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Mustapha Najib[a,b], Fabrice Bray[d], Simon Khelissa[c], Stephanie Flament[d], Elodie Richard[e], Khaled El Omari[a], Christian Rolando[d], Guillaume Delaplace[c], Monzer Hamze[a], Nour-Eddine Chihib[c*]

[a]*Health and Environment Microbiology Laboratory, Doctoral School of Sciences and Technology, Faculty of Public Health, Lebanese University, Tripoli, Lebanon*

[b]*Hallab 1881 s.a.l., Tripoli, Lebanon*

[c]*Univ Lille, CNRS, INRAE, Centrale Lille, UMR 8207 - UMET - Unité Matériaux et Transformations, F-59000 Lille, France*

[d]*Miniaturisation pour la Synthèse, l'Analyse & la Protéomique (MSAP), CNRS, USR 3290, Université de Lille ; Biochimie Structurale & Fonctionnelle des Assemblages Biomoléculaires, CNRS, FR 3688, FRABIO, Université de Lille and Institut Eugène-Michel Chevreul, CNRS, FR 2638, Université de Lille, 59000 Lille, France*

[e]*BiCel Campus cité scientifique, FRABIO, Université de Lille, Villeneuve d'Ascq*

* Corresponding author. Tel.: +33 (0)3 59 63 22 46

E-mail address: Nour-Eddine.chihib@univ-lille.fr (N.-E. Chihib)

ABSTRACT

Protein-protein cross-linking and protein-fat interactions in the traditional Lebanese dairy product, Qishta, were investigated. Liquid chromatography coupled to mass spectrometry was used for the detection of lysinoalanine (LAL) and lanthionine (LAN) during the production of Qishta, which is made by heating whole milk in an open shallow vessel for more than 2 h and harvesting the surface aggregates formed. LAL and LAN cross-links were seen in Qishta at concentrations higher than those in milk, indicating their importance in the gel formation and therefore their impact on Qishta texture. Disulphide bridges were also involved in Qishta formation. The amino acid residues (cysteine, serine or threonine) involved in the β-elimination and in dehydroalanine formation were identified. Confocal scanning laser microscopy showed the role of fat globules in the gel structure.

1. Introduction

Heat treatment of milk during the process of dairying, either for cheese making, cream separation or any milk by-product formation, results in a number of interactions involving milk components, such as proteins, minerals and fat. According to the process and the severity of heat treatment applied, a large number of chemical, physical and biochemical reactions occur in milk (Al-Saadi, Easa, & Deeth, 2013). These reactions are of high importance, since they determine both the texture and the organoleptic properties of the final product. The major reactions during milk heat treatment are denaturation and aggregation of proteins, fat coalescence, interactions between fat globules and proteins, and Maillard reactions (Corredig & Dalgleish, 1996).

Qishta is a heat-treated dairy product widely consumed in Lebanon (Najib et al., 2020). In the Middle East, different spellings are used for Qishta, such as Kishta, Kashta or Ghishta (Al-Saadi et al., 2013). Qishta, defined as a dairy product containing equal amounts of fat and protein, is obtained by heating whole milk in an open shallow vessel for 2 to 3 h. At the end of the process, the coagulum formed at the milk surface will be gathered and drained to form Qishta.

Protein cross-linking has been studied during the preparation of some food products. Al-saadi et al. (2013) defined cross-linking as covalent interactions within the same protein (intramolecular) or between two different proteins (intermolecular). It has been demonstrated that protein cross-linking can improve gel network of yoghurt (Lauber, Klostermeyer, & Henle, 2001) and can prohibit age gelation in UHT milk (Datta & Deeth, 2001). Disulphide bonds were the first type of cross-links detected in food (Gerrard, 2002). Sulphydryl-disulphide linkages were also reported to have an impact on the protein-fat interactions. Ye, Singh, Taylor, and

66 Anema (2004) showed that disulphide bridge associated β-lactoglobulin (β-Lg) and α-
67 lactalbumin (α-LA) to the milk fat globule membrane (MFGM) during heat treatment of milk.
68 Xenobiotic cross-links such as lysinoalanine (LAL), lanthionine (LAN) and
69 histidinoalanine (HAL) constitute new type of cross-links found in food matrix and dairy
70 products (Anema & Li, 2003; Buchert et al., 2010; Cartus, 2012; Hasegawa, Mukai, Gotoh,
71 Honjo, & Matoba, 1987; Miller, Spinelli, & Babbitt, 1983; Rombouts, Lambrecht, Carpentier, &
72 Delcour, 2015; Sieber, Bütikofer, Kaldas, & Rehberger, 2007). Their formation involved two
73 steps: β-elimination yielding dehydroalanine (DHA), and condensation reactions (Friedman,
74 1999). The presence of LAL has been confirmed in a large variety of milk products such as
75 pasteurised milk, UHT milk and whey protein concentrate, but at different levels (Annan &
76 Manson, 1981; Faist, Drusch, Kiesner, Elmadfa, & Erbersdobler, 2000; Friedman, 1999). For a
77 long time, the presence of LAL has been considered as undesirable and its presence has been
78 suggested to have a toxic effect. In fact, histopathological changes in renal cells, also known as
79 nephrocytomegaly, have been observed in rat experiments after consuming soy protein
80 containing LAL (Woodard & Short, 1973). However, Sieber et al. (2007) reported that the
81 presence of cross-links connecting the proteins, such as LAL, does not have a toxic effect on
82 human health. In addition, LAL has been identified in a large variety of food products, such as
83 legume protein (Friedman, 1999), meat protein (Visser, Slangen, & Rollema, 1991), fish protein
84 (Miller et al., 1983) and infant formulas (Anema, 2000).
85 The aim of this study was to understand the mechanisms of Qishta formation and to
86 understand the interactions between proteins and also between proteins and milk fat globules.
87 For that, the presence of protein crosslinks such as disulphide bonds, LAL and LAN in Qishta
88 was investigated.

2. Materials and methods

2.1. Materials

The stainless steel shallow flat surface used in Qishta production has a diameter of 50 cm, a capacity of 3 L and a thickness of 2 mm. It was provided by Nafco (Lebanon) and the burner from AEM (Chelles, France). UHT milk was purchased from Lactel (France). Lactic acid was purchased from Fischer Scientific (UK).

2.2. Qishta preparation procedure

Milk (3 L) was prepared by adding 1 mL of lactic acid, which is the amount needed to decrease the pH from 6.7 to 6.4 (procedure applied by the "Hallab 1881" company, located in Tripoli, Lebanon). The traditional process consists of heating milk for 2 to 3 h and gathering the aggregates formed at the milk's surface. During the heat treatment, and depending on the evaporation rate, milk is usually added to readjust its level in the plate. In this study, milk was not added to keep the milk's composition constant, and therefore the process was interrupted after 25 min of heating.

2.3. SDS-PAGE analysis

111 Samples of UHT milk (Lactel) and Qishta were analysed by SDS-PAGE under reduced
112 and non-reduced conditions according to the method described by Anema (2000). The resolving
113 and stacking gel contained 12–15% and 4% acrylamide, respectively. Electrophoresis was
114 performed using a vertical electrophoretic unit type TV200YK twin-plate, associated with the
115 source voltage EV202. SDS PAGE was performed at 30 V until the samples had completely left
116 the stacking gel, then the voltage was increased to 90 V until the tracking dye reached 80% of
117 the gel. Staining of gels was performed in 0.23% solution of Coomassie Blue R-250, containing
118 3.9% (w/v) TCA, 6% (v/v) acetic acid and 17% (v/v) methanol, for 90 min. The
119 electropherogram obtained from SDS-PAGE was converted by image converter software and
120 then analysed using the ImageJ 1:46 software. Each protein was identified by comparing the
121 migration distance of each band with that of a protein marker having known molecular mass. The
122 intensity of the patterns corresponds to a grey value that varies between 0 and 250.
123
124 *2.4. Sample preparation for quantification and cross-links analysis*
125
126 Milk samples (1 mL) or 10 mg of Qishta were added separately into 15 mL tubes. To
127 denature the samples, 1 mL of 8 M urea, 150 mM NaCl and 100 mM ammonium bicarbonate was
128 added. The mixtures were then vortexed for 30 min at 4 °C with Vortex-Genie 2 ™ (Scientific
129 Industries, Bohemia, USA). Eight millilitres of chloroform methanol (1:2, v/v) were added to the
130 samples and the mixture was vortexed for 30 min. A triphasic solution with protein interphase
131 was produced. To maximise the separation of phases, the tubes were centrifuged at 4032 × *g*, 4
132 °C for 30 min using Centrifuge Allegra® 64R (Beckman Coulter, Brea, USA). Both upper and
133 lower phases were carefully discarded. The interphase was dried under air vacuum using a

sample concentrator for 60 min (SBH130, Stuart, Staffordshire, UK). The dry interphase, containing protein, was resuspended by adding 1 mL of 6 M urea, 150 mM NaCl, 100 mM ammonium bicarbonate and the mixture was vortexed overnight at 4 °C. Protein extracts were stored at –80 °C until use.

