However, H$_2$S was detected in both heated pure β-LG and the whey model system (α-LA + β-LG) with heating at ≥ 80 °C (10 min). Interestingly, more H$_2$S was generated in the heated whey model (9.5 ± 0.6 μM) and β-LG (7.3 ± 0.2 μM) solutions at 90 °C for 10 min than in WPI (4.0 ± 0.1 μM), whereas a higher H$_2$S content was found in WPI at higher heat loads (90 °C for 120 min and the UHT-like treatment) compared to the whey model and β-LG (Table 1). Regardless of the heat treatment, H$_2$S was absent in all heated samples of pure α-LA at a concentration similar to that of the (3 %) WPI sample (2.45 mg/mL) indicating that H$_2$S was mainly formed from β-LG in the whey model and WPI. Increasing H$_2$S concentrations were observed in Cys-containing amino acid solutions, i.e. Cys and the sulfur amino acid mixture (Cys + Met), with increasing heat load, whereas no H$_2$S was detected in any of the heated solutions containing only Met (Table 1). Altogether, these results showed that β-LG and Cys were the key precursors of heat-induced H$_2$S in WPI, which was in line with the findings of Gaffar (1987), where H$_2$S formation was positively correlated with the quantity of free Cys residues and the heat load. Because free Cys amino acids were not detected in WPI (Jansson et al., 2020), β-LG, being the primary source of free Cys residue (with free thiol groups) in WPI, is therefore suggested to be responsible for the detected H$_2$S in heated WPI.

In addition, according to the proteomic analysis, signals of protein-bound DHA were identified site-specifically at all Cys residues in β-LG, with significant increases after severe heat treatments (i.e. 90 °C for 120 min and the UHT-like treatment) (Table 2). This increasing DHA signal was also detected at Cys66 and Cys160 on β-LG in both mixed protein systems (i.e. the whey model and WPI). Hence, H$_2$S released from the heated whey protein solutions was suggested to be generated from (protein-bound) free Cys residues (with free thiol groups), presumably via Cys β-elimination (Fig. 1a) (Gaffar, 1987; Jo et al., 2018; Walzra et al., 2005). The frequently-used unspecific method for DHA determination that relies on conversion of DHA into pyruvic acid was not used in the present study, because DHA could also be formed at other amino acid side chains, e.g. serine (Ser), via β-elimination, without generating H$_2$S (Romboutz, Lagrain, Brijs, & Delcour, 2011).

### 3.2. The total content of Cys and cystine affects H$_2$S formation

To examine the effect of protein concentration on H$_2$S formation, the GC-FPD based H$_2$S analysis was also carried out on a concentrated (16 mg/mL) α-LA sample. After heating at 90 °C for 10 min, a limited concentration (1.5 ± 0.2 μM) of H$_2$S was detected, yet it was above the reported sensory detection threshold (ca. 0.3 μM) and perceived by smell (Rychlik et al., 1998). The level of H$_2$S was surprisingly high in the UHT-like treated concentrated α-LA (52 ± 18 μM), and was significantly higher than the H$_2$S formed in the UHT-like-treated β-LG (30 ± 3 μM) with a comparable protein concentration (w/v) (Table 1). It was therefore speculated that the total content of Cys residues (including both free Cys residues and Cys present as disulfides) within the protein system was important for the formation of H$_2$S; free Cys residues were absent in the unheated α-LA, but α-LA contained higher quantity of total Cys residues as compared to (the free Cys-containing) β-LG at the same protein concentration (w/v).

### 3.3. Beta-Elimination of cystine as a pathway of free Cys residue release

The total content of free Cys residue(s) in the protein samples was determined as free thiol concentration (Table 3). The increased thiol concentration observed in α-LA after heating at 90 °C for 120 min and the UHT-like condition suggested that disulfide bonds were cleaved in these samples, which was in agreement with a previous study (Nielsen, Lund, Davies, Nielsen, & Nielsen, 2018). These results also supported the findings from our previous study, in which disulfide rearrangement was observed in α-LA and pre-alkylated β-LG protein solutions heated at 90 °C for 120 min, but not after heating for 10 min (up to 90 °C) (Li, Engholm-Keller, & Lund, 2022). In UHT-like-treated β-LG-containing protein samples, a significant increase of free Cys residue (as free thiol) concentration was also observed (Table 3).