2.5. In-gel digestion and LC-MS/MS analysis

The protein bands were excised from the gel and digested using trypsin digestion. All digestion were analysed by LC-MS/MS. LC-MS/MS protein analysis was performed on an Orbitrap Q-Exactive plus Mass Spectrometer hyphenated to a U3000 RSLC Microfluidic HPLC System (Thermo Fisher Scientific, Waltham, MA, USA) (Helle et al., 2018). For details, see Supplementary material A.

2.6. Digestion and analysis in LC-MS/MS

All samples were prepared using a modified enhanced Filter Aided Sample Preparation (eFASP). eFASP was used to increase proteome coverage and sample recovery for quantitative proteomic experiments (Erde, Loo, & Loo, 2014; Helle et al., 2019). For details, see Supplementary material A.

2.7. Bioinformatics identification and quantification

Raw files from in-gel digestion were analysed using the Proteome Discoverer™ software (Thermo Scientific, version 2.2). The SEQUEST algorithm was used for database searches with the UniProtKB/Swiss-Prot Bovine database (*Bos taurus*, January 2018, Sequences: 6014). For details, see Supplementary material A.

Analysis of milk samples using LC-MS/MS from eFASP digestion data was performed using MaxQuant *(version 1.5.3.30*). Cox and Mann (2008) search was used for database searching against the UniProtKB/Swiss-Prot Bovine (*Bos taurus*, January 2018, Sequences: 6014). For details, see Supplementary material A.

2.8. Identification of cross-links

Raw files from eFASP and in-gel digestion were analysed using Mass Spec Studio v 2.1.2.3107 (Sarpe et al., 2016). LAL cross-links between serine and lysine were examined using a mass shift of −18.01056 Da due to the elimination of H_2O, while LAN cross-links between two cysteine residues as well as LAL between cysteine and lysine were searched using a mass shift of –33.9877 Da due to the elimination of H_2S. Carbamidomethyl cysteine and methionine oxidation was set as dynamic modification. Trypsin was selected as an enzyme with 3 missed cleavages. Error of MS and MSMS precursor was set to 10 ppm. The minimum charge of peptides was set to 3 and the maximum was set to 8. Peptide cross-links with a score higher than 18 were considered for further analysis.

2.9. Microstructure characterisation

Microstructure characterisation was performed using a confocal laser scanning microscope (CLSM). Samples were labelled with Nile Red and Fast Green to stain the fat globules and the proteins, respectively. Nile Red (10 µL) and Fast Green (5 µL) were added to 1 mL of milk or 0.8 g of Qishta. Samples were gently mixed to avoid structure degradation. Samples were kept at room temperature (20 °C) for, at least, 15 min before observations and then 100 µL of milk and 0.1 g of Qishta were placed in Lab-Tek chamber (Nunc™ Lab-Tek™ II Chambered Cover glass). Confocal laser scanning microscope (ZEISS LSM 780, Carl Zeiss Micro Imaging GmbH) was used to characterise emulsion microstructures. Images were acquired with a Plan Apochromat 40× /1.3 numerical aperture oil immersion objective, using Zen Software (Carl Zeiss Micro Imaging GmbH). Fluorophore excitations were performed using 561 nm laser line for Nile Red imaging and 633 nm laser line for Fast Green.

2.10. Statistical analysis

One-way analysis of variance (ANOVA) was conducted using the SPSS software for Windows (version 13.0, SPSS). A Duncan test was carried out to assess any significant differences between the means. Differences were considered statistically significant when $P \leq 0.05$.

3. Results and discussion

3.1. Chemical composition of Qishta and Lacte milk obtained by LCMS analysis

To understand the protein interactions that occur during the heat treatment of milk, it was important to understand the most abundant proteins present in milk and those involved in Qishta formation. In milk and Qishta, 174 proteins were identified and quantified using the label-free method. Holland, Deeth, and Alewood (2004) identified more than 150 proteins in whole bovine milk, while in other studies 186 proteins have been identified in Holstein and Jersey cows' milk (Jensen, Poulsen, Møller, Stensballe, & Larsen, 2012; Vincent et al., 2015). The number of proteins identified depends on the technique used (Jensen et al., 2012). Table 1 shows the average relative abundance of the major proteins quantified in both milk and Qishta samples. Our findings showed that caseins and whey proteins are the most abundant proteins present in both milk and Qishta, and represent more than 97% of total protein present in the samples. Among the 6 major proteins, β-casein (β-CN; 38,251.8) and α_{S1}-CN (27,499.9) were the most abundant while α-lactalbumin was the least abundant. Lactadherin, serum albumin and lactoferrin were present as traces. Our results were in line with those of Jensen et al. (2012) and Abd El-Salam (2014), who found that casein and whey proteins represent almost 95 % of total proteins in milk.

Table 2 shows the percentages of the four main caseins (α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN) and the two major serum proteins (β-Lg) and (α-LA) present in fresh raw milk, Swedish UHT milk and Lactel milk used during Qishta production. For fresh bovine milk, the level of proteins varies between 2.5% to 3.5%. This variation could be attributed to breeding, individual variation and nutrition status of the animals. The protein ratio casein/whey, in fresh milk is approximately 80/20 (Dalgleish, 1993; Gellrich, Meyer, & Wiedemann, 2014). Karlsson, Langton, Innings, Wikström, and Lundh, (2017) showed that the amounts of caseins and whey proteins present in UHT milk were 87% and 13%, respectively. These results are in line with our

findings; however, the percentages of the individual caseins were different from those reported in previous studies (Dalgleish, 1993; Heck, van Valenberg, Dijkstra, & van Hooijdonk, 2009; Karlsson et al., 2017).

The relatively high amount of κ-CN (15.9%) present in the UHT milk studied could be explained by an overestimation resulted from the interaction with α_{S2}-CN through disulphide bonds. In fact, Miller et al. (1983) showed that the complete separation between κ-CN and α_{S2}-CN is not always achieved. Fig. 1 shows the relative abundance of major proteins present in UHT Lactel milk and Qishta. No significant difference ($P < 0.05$) was observed between the percentage of each protein in milk and Qishta, indicating that the heat treatment applied did not promote the denaturation of specific proteins in preference to others. Concerning UHT milk, β-CN (37%) and α_{S1}-CN (27.5%) constitute the major parts (approximately 65%) of the casein while α-LA represents the minor percentage (≈ 5%). Regarding Qishta, the gap between the highest and the lowest percentage decreased and reached 18% (31% in UHT milk). β-CN represented the major protein present in Qishta; however, α_{S1}-CN, κ-CN and β-Lg have almost similar percentages (≈ 18%). The comparison between milk and Qishta showed that the percentages of β-CN and α_{S1}-CN in the Qishta decreased while those of β-Lg and α_{S2}-CN increased.

The high rate of β-Lg migration from milk to Qishta could be attributed to the high thermal sensitivity of this protein. In fact, when heating milk above 65 °C, β-Lg denatures (Anema, 2008). β-Lg has a free thiol group allowing interaction with casein micelles (Eigel et al., 1984). A complex between β-Lg and κ-CN can be formed through disulphide bridge during milk heat treatment, having a significant impact on Qishta's texture. The position of κ-CN on the surface of the micelles makes it more accessible for interaction with β-Lg (Anema, 2008).

248

249 *3.2. Protein-protein interaction*

250

251 *3.2.1. Identification of S-S crosslinks in Qishta by SDS-PAGE*

252 Fig. 2 shows a quantitative comparison between Qishta proteins under SDS-R and SDS-
253 NR. The electropherogram was converted by image converter software and then analysed using
254 ImageJ software. The intensity of the peak corresponding to α-LA increased from ≈ 215 under
255 SDS-NR to ≈ 245 under SDS-R; the same trend was observed for β-Lg and κ-CN. The major
256 increase was observed for β-Lg with ≈ 40% from SDS-NR to the SDS-R (185 to 255). The
257 intensity of high molecular weight aggregates (HMW) decreased from 250 under SDS-NR
258 conditions to 240 under SDS-R. The addition of β-mercaptoethanol in SDS-R resulted in
259 breaking of disulphide bonds between proteins. This change led to an increase in the intensity of
260 individual proteins (α-LA, β-Lg and κ-CN), with a major change especially occurring in β-Lg.
261 The decrease of the aggregate intensity indicates that these proteins interacted through disulphide
262 bridges. Heating milk above 65 °C causes the denaturation of whey proteins (Wijayanti, Bansal,
263 & Deeth, 2014). According to the temperature intensity, time of heating, pH and protein
264 concentration, the major denatured whey protein (β-Lg) will form a complex with casein
265 micelles, which will directly impact the characteristics of the dairy product formed (Anema & Li,
266 2003; Cho, Singh, & Creamer, 2003; Singh & Creamer, 1991).