The release of free Cys residues from disulfide bonds in α-LA, which contained no free Cys residue in the unheated structure, could be due to β-elimination occurring at disulfide bonds, as previously studied in other proteins under thermal treatment (Lagrain et al., 2010; Romboutz, Lagrain, Brijs, & Delcour, 2010; Volkin & Klibanov, 1987). The formed (protein-bound) persulfide from cystine β-elimination can then be converted into free Cys residue with the potential release of H$_2$S (Fig. 1c) (Friedman, 1999). According to the sensitive LC-MS/MS-based proteomic analysis, the two main (intermediate) reaction products of cystine

| Sample | Heat Treatments | Unheated (80 °C) | 90 °C (10 min) | 90 °C (120 min) | UHT-like | LOD 2.9 μM |
|---------|----------------|-----------------|---------------|----------------|----------|------------|
| α-LA    | <LOD           | NT              | <LOD          | <LOD           | <LOD     | NT         |
| β-LG    | 2.0 ± 0.1 μM   | 7.3 ± 0.2 μM    | 15.4 ± 0.1 μM | 22.2 ± 1.8 μM  | **a**    |
| Whey model | 2.9 ± 0.5 μM | 9.5 ± 0.6 μM | 18.7 ± 1.0 μM | 30.1 ± 3.0 μM | **a** |
| WPI     | <LOD | 4.0 ± 0.1 μM | 23.3 ± 0.4 μM | 38.1 ± 8.6 μM | **a** |
| Concentrated α-LA | <LOD | NT | 4.0 ± 0.1 μM | 51.6 ± 17.9 μM | **a** |
| Cys     | <LOD | NT | 3.8 ± 0.3 μM | 10.7 ± 4.8 μM | **a** |
| Met     | <LOD | NT | <LOD | NT | **a** |
| Mixed amino acid | 4.3 ± 0.6 μM | 11.6 ± 0.8 μM | **a** |