267 Anema and Li (2003) reported that, during heat treatment of skim milk, the size of the
268 casein micelles increased with the increase of denatured whey proteins, suggesting, therefore, the
269 probable association between β-Lg and κ-CN. Cho et al. (2003) reported that the heat treatment
270 exposed the hidden cysteine residue in β-Lg, consequently creating a reactive sulphydryl group

that is able to interact through thiol-disulphide exchange reaction with κ-CN. This hypothesis was confirmed by the addition of thiol-blocking agents that prevent interactions between these two proteins (Purkayastha, Tessier, & Rose, 1967). In addition to the disulphide bonds, it was reported that hydrophobic and ionic interactions could play an important role in complex formation between β-Lg and κ-CN. α-LA does not associate with casein micelles on its own like β-Lg; instead, it has to form complexes with β-Lg to be later associated with casein micelle during prolonged heating (Thakur & Balaram, 2009). According to the results shown in Fig. 2, it can be concluded that disulphide bonds are involved in the coagulum formation. However, the presence of HMW aggregates after the addition of β-mercaptoethanol suggests the involvement of other types of cross-links in Qishta formation.

The LC-MS/MS Proteomic analysis of these HMW patterns, specifically those having a molecular weight between 50-60 kDa, showed the presence of α_{S1}-CN, α_{S2}-CN, κ-CN, BSA, α-LA, β-Lg, lactoferrin, lactotransferrin. α_{S1}-CN and α_{S2}-CN were the two major proteins present in Qishta and milk samples analysed under the two conditions. However, the percentage relative abundance and the number of identified peptides of these proteins was higher under NR conditions, confirming the hypothesis that disulphide bonds are not the only cross-link that exists between the proteins forming the coagulum (Supplementary material B Table S1).

3.2.2. Identification of LAL and LAN in milk and Qishta

The mechanism of LAL and LAN formation consists of two steps (Fig 3): (i) DHA formation resulting from β-elimination of cysteine, serine or threonine; and (ii) Michael addition of lysine or cysteine to the DHA formed, leading to the formation of LAL or LAN, respectively (Faist et al., 2000; Friedman, 1999; Miller et al., 1983; Ritota, Costanzo, Mattera, & Manzi,

294 2017; Rombouts et al., 2015; Thakur & Balaram, 2009). The objective of our study was to
295 investigate the presence of LAL and LAN in Qishta then to locate the peptide sequences
296 involved in cross-link formation (β-elimination of cysteine, serine or threonine). This will assist
297 in explaining the mechanism of Qishta formation and therefore show the impact of cross-links on
298 the network structure formed during heat treatment of milk. The *m/z* ratio of the crosslinking
299 peptides detected were compared with the theoretical ones. The high accuracy of Q-Exactive
300 plus mass spectrometer (less than 5 ppm) allowed identification of the cross-links. Finally, the
301 confirmation was done by the MS/MS spectra.
302 A crosslink analysis was performed on the in-gel digestion of the HMW Qishta bands and
303 showed the presence of cross-links. Two *m/z* values of compounds present in Qishta matched
304 with the theoretical *m/z* values of two peptide chains linked with LAL between serine, threonine
305 and lysine residues. Analyses show the presence of LAL between α_{S2}-CN (Table 5). The first
306 internal crosslink was observed at *m/z* 677.3096, z = 3, between the serine of the peptide
307 NMAINPSK (position 25-32) and the lysine residue of the peptide ENLCSTFCK (position 33-
308 41) from α_{S2}-CN. Another internal crosslink was identified at *m/z* 885.1151, z = 3, between the
309 threonine of the peptide LTEEEK (position 153–158) and the lysine of the peptide
310 HYQKALNEINQFYQK (position 77–91) from α_{S2}-CN. The cross-link at *m/z* 677.3096 was also
311 identified in eFASP digestion. The identification of cross-links after in-gel digestion was more
312 complex because peptides were difficult to extract from gels. Cross-link analysis is more
313 efficient when the eFASP method was used on liquid samples.
314 Crosslink analysis from eFASP digestion shows six *m/z* values of compounds present in
315 milk or Qishta matched with the theoretical *m/z* values of two peptide chains linked with LAL
316 between serine and lysine residues. Four *m/z* values (925.8921, 677.3094, 648.5323, 465.9223)

317 were present in both milk and Qishta (Table 3; peptides 1, 4, 5 and 6). However, two *m/z* values
318 (921.8939; 957.9172) were only present in Qishta samples (Table 3; peptides 2 and 3). The basic
319 Local Alignment Search Tool (BLAST) was used to identify the peptide sequences involved in
320 LAL cross-link formation and confirmed that the sequences were present exclusively in bovine
321 proteins (α-LA, α_{S2}-CN and β-CN). Four of these inter-chain cross-links connected 2 α-La
322 proteins, one connected 2 α_{S2}-CN and the last one connected α_{S2}-CN with β-CN. Peptides 1, 2, 3
323 and 5 engaged an identical peptide sequence 1 (DDQNPH**SS**NI**C**NI**S**CDK) from α-LA. It was
324 not possible to identify which serine residue was involved in cross-link formation, since protein
325 sequence 1 contains three serine residues. Fig. 4 shows the MS/MS spectra of quadruple charged
326 double-chain peptide having a theoretical Mr of 3683.5756, originating from two peptide
327 sequences linked with LAL residue between one of the three serine residues and lysine. The
328 fragmentation of the parent ion yielded 16 y-fragments, 16 b-fragments, and 4 internal
329 fragments.
330 Two detected *m/z* values of compounds detected only in UHT milk and Qishta (498.5855
331 and 619.2674) matched with the theoretical *m/z* values of two peptide chains linked by LAN
332 between two cysteine residues (Table 3; peptides 7 and 8). BLAST confirmed that the two
333 sequences were present only in milk protein (α_{S2}-CN and α-LA). These two intra-chain cross-
334 links occurred in both casein and whey protein (α_{S2}-CN and α-LA, respectively). No lanthionine
335 was found resulting from the interaction between cysteine and DHA resulting from the *β*-
336 elimination of serine residue. The comparison of theoretical fragmentation patterns with the
337 MS/MS spectra obtained allowed the confirmation of the cross-links identified. Figure 5 shows
338 the MS/MS spectra of the triply charged intra-chain peptide having a theoretical Mr of 1492.76
339 and originating from LAN linkage between one of the three serine residues and lysine in the

340 ENLCSTFCKEVVR sequence. The fragmentation of the parent ion yielded 11 y-fragments and
341 2 b-fragments.
342 Table 4 shows the intensity of 6 LAL (serine-lysine) cross-links found in UHT milk and
343 Qishta. The average intensity of LAL detected was higher in Qishta than in milk. In fact, average
344 intensities of LAL 1 and 4 in Qishta were of relative intensity 4×10^6 and 4.32×10^6
345 respectively, while these values were 3.35×10^6 and 5.22×10^5 in milk, respectively. LAL
346 number 2 and 3 could not be detected in milk. LAL intensity has been used as an indicator of the
347 severity of heat treatment applied during milk processing (Annan & Manson, 1981; Faist et al.,
348 2000; Friedman, 1999; Sieber et al. 2007).
349 These results were in line with those of Faist et al. (2000) and Hasegawa et al. (1987),
350 who detected the presence of LAL in UHT milk Sieber et al. (2007) did not find the LAL
351 peptide in UHT milk containing 2.7% fat. However, they found LAL in two cheese samples.
352 Faist et al. (2000) demonstrated that the amount of LAL increased progressively with the
353 temperature applied during milk processing; the amount of LAL was highest in sterilised milk
354 and the lowest in raw cows' milk. Their hypothesis was confirmed with the analysis of cheese
355 samples, where they found that the amount of LAL increased according to the severity of the
356 process applied during cheese manufacturing.
357 The use of mass spectrometry analysis, to detect the presence of cross-links and to locate
358 the peptides involved in their formation, is a new approach. To the best of our knowledge, this
359 analysis has not been applied in milk products except for the study conducted by Rombouts et al.
360 (2015) who succeeded in quantifying and locating LAL in BSA using tandem mass spectrometry
361 coupled with higher energy collisional dissociation. As discussed above, two types of LAL were
362 detected in Qishta; however, they were absent from UHT milk. LC-ESI-MS/MS analysis

363 succeeded in identifying the peptide residue (serine) involved in the β-elimination prior to the
364 Michael addition with lysine. These identifications were impossible in previous studies which
365 applied high-performance chromatography methods to quantify the amount of cross-links present
366 in the samples tested. The analysis of the residual milk during Qishta production (results not
367 shown) have shown that the intensities of LAL and LAN detected in heated milk were much
368 higher than that present in UHT milk used for Qishta production. LAL can be used therefore as
369 an indicator of the heat treatment applied during milk processing.
370 The last cross-link peptide identified was LAL resulting from β-elimination of cysteine
371 residue. Its *m/z* value (587.3213) matched with the theoretical *m/z* values of two peptide chains
372 linked by LAL between two cysteine and lysine residues. A BLAST search confirmed that the
373 two sequences were present only in milk bovine serum albumin (BSA). This interchain cross-
374 link connected two BSA molecules. Fig. 6 shows the MS/MS spectra of the triply charged
375 double-chain peptide having a theoretical Mr of 1758.9639, originating from two peptide
376 sequences linked with LAL residue between cysteine residue and lysine residues. The
377 fragmentation of the parent ion yielded 3 y-fragments and 3 b-fragments. Cross-linked amino
378 acid (CLAA) formation depends on heat treatment, the pH, and the processing time applied
379 (Donato & Guyomarc, 2009). Nisin and duramycin are examples of protein antibiotics in which
380 LAN, LAL and HAL can be found naturally (Cartus, 2012). In food matrices, and especially in
381 milk and dairy products, the presence of DHA resulting from β-elimination of cysteine, serine
382 and threonine and the extent of use of heat treatment, have led to the increase of the amount of
383 cross-links present in these kind of products. Friedman (1999) noted the presence of LAL in
384 different types of milk and succeeded in detecting this cross-link in raw milk but at low
385 concentration (15 µg g^{-1} protein).

386 The influence of the presence of cross-links has not been well studied, especially in the
387 dairy field. Disulphide bonds either present as inter or intra molecular scale have been proved to
388 have an important impact on the gel strength, firmness, viscosity and elasticity; consequently,
389 they have a significant impact on the texture of the final product (Buchert et al., 2010). Gerrard
390 et al. (1998) studied the effect of transglutaminase on casein cross-linking and on gel strength
391 during yogurt preparation. Lauber and Klostermeyer (2000) also studied the effect of this
392 enzyme on the dough of white pan bread.
393 Identifying the presence of cross-links such as LAL and LAN and then locating the
394 amino acids and the peptide sequences involved in such links is of high importance since it
395 allows the understanding of the mechanism of Qishta formation and confirms the presence of
396 links other than the disulphide bridges leading to the formation of this product. Further studies
397 should examine the effect of cross-links on the digestibility of food products, since it has been
398 reported that increasing the protein network has a negative effect on protein digestibility (Hunt &
399 Dalgleish, 1994).
400 This report represents the first work demonstrating the presence of cross-links in Qishta
401 and also allowing the identification of the amino acids sequences involved in β-elimination and
402 Michael addition. Rombouts et al. (2015) demonstrated and located these cross-links in two
403 different matrices: wheat gliadin and bovine serum albumin, without determining the sequences
404 involved. LC MS/MS coupled with HCD had the advantage of locating the amino acids involved
405 in the cross-link formation, allowing us to distinguish between the different sources of DHA and
406 between the amino acids involved in β-elimination. This technique has been demonstrated to
407 allow a maximum number of cross-links identified comparing with collision-induced
408 dissociation (CID), and electron-transfer dissociation (ETD) methods. However, it results in the

409 lowest coverage distribution for the αpeptide (≈ 50%). This issue can be solved by combining
410 HCD with ETD, which has been proved to give the highest sequence coverage (Kolbowski,
411 Mendes, & Rappsilber, 2017; Liu, Lössl, Scheltema, Viner, & Heck, 2017).
412
413 *3.3. Protein-fat interactions*
414
415 *3.3.1. Milk characterisation before and after heat treatment*
416 Changes in the microstructure of milk during Qishta process were monitored by confocal
417 microscopy to examine the effect of temperature on interactions between proteins and fat
418 globules. To achieve this, samples of milk were taken every 2 min, until the formation of Qishta.
419 It is important to mention that the samples of milk were taken from the same area located near
420 the flame where temperature varied between 90 °C and 99 °C (known to be its highest). The
421 kinetic of aggregate formation is shown in Fig. 7. The CLSM image of UHT milk showed a
422 homogeneous distribution of both proteins and fat globules. Since homogenised UHT milk was
423 used for Qishta production, small amounts of large fat globules were observed. After 2 min of
424 heating (at 52 °C), the appearance of new structures was noticed as a result of protein and fat
425 interaction. At this stage, fat globules had a bigger diameter than that in milk and a spherical
426 regular shape (Fig. 7B). After 4 min of heating, the amount of aggregates increased and fat
427 globules had a bigger and more regular spherical shape (Fig. 7C). After six min of heating, the
428 temperature reached 80 °C and the size of the complexes kept on increasing (Fig. 7D). Since Fast
429 Green dye labelled both casein and whey protein, it was not possible to distinguish between these
430 two types of proteins. Whey proteins are known to be more sensitive to temperature than caseins

(Donato & Guyomarc, 2009), and caseins a can withstand heating at 140 °C for more than 20 min (Fox, 1981).

During the heat treatment of whole milk, casein and whey proteins are in competition to adhere on the milk fat globule membrane (Dalgleish, 1997; Hunt & Dalgleish, 1994; Ye, 2008). According to the protein concentration, the adhesion reaction between fat globules and milk proteins will occur. At low protein concentrations, whey proteins will be adsorbed preferentially; however, at higher protein concentrations (above than 3%), caseins will adhere (Hunt & Dalgleish, 1994; Ye, 2008). The milk used in our study during Qishta formation contains 3.2% protein which probably means that casein adsorption to the fat globule will be more pronounced.

3.3.2. Mechanism of Qishta formation

After 8 min of heating, the aggregates forming Qishta became visible. The CLSM images showed a large compact gel of proteins exhibiting an irregular form with a large fat droplet indicating the coalescence phenomena (Fig. 8A). The individual distribution of proteins showed that they form a matrix connecting and entrapping fat globules (Fig. 8B,C).

The kinetics of Qishta formation can be summarised as follows; an intense evaporation of water due to the increase in milk temperature occurs, followed by the formation of aggregates resulting from the interaction between denatured proteins and coalesced fat globules. The CLSM images showed that aggregate size increased with time, and that the maximum size was reached after 8 min heating, which corresponds to either a visible gel or to Qishta formation. At this level, Qishta can be defined as a dehydrated gel consisting of a complex of proteins entrapping the fat globules. In addition to these complexes, which represent the majority of Qishta, the CLSM images showed that the structures also obtained at 0, 2, 4 and 6 min were present. These

observations could be explained by the heterogenous temperature distribution and by the specific techniques of heating and skimming used during the production process of Qishta.

4. Conclusions

Qishta is a widely consumed Lebanese dairy product with an inconsistent composition that varies depending on handlers, raw material, and the process applied for its production. Qishta's texture is one of these parameters that varies from a producer to another. Understanding the mechanisms leading to Qishta formation is of high importance since it helps to improve its process. This study succeeded in identifying the presence of cross-links, other than the disulphide bridges, such as LAL and LAN, and then locating the amino acids and the peptide sequences involved in such links. Further studies are required to study the effect of cross-links on the digestibility of food products, since it has been reported that increasing the protein network has a negative effect on protein digestibility and on the relation between the presence of these cross-links and the strength of the coagulum. Finally, this study demonstrated that the presence of cross-links could be used as an indicator of the severity of the heat treatment applied, and this should be further investigated.

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478

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Figure legends

Fig. 1. Individual percentages of the 6 major proteins present in UHT milk (■) and Qishta (□) obtained by mass spectrometry analysis; the percentage of each protein was obtained by dividing the individual abundance by the total abundance.

Fig. 2. The difference in protein intensity (grey value) of Qishta analysed under reducing conditions (——) and under non-reducing conditions (----). The electropherogram was converted by image converter software and then analysed using ImageJ software.

Fig. 3. The mechanism of LAL and LAN formation: β-elimination of cystine, cysteine or serine resulting from dehydroalanine formation, Michael addition of lysine or cysteine yielding LAL or LAN, respectively.

Fig. 4. MS/MS spectrum of double peptide sequence DDQNPHSSNICNISCDK and FLDDDLTDDIMCVK linked by LAL derivative from serine and lysine interaction. These peptide sequences were found in α-lactalbumin. Amino acids are referred to with their abbreviation code.

Fig. 5. MS/MS spectrum of the intra chain LAN cross-link between two cysteines in the peptide sequence. This peptide sequence was found in α_{S2}-CN in Qishta. Amino acids are referred to with their abbreviation code.

Fig. 6. MS/MS spectrum of the double peptide sequence NECFLSHK and SLGKVGTR linked by LAL derivative from cysteine and lysine interactions. These peptide sequences were found in the bovine serum albumin in Qishta. Amino acids are referred to with their abbreviation code.

Fig. 7. Visualisation of milk fat globules and proteins with confocal microscopy before heat treatment (A) and after 2, 4, and 6 min of heating (B, C and D, respectively). Fat globules appear in red while protein appears in green.

Fig. 8. Visualisation of proteins (green) and fat (red) in Qishta (A); protein distribution (B) and fat droplet distribution (C).