* Protein concentrations of samples: α-LA: 2.45 mg/mL (0.17 mM); β-LG: 15.88 mg/mL (0.87 mM); whey model: 2.45 mg/mL (0.17 mM) α-LA: 15.88 mg/mL (0.87 mM) β-LG; WPI: 30 mg/mL and conc. α-LA: 16 mg/mL (1.11 mM). Amino acid concentrations of samples: Cys: 0.69 mg/mL (5.7 mM); Met: 0.54 mg/mL (3.6 mM) and mixed amino acid: 0.69 mg/mL (5.7 mM) Cys + 0.54 mg/mL (3.6 mM) Met. Values in the same row followed by different number of asterisks (*) are significantly different between treatments, and values in the same column followed by different number of asterisks are significantly different between samples (one-way ANOVA with a Tukey-Kramer HSD post-hoc test, p < 0.05). Tests were conducted with at least two independent replicates, while only one measurement was performed for the values without standard deviation. NT, not tested; LOD = 1.3 μM.
| Protein | Cys residue | Modification | Sample | Heat Treatment | Unheated | 80 °C, 10 min | 90 °C, 10 min | 90 °C, 120 min | UHT-like (160 °C, 160 s) |
|---------|-------------|--------------|--------|---------------|----------|--------------|--------------|--------------|-------------------|
| α-LA    | Cys6        | Persulfide   | α-LA   | 4.5 ± 0.4 %   | 5.7 ± 0.9 % | 6.5 ± 0.5 % | 19.3 ± 2.2 % | 100.0 ± 11.7 % |
|         |             |              | whey model | 14.5 ± 3.8 % | 7.0 ± 1.1 % | 13.0 ± 1.5 % | 34.7 ± 4.2 % | 75.3 ± 13.0 % |
|         |             |              | WPI     | ND            | 3.7 ± 0.8 % | 9.9 ± 1.9 % | 26.9 ± 3.3 % | 81.7 ± 8.8 %   |
| Cys28   | DHA         | α-LA         | ND      | ND            | ND        | 9.1 ± 1.6 % | ND            | 100.0 ± 13.3 % |
|         |             |              | ND      | ND            | ND        | ND           | ND            | ND             |
| Cys61   | DHA         | α-LA         | ND      | 0.2 ± 0.3 %   | 1.4 ± 0.4 % | 3.2 ± 0.5 % | 21.1 ± 4.0 % | 100.0 ± 20.7 % |
|         |             |              | ND      | 0.1 ± 0.0 %   | 1.2 ± 0.2 % | 2.6 ± 0.4 % | 11.1 ± 1.7 % | 56.0 ± 11.0 %  |
|         | Persulfide  | α-LA         | ND      | 0.7 ± 0.1 %   | 1.8 ± 0.2 % | 2.7 ± 0.2 % | 16.4 ± 1.6 % | 100.0 ± 11.8 % |
|         |              |              | ND      | 0.9 ± 0.5 %   | 6.4 ± 0.7 % | 7.0 ± 1.1 % | 10.4 ± 1.1 % | 74.4 ± 11.9 %  |
| Cys73/Cys77 | DHA and Alkylation | α-LA | 0.0 ± 0.0 % | 3.8 ± 0.5 % | 8.3 ± 0.7 % | 13.1 ± 1.7 % | 62.9 ± 6.5 % |
|         |             |              | ND      | 0.6 ± 0.1 %   | 2.7 ± 0.3 % | 9.3 ± 1.1 % | ND            | 100.0 ± 9.6 %  |
|         |              |              | ND      | ND            | ND        | ND           | 52.2 ± 10.9 % | ND             |
|         | 2 × DHA     | α-LA         | 0.1 ± 0.0 % | 0.2 ± 0.1 %   | 0.5 ± 0.1 % | 10.6 ± 2.5 % | 100.0 ± 26.8 % |
|         |              |              | ND      | ND            | ND        | ND           | ND            | ND             |
| Cys91   | DHA         | α-LA         | 0.2 ± 0.0 % | 1.2 ± 0.1 %   | 3.0 ± 0.2 % | 11.5 ± 0.8 % | 100.0 ± 9.4 % |
|         |             |              | ND      | 0.2 ± 0.4 %   | 3.4 ± 0.5 % | 11.9 ± 1.0 % | 53.8 ± 4.0 %  |
| Cys111  | DHA         | α-LA         | 0.4 ± 0.2 % | 1.3 ± 0.1 %   | 2.2 ± 0.2 % | 10.5 ± 0.7 % | 77.5 ± 6.1 %  |
|         |             |              | ND      | 0.7 ± 0.0 %   | 1.6 ± 0.1 % | 6.9 ± 0.5 % | 74.5 ± 10.8 % |
| Persulfide | α-LA       | 1.3 ± 0.5 %  | ND      | 2.40 ± 0.49 % | 4.20 ± 0.4 % | 17.3 ± 1.4 % | 100.0 ± 8.7 % |
|         |              |              | ND      | ND            | ND        | ND           | ND            | ND             |
| Cys120  | DHA         | α-LA         | 1.2 ± 0.2 % | 1.6 ± 0.2 %   | 2.3 ± 0.3 % | 9.1 ± 1.2 % | 66.6 ± 10.4 % |
|         |             |              | ND      | 2.5 ± 0.4 %   | 3.9 ± 0.3 % | 12.2 ± 1.9 % | 61.1 ± 8.3 %  |
| Persulfide | α-LA       | 6.8 ± 1.3 %  | ND      | 11.2 ± 2.6 % | 8.5 ± 1.6 % | 14.7 ± 2.7 % | 71.2 ± 14.6 % |
|         |              |              | ND      | 36.1 ± 12.1 % | 14.1 ± 2.8 % | 11.4 ± 2.1 % | 100.0 ± 25.3 % |
|         | Persulfide  | β-LG         | ND      | 9.9 ± 2.3 %   | 4.89 ± 0.9 % | 4.70 ± 1.0 % | 10.6 ± 1.9 % | 87.3 ± 19.5 %  |
| Cys66   | DHA         | β-LG (Glu-C) | ND      | 10.0 ± 1.8 % | 14.9 ± 3.2 % | 46.3 ± 7.9 % | 100.0 ± 23.0 % |
|         |              |              | ND      | 0.3 ± 0.1 %   | 1.8 ± 0.2 % | 13.9 ± 2.4 % | 100.0 ± 15.9 % |
| Persulfide | β-LG (Glu-C) | 15.2 ± 2.9 % | ND      | 23.2 ± 4.5 % | 29.9 ± 5.6 % | 42.0 ± 8.1 % | 100.0 ± 26.0 % |
| Cys106  | DHA         | β-LG (Glu-C) | ND      | ND            | ND        | ND           | ND            | ND             |
|         |              |              | ND      | ND            | ND        | ND           | ND            | ND             |
| Cys119 & Cys121 | DHA and Alkylation | β-LG (Glu-C) | ND      | ND            | ND        | ND           | ND            | ND             |
|         |              |              | ND      | ND            | ND        | ND           | ND            | ND             |
| Cys160  | DHA         | β-LG (Glu-C) | ND      | ND            | ND        | ND           | ND            | ND             |