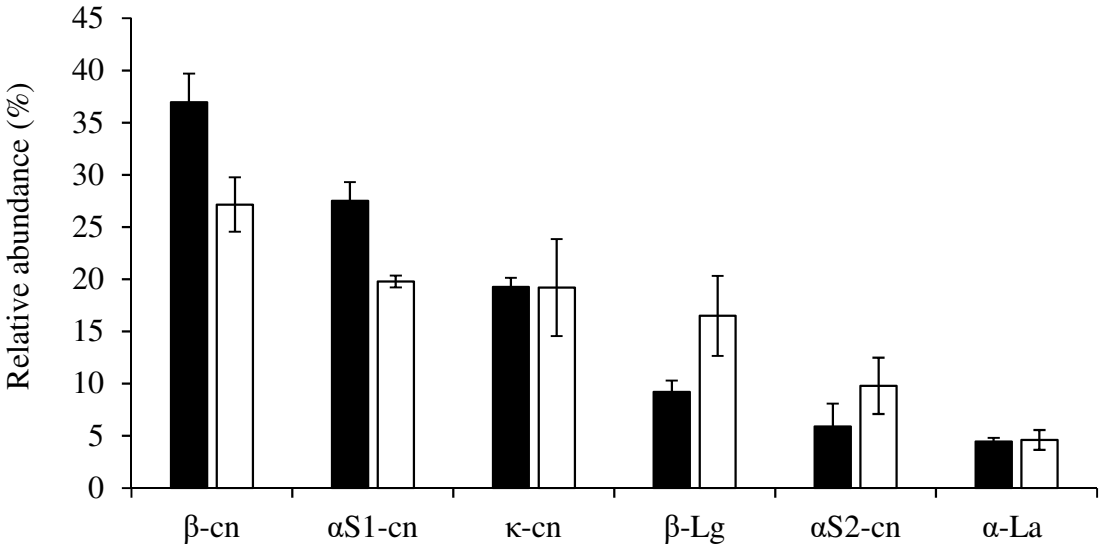
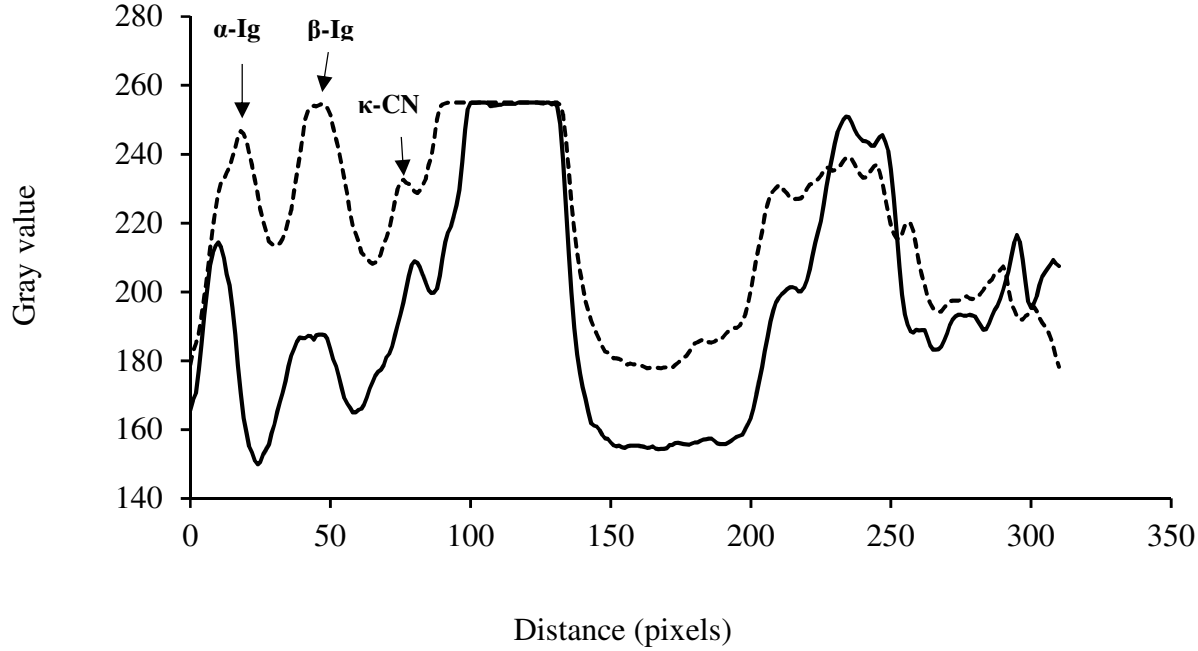

Relative abundance (%)
45
40
35
30
25
20
15
10
5
0
β-cn
αS1-cn
κ-cn
β-Lg
αS2-cn
α-La

Figure 1


α-Ig
β-Ig
κ-CN
Gray value
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220
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180
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140
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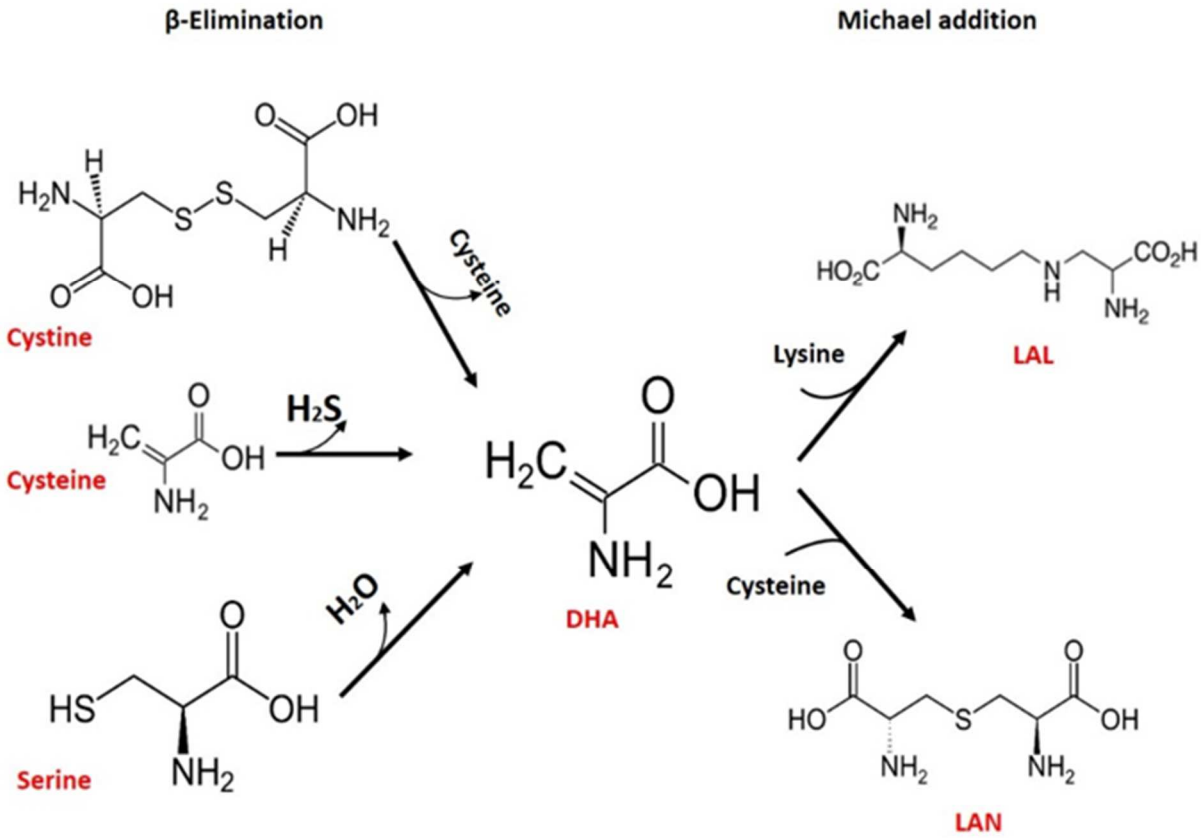
100
150
200
250
300
350
Distance (pixels)