**Notes:**

- The results of DHA and persulfide residues are presented as percentage values (the normalized peak area of the DHA- or persulfide-containing peptide for a particular treatment divided by the highest normalized peak area of the same residue peptide among all treatments and samples that followed the same digestion process) with their respective standard deviations (SDs) from the non-reduced sample group. Data from β-LG was Glu-C digested, as indicated as “β-LG (Glu-C)”, while all other samples were digested by Lys-C and trypsin. Values from β-LG (Glu-C) in the same modification (row) followed by different letters are significantly different between treatments. Values other than the same modification at the same Cys residue(s) followed by different letters are significantly different between treatments and systems. (One-way ANOVA with a Tukey-Kramer HSD post-hoc test, p < 0.05, n = 3). ND, not detected; Alkylation, Cys carbamidomethylation (+57 Da). 

β-elimination, DHA, and persulfide (Nashef et al., 1977), were site-specifically detected in peptides originating from both α-LA and β-LG in all heated protein samples under the non-reduced conditions (Table 2). More specifically, DHA was identified in both heated single whey proteins (i.e. α-LA and β-LG) at almost all Cys residues with increasing levels as the heat load increased, being significant at the intense conditions (i.e. 90 °C, 120 min and the UHT-like treatment). Persulfide was found at many Cys residues and shared a similar increasing pattern with the DHA signal (Table 2). Most of the DHA signals found in the single whey proteins were also detected in the mixed protein systems, i.e. the whey model and WPI, except on two relatively long Cys-containing peptides, i.e. [17–58] from α-LA and [102–124] from β-LG. It was hypothesized that these long peptides, having multiple reactive amino acids, may have a high possibility to undergo different modifications (e.g. oxidation) simultaneously under elevated temperatures, leading to a lower probability of having only Cys modified to DHA, especially in α-LA (Li, Nielsen, Engholm-Keller, & Lund, 2022). In addition, the triptic β-LG tri-Cys peptide [102–124] from mixed protein
systems under the non-reduced condition was likely to be involved in disulfide-linked peptides, resulting in the absence of signal of the targeted linear DHA-modified peptide in the present proteomic analysis. The matrix effect in these two complex samples (i.e. the whey model and WPI), as compared to the single protein solutions, potentially also suppressed the detection of DHA and persulfide on these two long peptides and the cross-linking site of LAL, LAL-linked peptides were expected to be found in relatively long mis-cleaved peptides. Thus, only one LAL was identified in heated α-LA (Cys91-Lys98), while a histidinoalanine (HAL) cross-link, as a Michael addition product of DHA and a His residue, was also detected in the intensely heated α-LA, linking Cys91 and His107. The detection of HAL further supported the occurrence of β-elimination at cystine (and/or Cys) residues in the heated protein systems.