Figure 2


β-Elimination
Michael addition
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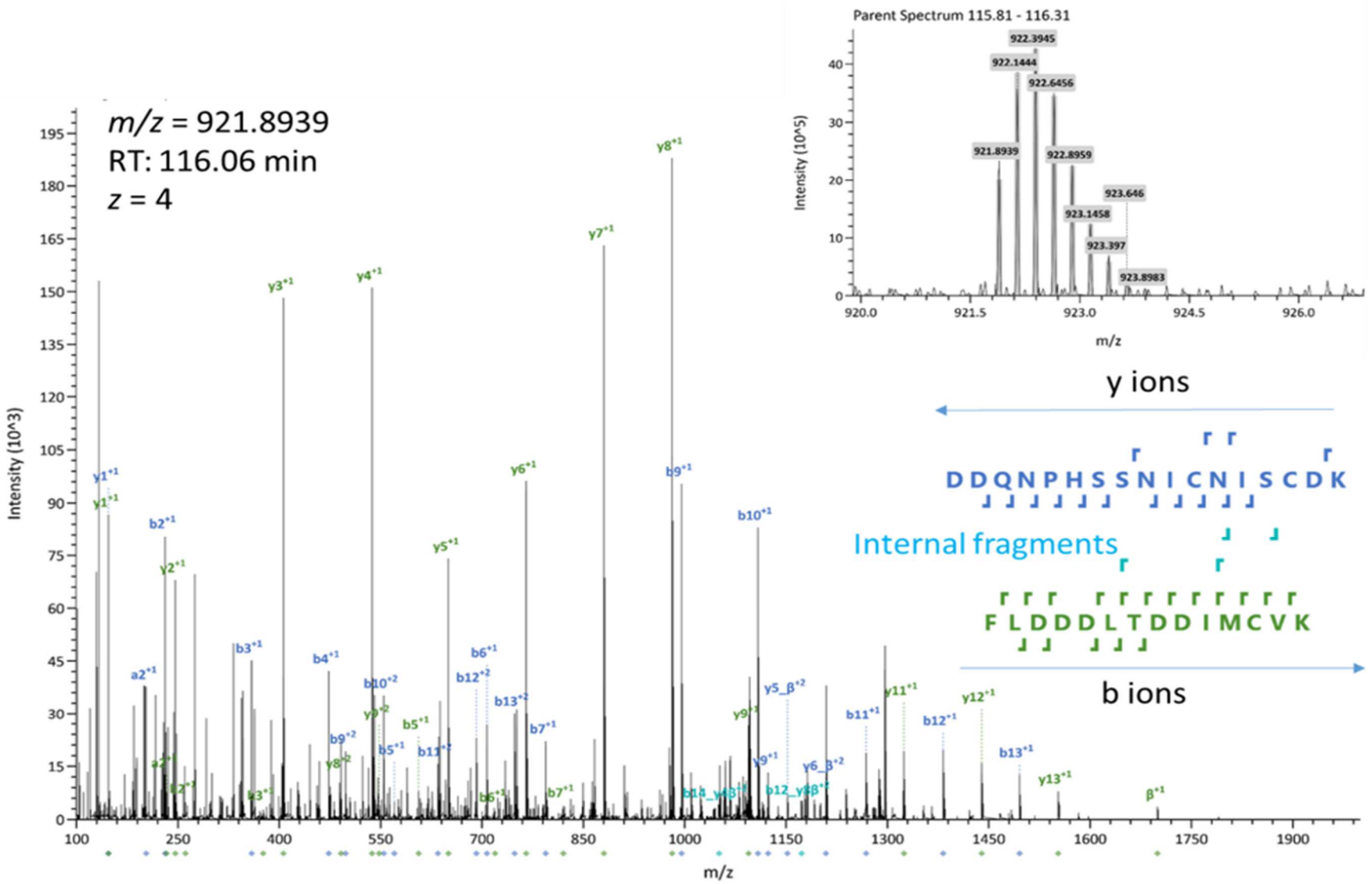
Cysteine
Cysteine
H₂S
Serine
H₂O
DHA
Lysine
LAL
Cysteine
LAN

Figure 3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m/z
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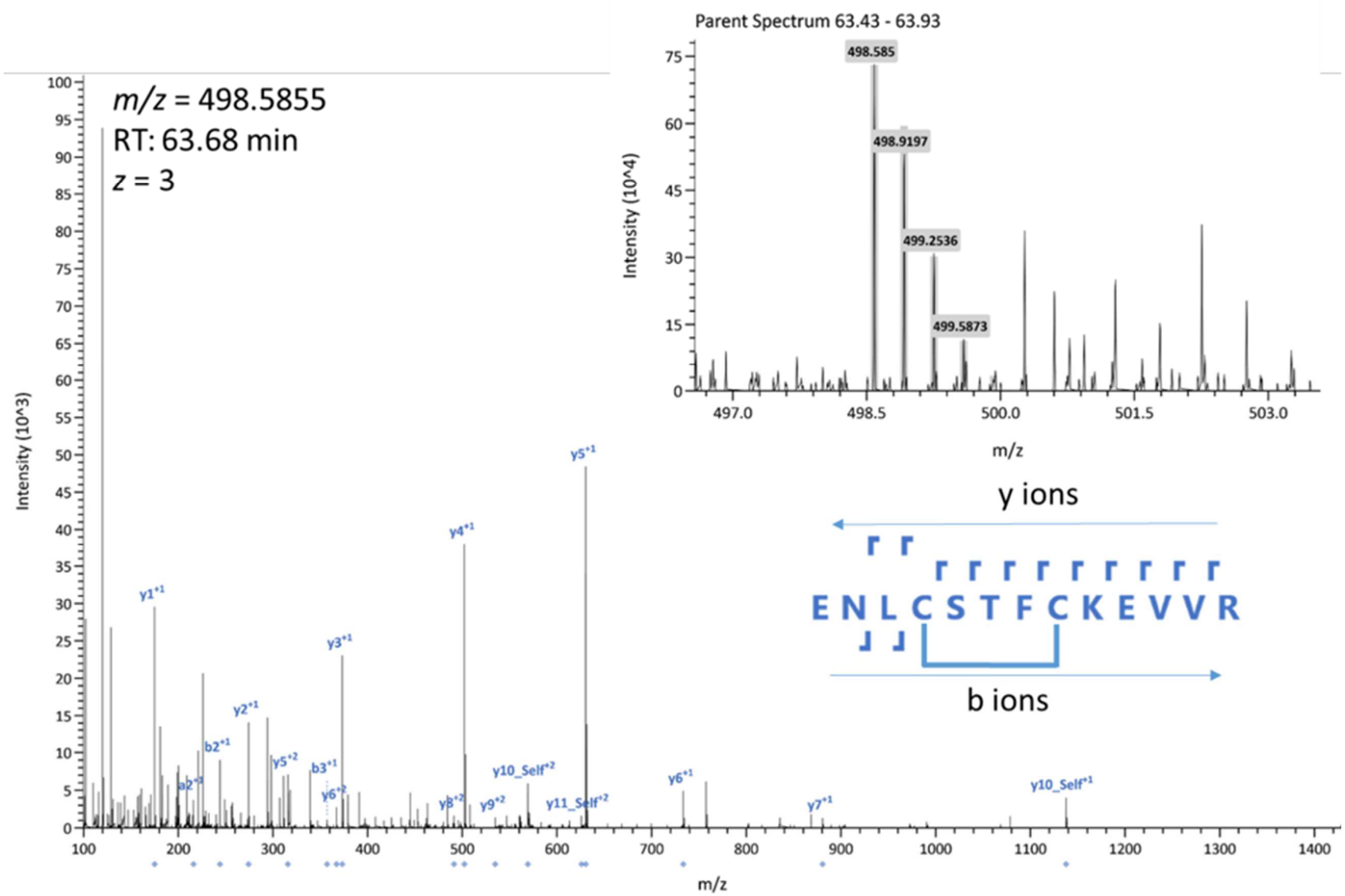
922.6456
921.8939
922.8959
923.646
923.1458
923.397
923.8983
Intensity (10^5)
y ions
D D Q N P H S S N I C N I S C D K
Internal fragments
F L D D D L T D D I M C V K
b ions