The absolute quantities of LAN were also determined in acid-hydrolyzed protein samples by LC-MS with standards in known concentrations (Table 4). The content of LAN was found to increase with increasing heat load in all protein samples, which was in agreement with the findings from the proteomic analysis. LAN concentrations detected in the whey model and WPI were significantly higher than in β-LG, and much higher than in α-LA, after intense heat treatments, implying different extents of β-elimination in different protein systems. The formation of H₂S and LAN in heated protein solutions followed the same trend (Supporting Information, Figure S2). The formation of LAN was likely to be an important reaction for the DHA that was formed via Cys β-elimination accompanied by the formation of H₂S. However, DHA could also be formed on other amino acid residues than Cys (e.g. Ser) without generating H₂S. It was found that the concentration of the released free Cys residues (mM level) (Table 3) was significantly (~1,000 times) higher than the measured LAN concentration (μM level) (Table 4) after heat treatments. It was therefore suggested that other possible pathways existed for disulfide breakage and release of free Cys residues (without generating DHA) in heated whey proteins, and/or that other reaction(s) of the newly-formed DHA took place (e.g. formation of LAL). For example, oxidative breakage of the amide bond (Supporting Information, Figure S1) was reported to occur at protein-bound DHA (Hellwig, 2019; Hellwig, Löbmann, & Orywol, 2015; Herbert et al., 2003; Tian, Liu, Sun, Fu, & Yang, 2018), leading to protein fragmentation as observed previously by SDS-PAGE (Li, Nielsen, Engholm-Keller, & Lund, 2022). The resulting amide and pyruvyl groups of this proposed reaction were both specifically identified at Cys residues (i.e. Cys6, Cys28, Cys73, Cys77, Cys91 and Cys120 on α-LA, and Cys66, Cys106, Cys119 and Cys121 on β-LG) in our current study with signals significantly increased in protein systems after the UHT-like treatment (data not shown).

### 3.5. The release and consumption of free Cys residues

β-Elimination has previously been proposed to take place at both Cys and cystine residues under heat and/or alkaline conditions (Nashef et al., 1977; Volkin & Kilibanov, 1987; Whitaker & Feeney, 1983). Because Cys and cystine residues are interconvertible via disulfide

### Table 3

| Samples | Heat Treatments | Thiol Concentrations (mM) |
|---------|----------------|--------------------------|
| Unheated | 70 °C, 10 min | 80 °C (10 min) | 90 °C (10 min) | 90 °C (120 min) | UHT-like (160 °C, 160 s) |
| α-LA    | <LOD           | <LOD                     | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| Conc. α-LA | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| β-LG    | 0.78 ± 0.06   | 0.77 ± 0.06              | 0.73 ± 0.06 | 0.48 ± 0.10 | 1.20 ± 1.00 |
| Whey    | 0.77 ± 0.06   | 0.77 ± 0.06              | 0.70 ± 0.05 | 0.45 ± 0.10 | 1.07 ± 1.00 |
| WPI     | 0.77 ± 0.06   | 0.76 ± 0.06              | 0.78 ± 0.06 | 0.60 ± 0.10 | 1.09 ± 1.00 |

α-Protein concentrations of samples: α-LA: 2.45 mg/mL (0.17 mM); conc. α-LA: 16 mg/mL (1.11 mM); β-LG: 15.88 mg/mL (0.87 mM); whey model: 2.45 mg/mL (0.17 mM) α-LA + 15.88 mg/mL (0.87 mM) β-LG, and WPI: 30 mg/mL. Values in the same row followed by different number of asterisks (*) are significantly different between treatments, and in β-LG-containing samples, values in the same column followed by different letters are significantly different between different β-LG-containing samples (one-way ANOVA with a Tukey-Kramer HSD post-hoc test, p < 0.05, n = 3). NT, not tested. LOD = 0.003 mM.

### Table 4

| Sample | Heat treatment | LC-MS/MS Quantification of LAN (μM) in Heat-Treated Protein Samples after Acid Hydrolysis.* |
|--------|----------------|-------------------------------------------------------------|
| Unheated | 80 °C (10 min) | 90 °C (10 min) | 90 °C (120 min) | UHT-like (160 °C, 160 s) |
| α-LA    | <LOD           | <LOD                     | <LOD                     | 1.0                      |
| β-LG    | <LOD           | <LOD                     | 3.9 ± 0.5                | 4.2 ± 0.9***             |
| Whey    | 1.6 ± 0.6      | 2.0 ± 0.4               | 11.8 ± 1.7              | 14.4 ± 2.1***          |
| WPI     | 1.3 ± 0.1      | 2.6 ± 0.6               | 11.5 ± 1.3              | 21.4 ± 5.0**            |