Figure 4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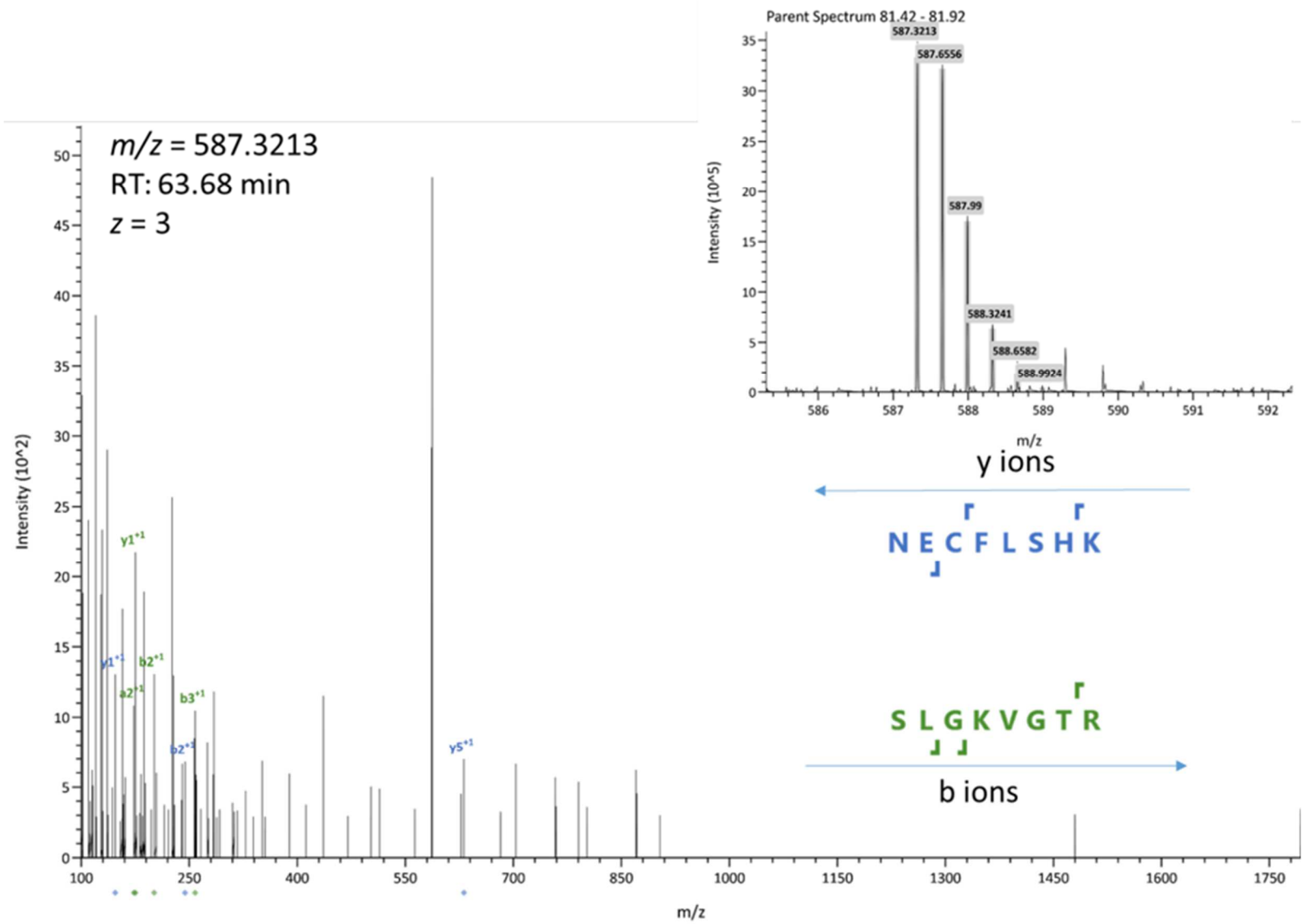
m/z
y ions
E N L C S T F C K E V V R
b ions

Figure 5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y5+1
Parent Spectrum 81.42 - 81.92
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587.6556
587.99
588.3241
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m/z
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N E C F L S H K
S L G K V G T R
b ions

Figure 6

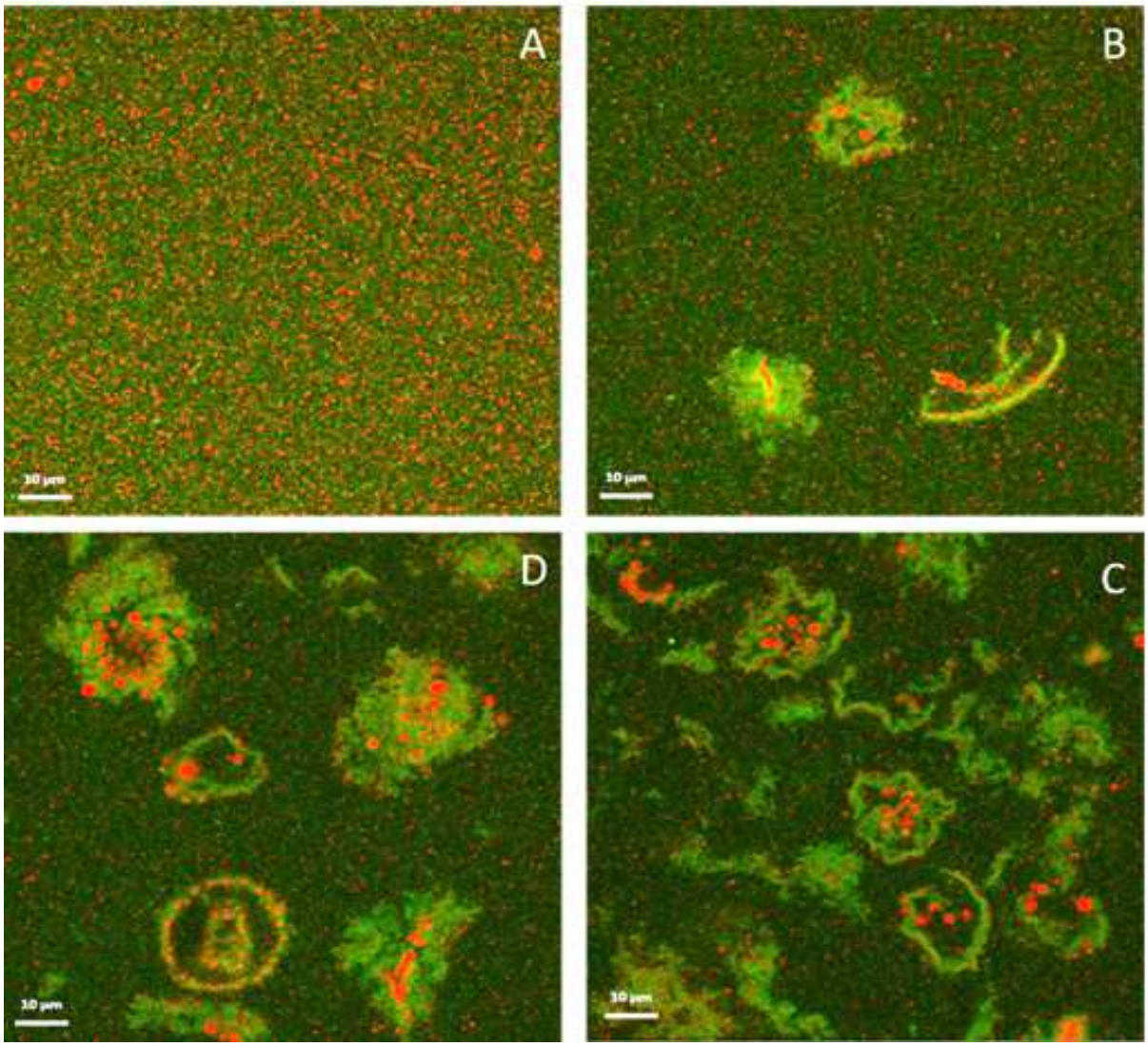

Figure 7

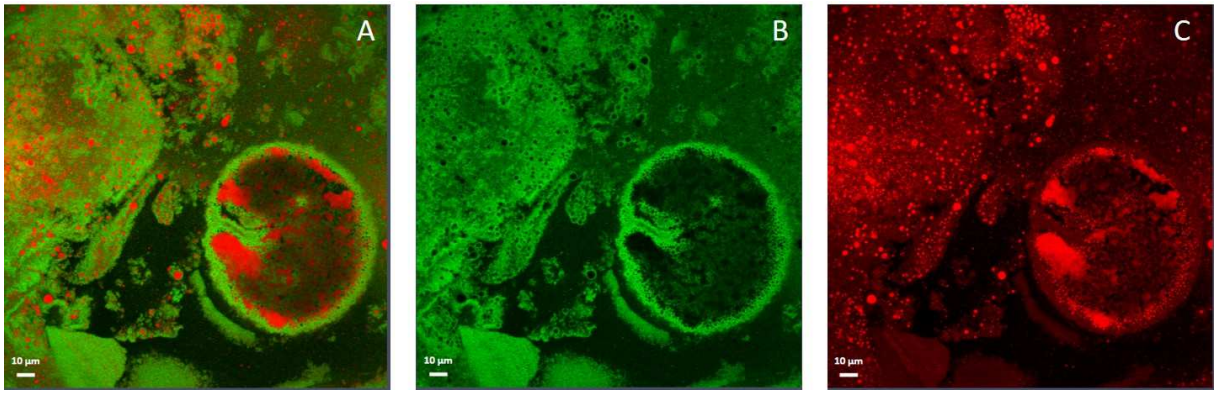

Figure 8

Table 1

Average relative abundance of the proteins present in milk and Qishta. [a]

Protein	UHT milk	Qishta
β-Casein	382518 ± 27334	348378 ± 96824
α_{S1}-Casein	274999 ± 58068	186559 ± 5682
κ-Casein	178523 ± 8670	198968 ± 46452
β-Lactoglobulin	96122 ± 11083	123990 ± 38322
α_{S2}-Casein	52432 ± 21994	76506 ± 26965
α-Lactalbumin	41804 ± 3573	39050 ± 9497
Lactadherin	863 ± 27	948 ± 231
Serum albumin	394 ± 14	450 ± 70
Lactoferrin	364 ± 29	435 ± 123

[a] Each sample was analysed in triplicate.

Table 2

Casein and whey protein distribution in UHT milk obtained by our mass spectrometry analysis compared with those in fresh raw milk and Swedish UHT milk. [a]

Protein	UHT milk (%)	Swedish UHT (%)	Fresh raw milk (%)
Casein	86.4	87.4	83.0
β-Casein	37.0	44.1	27.1
α_{S1}-Casein	27.5	30.5	26.0
κ-Casein	15.9	4.2	10.0
α_{S2}-Casein	6.0	8.6	10.0
Whey protein	13.6	12.6	17.0
β-Lactoglobulin	9.2	9.4	11.0
α-Lactalbumin	4.4	3.2	4.3

[a] Data from Dalgleish (1993), Heck et al. (2009), and Karlsson et al. (2017).

Table 3
Double chain peptides containing LAL and LAN. [a]

Sample	Theorical Mr and charge state	Detected *m/z*	Elution time	Error (ppm)	Protein 1	Peptide sequence 1	Theorical Mr monoisotopic of peptide sequence 1	Localisation on protein 1	Protein 2	Peptide sequence 2	Theorical Mr monoisotopic of peptide sequence 2	Localisation on protein 2	Crosslink site 1	Crosslink site 2
LAL between serine and lysine														
Q1-1	(1) 925.8921	925.8931	105.48	-1.08	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S**S**N**I**C**N**I**S**C**DK	2002.8108	63-79			1714.7429	80-93	S7	K12
Q1-2	(z = 4)	925.8931	105.66	-1.08					sp|P00711|20-142|LALBA_BOVIN	FLDDDLTDDI**M**C**V**K			S8	
Q1-3		925.8929	105.66	-0.86									S14	
LF-1		925.8934	105.31	-1.40										
LF-2		925.8931	106.36	-1.08										
LF-3		925.8932	106.56	-1.19										
Q1-1	(2) 921.8939	921.8944	115.92	-0.54			2002.8108	63-79	sp|P00711|20-142|LALBA_BOVIN	FLDDDLTDDIM**C**V**K**	1698.7480	80-93	S7	K12
Q1-2	(z = 4)	921.8942	116.39	-0.33	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S**S**N**I**C**N**I**S**C**DK							S8	
Q1-3		921.8943	116.31	-0.43									S14	
LF-1		x	x	x										
LF-2		x	x	x										
LF-3		x	x	x										
Q1-1	(3) 957.9172	957.9168	95.45	0.42	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S**S**N**I**C**N**I**S**C**DK	2002.8108	63-79	sp|P00711|20-142|LALBA_BOVIN		1842.8379	80-94	S7	K12
Q1-2	(z = 4)	x	x	x						FLDDDLTDDI**M**C**V**K**K**			S8	
Q1-3		957.9168	95.04	0.42									S14	
LF-1		x	x	x										
LF-2		x	x	x										
LF-3		x	x	x										
Q1-1	(4) 677.3094	677.3095	59.92	-0.15										
Q1-2	(z = 3)	677.3099	60.39	-0.74										
Q1-3		677.3095	59.45	-0.15										
LF-1		677.3094	59.47	0										
LF-2		677.3094	60.07	0										
LF-3		677.3094	60.06	0	sp|P02663|16-222|CASA2_BOVIN	N**M**AINP**S**K	889.4327	25-32	Isp|P02663|16-222|CASA2_BOVN	ENL**C**STF**C**K	1157.4844	33-41	S7	K9
Q1-1	(5) 648.5323	648.5322	50.86	0.15				63-79			605.2995	59-62	S7	K4
Q1-2	(z = 4)	648.5322	51.25	0.15			2002.8108						S8	
Q1-3		648.5322	50.29	0.15	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S**S**N**I**C**N**I**S**C**DK			sp|P00711|20-142|LALBA_BOVIN	IW**C**K			S14	
LF-1		648.5322	50.32	0.15										
LF-2		648.5322	50.91	0.15										
LF-3		648.5322	50.97	0.15										
Q1-1	(6) 465.9223	465.9223	30.54	0		I**S**QR	502.28639	167-170		EAMAP**K**HK	910.4695	100-107	S2	K6
Q1-2	(z = 3)	465.9223	30.65	0	sp|P02663|16-222|CASA2_BOVIN									
Q1-3		465.9224	30.26	-0.21					sp|P02666|16-224|CASB_BOVIN					
LF-1		465.9223	29.54	0										
LF-2		465.9223	29.60	0										
LF-3		465.9223	30.30	0										
LAN between two cysteines														
Q1-1	(7) 498.5855	498.5854	63.81	0.20			1526.7221	33-45					C4	C8
Q1-2	(z = 3)	498.5854	63.92	0.20	sp|P02663|16-222|CASA2_BOVIN	ENL**C**STF**C**KEVVR								
Q1-3		498.5855	63.67	0										
LF-1		498.5854	63.67	0.20										
LF-2		498.5854	64.11	0.20										
LF-3		498.5854	64.08	0.20										
Q1-1		619.2674	37.66	0										
Q1-2	(8) 619.2674	619.2675	37.74	-0.16										
Q1-3	(z = 3)	619.2673	37.32	0.16										
LF-1		619.2673	37.12	0.16										
LF-2		619.2673	37.49	0.16										
LF-3		619.2673	37.40	0.16	sp|P00711|20-142|LALBA_BOVIN	DDQNPHSSN**I**C**N**IS**C**DK	1888.7680	63-79					C11	C15
LAL cysteine and lysine														
Q1-1	(9) 587.3213	587.3198	80.80	2.55		NE**C**FLSHK	976.4436	123-130		SLG**K**VGTR	816.4817	452-459	C3	K4
Q1-2	(z = 3)	587.3198	81.75	2.55	sp|P02769|25-607|ALBU_BOVIN				sp|P02769|25-607|ALBU_BOVIN					
Q1-3		587.3199	81.43	2.38										
LF-1		587.3198	80.75	2.55										
LF-2		587.3199	81.41	2.38										
LF-3		587.3201	81.60	2.04										

[a] The error was calculated from the absolute difference between the theoretical and detected molecular masses (Mr) divided by the theoretical Mr and multiplied by 106. Amino acids in bold red font are those involved in cross-links; post translational modifications are highlighted in bold black font.

Table 4
Intensity of the LAL cross-links, resulted from the β-elimination of serine and condensation with lysine, present in UHT milk and Qishta. [a]

m/z	Number	Qishta intensity	Average	St dev	Milk intensity	Average	St dev
925.8931	1	7.95E+06	4.00E+06	2.64E+06	3.08E+06	3.35E+06	5.78E+05
		1.83E+06			2.76E+06		
		2.21E+06			4.22E+06		
921.8943	2	4.60E+06	3.60E+06	1.28E+06	0.00	0.00E+00	0.00E+00
		1.68E+06			0.00		
		4.53E+06			0.00		
957.9168	3	2.27E+06	1.12E+06	7.69E+05	0.00	0.00E+00	0.00E+00
		0.00			0.00		
		1.08E+06			0.00		
677.3095	4	8.72E+06	4.32E+06	2.93E+06	5.18E+05	5.22E+05	6.47E+04
		6.83E+05			6.19E+05		
		3.56E+06			4.29E+05		
648.5322	5	8.54E+06	3.21E+06	3.55E+06	1.32E+06	1.20E+06	2.68E+05
		4.25E+05			7.98E+05		
		6.58E+05			1.48E+06		
465.9223	6	3.68E+06	2.49E+06	7.96E+05	8.42E+05	1.30E+06	4.04E+05
		2.14E+06			1.91E+06		
		1.64E+06			1.16E+06		

[a] Analyses were done in triplicate.

Table 5
Double chain peptides containing LAL. [a]

Theorical Mr and charge state	Detected *m/z*	Elution time	Error (ppm)	Protein 1	Peptide sequence 1	Theorical Mr monoisotopic of peptide sequence 1	Localisation on protein 1	Protein 2	Peptide sequence 2	Theorical Mr monoisotopic of peptide sequence 2	Localisation on protein 2	Crosslink site 1	Crosslink site 2
(1) 677.3117 ($z = 3$)	677.3096	47.88	3.10	p|P02663|16-222|CASA2_BOVIN	NMAINP**S**K	889.4327	25-32	sp|P02663|16-222|CASA2_BOVIN	ENL**C**STF**C**K	1157.4844	33-41	S7	K9
(2) 671.9781 ($z = 3$)	671.9780	55.60	0.15	sp|P02663|16-222|CASA2_BOVIN	NMAINP**S**K	873.4379	25-32	sp|P02663|16-222|CASA2_BOVIN	ENL**C**STF**C**K	1157.4844	33-41	S7	K9
(3) 885.1151 ($z = 3$)	885.1146	76.28	0.56	sp|P02663|16-222|CASA2_BOVIN	L**T**EEEK	747.3651	153-158	sp|P02663|16-222|CASA2_BOVIN	HYQ**K**ALNEINQFYQK	1922.96394	77-91	T2	K4

[a] The error was calculated from the absolute difference between the theoretical and detected molecular masses (Mr) divided by the theoretical Mr and multiplied by 106. Amino acids in bold red font are those involved in cross-links; post translational modifications are highlighted in bold black font.