* Protein concentrations of each sample: α-LA: 2.45 mg/mL (0.17 mM); β-LG: 15.88 mg/mL (0.87 mM); whey model: 2.45 mg/mL (0.17 mM) α-LA + 15.88 mg/mL (0.87 mM) β-LG, and WPI: 30 mg/mL. Values in the same row followed by different number of asterisks (*) are significantly different between treatments, and in the same column followed by different letters are significantly different between samples (one-way ANOVA with a Tukey-Kramer HSD post-hoc test, p < 0.05), values of α-LA were obtained from the mixture of five independent heat treatments. LOD = 0.0 μM.
rearrangement, it is not possible to state whether the detected DHA signal on Cys residues in our study originated from direct β-elimination of Cys residues or (re-arranged) cystine residues. However, by comparing the release of free Cys residues, as a result of cystine breakage, and the generated H\(2\)S content after heat treatments, the extent of H\(2\)S formation from Cys residues (likely via β-elimination) (Eq. (1) and (2)) and of disulfide-linked cystine breakage (Eq. (3)) can therefore be evaluated in different heated protein systems. In this case, the free Cys content can be monitored via the concentration of free thiol (Table 3). The estimated extent of disulfide cleavage was found to be comparable in the heated α-LA, regardless of protein concentration (Table 5a), although H\(2\)S was only detected in intensely heated (≥90 °C) concentrated α-LA (Table 1). It was therefore assumed that the released free Cys residue content in the diluted α-LA sample with a concentration similar to the 3 % WPI (2.45 mg/mL) was not high enough to form detectable amounts of H\(2\)S, and/or that other Cys reactions were more favorable under the applied heat treatments. Such other Cys reactions, e. g. thiol-disulfide exchange, Cys oxidation, and LAN formation, were likely competing with H\(2\)S formation for the limited free Cys residues within the system (Li, Engholm-Keller, & Lund, 2022; Li, Nielsen, Engholm-Keller, & Lund, 2022). This was supported by the fact that the much higher initial free Cys concentration in the pure Cys amino acid solution did not always generate higher levels of H\(2\)S after heat treatment compared to WPI that was heated under the same conditions (Table 1). In addition, the significant loss of free thiol in all β-LG containing samples heated at 90 °C for 120 min was clearly higher than the concentration of formed H\(2\)S after the same treatment (Tables 1 and 3), which supported the suggestion of other Cys reactions occurring in parallel. As calculated from Equations (1) and (2), the formation of H\(2\)S only accounted for a small (<5%) fraction of all Cys reactions, and the extent of this reaction was estimated to be higher in the more complex samples that contained β-LG (WPI > whey model > β-LG) under severe heat treatments (90 °C, 120 min and the UHT-like treatment) (Table 5b). However, the significantly higher calculated extent of H\(2\)S formation from free Cys residues in the intensely heated concentrated α-LA sample, compared to other whey protein solutions following the same heat treatments (Table 5b), was likely to suggest other pathways of H\(2\)S formation, besides Cys β-elimination. One possible explanation for this H\(2\)S release could be persulfide degradation (Fig. 1c), but this requires further investigation.

In addition, the presence of α-LA in the mixed protein systems (i.e. the whey model and WPI) significantly facilitated LAN formation (Table 4), while a slightly higher concentration of H\(2\)S was also detected after heating (Table 1) as compared to the pure β-LG sample. Together with the higher H\(2\)S content detected in the concentrated α-LA (51.6 ± 17.9 \(\mu\)M) than in β-LG (22.2 ± 1.8 \(\mu\)M) after the UHT-like treatment, it was speculated that the total Cys content (including Cys and cystine) within the protein system promoted the formation of H\(2\)S.

### 4. Conclusion

H\(2\)S was detected in 3 % WPI solution heated at 90 °C (10 min), and the concentration of H\(2\)S increased significantly after more severe heat treatments (i.e. 90 °C for 120 min and the UHT-like treatment). The detected H\(2\)S concentrations were not only above the reported odor threshold from a previous sensory analysis (Rychlik et al., 1998), but also much higher than the previously published values detected in UHT-treated milk systems, which implied a previously neglected importance of H\(2\)S in the sulfur odor of heated whey solutions. However, a 10 °C decrease (to 80 °C, 10 min) significantly reduced the formation of H\(2\)S to below the detection limit in WPI. In the present study, protein-bound Cys residues were suggested to be the primary source of H\(2\)S in heated WPI, potentially formed via β-elimination, since H\(2\)S was detected in all β-LG-containing and Cys-containing systems, while being absent in α-LA and Met solutions with comparable concentrations to the 3 % WPI solution. The heat-induced H\(2\)S formation, together with its sulfurous off-flavor, is likely to have higher significance in WPI-based beverages due to a higher protein-bound Cys concentration in these products as compared to milk-based systems with a comparable protein concentration.

### Disulfide cleavage, likely occurring via β-elimination of cystine residues, was also observed in intensely (≥90 °C) heated protein samples, where the increased content of free Cys residues presumably facilitated the formation of H\(2\)S. Under the UHT-like treatment, disulfide cleavage in β-LG-containing systems (disulfide cleavage extent: β-LG > WPI) was speculated to be more favorable compared to Cys residues being converted into H\(2\)S (extent of H\(2\)S formation: WPI > whey model system > β-LG). However, due to the reactive nature of Cys residues, β-elimination of both Cys and cystine was speculated to compete with other reactions, which potentially affected the formation of H\(2\)S.

### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marianne Nissen Lund reports financial support was provided by Innovation Fund Denmark. Chengkang Li reports financial support was provided by China Scholarship Council. Søren Bang Nielsen reports a relationship with Arla Foods Ingredients that includes: employment. Peter Aasted Pauslen reports a relationship with Arla Foods Ingredients that includes: employment. The remaining 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.
Ellman, G. L. (1959). Tissue sulfhydryl groups. Biochemistry, 8, 729-735.

Belitz, H. D., Grosch, W., & Schieberle, P. (2009). Food Chemistry (4th revised and extended ed.). Berlin (DE): Springer.

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O’Mahony, J. A. (2015). Dairy Chemistry and Biochemistry (2nd ed.). Springer International Publishing. https://doi.org/10.1007/978-3-319-14892-2.

Friedman, M. (1999). Chemistry, biochemistry, nutrition, and microbiology of food. CRC Press.

Hellwig, M. (2019). The chemistry of protein oxidation in food. Angewandte Chemie International Edition, 58, 2-24. https://doi.org/10.1002/anie.201814142.

Hellwig, M., Löbmann, K. & Öryelov, T. (2015). Peptide backbone cleavage by a -amidation is enhanced at methionine residues. Journal of Peptide Science, 21(1), 17-23. https://doi.org/10.1007/s10930-014-9934-1.

Herbert, B., Hopwood, F., Oxley, D., McCarthy, J., Laver, M., Griner, J., … Righetti, P. G. (2003). -elimination: An unexpected artefact in proteome analysis. Proteomics, 3(6), 826-831. https://doi.org/10.1002/pmic.200300414.

Hutton, J. T., & Patton, S. (1952). The origin of sulphydryl groups in milk proteins and their contributions to "cooked" flavor. Journal of Dairy Science, 35(8), 699-705. https://doi.org/10.3168/jds.s0022-0355(52)70165-8.

Jansson, T., Nielsen, S. B., Petersen, M. A., & Lund, M. N. (2020). Temperature dependency of unwanted aroma formation in reconstituted whey protein isolate solutions. International Dairy Journal, 104, Article 104653. https://doi.org/10.1016/j.idairyj.2020.104653.

Jessome, L. L., & Volmer, D. A. (2006). Ion suppression: A major concern in mass spectrometry. LC GC North America, 24(5), 498-510.

Jo, Y., Bentois, D. M., Barbanco, D. M., & Drake, M. A. (2018). Flavor and flavor chemistry differences among milks processed by high-temperature, short-time pasteurization or ultra-pasteurization. Journal of Dairy Science, 101(5), 3812-3828. https://doi.org/10.3168/jds.2017-14971.

Jo, Y., Carter, B. G., Barbanco, D. M., & Drake, M. A. (2019). Identification of the source of volatile sulfur compounds produced in milk during thermal processing. Journal of Dairy Science, 102(10), 8658-8669. https://doi.org/10.3168/jds.2019-16007.

Lagrain, B., Vienschovser, K. D., Rombouts, I., Brijs, K., Hendrickx, M. E., & Delcour, J. A. (2010). The kinetics of -elimination of cystine and the formation of lanthionine in gliadin. Journal of Agricultural and Food Chemistry, 58(19), 10761-10767. https://doi.org/10.1021/jf100527r.

Li, C., Engholm-Keller, K., & Lund, M. N. (2022). Site-specific characterization of heat-induced disulfide rearrangement in beta-lactoglobulin by liquid chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry, 70(3), 847-856. https://doi.org/10.1021/acs.jafc.1c06644.