International Journal of Dairy Technology

SDT Society of Dairy Technology

Check for updates

doi: 10.1111/1471-0307.13156

RESEARCH ARTICLE

Real-time measurement of heat stability of skim milk using attenuated total reflectance (ATR)-FTIR spectroscopy

ELENA HAYES,[1,2] LIAM KELLY,[1] TUGCE AYDOGDU,[1] NORAH O'SHEA,[1] COLM O'DONNELL,[1,2] DEREK GREENE[3] and MARK A FENELON[1,2,*]

[1]*Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork P61C996, Ireland,* [2]*School of Biosystems and Food Engineering, University College Dublin, Dublin D04V1W8, Ireland, and* [3]*School of Computer Science, University College Dublin, Dublin D04V1W8, Ireland*

Milk proteins are susceptible to denaturation and aggregation upon heating, affecting product quality and shelf-life. Understanding the underlying molecular changes during heating is important to the dairy industry for process optimisation and product functionality. This study used Attenuated Total Reflectance (ATR)-Fourier Transform Infrared (FTIR) spectroscopy to non-destructively measure changes in protein molecular structure as the precursor to heat-induced aggregation in milk. Raw skim milk was divided into three subsamples, adjusted to pH 6.2, native pH or pH 7. Each sample was heated at 85°C on a BioATR crystal, with scans taken at 1-min intervals over 20 min using FTIR to measure protein denaturation and aggregation. The second derivative of the amide I region was used to measure changes in protein structure, with the spectra for pH 6.2 samples changing faster than pH 6.8 or pH 7 samples, indicating a higher rate of denaturation. The peak at 1072 cm^{-1} related to colloidal calcium phosphate (CCP) increased with increasing temperature and pH. More extensive changes in CCP between colloidal and serum phases and protein denaturation/aggregation correlated with lower heat stability in milk. This study highlights the potential of ATR-FTIR spectroscopy for assessing the heat stability of milk via in situ measurement of changes in protein structure and CCP.

Keywords Dairy, Milk processing, Heat stability, Spectroscopy, Protein structure, β-lactoglobulin.

Author for correspondence. E-mail: mark.fenelon@teagasc.ie

INTRODUCTION

Heat treatment is one of the dairy sector's most widely used unit operations. Heat stability of milk can be defined as the length of time taken for milk to coagulate at a constant temperature in an oil bath. Milk can be separated into two types based on its heat stability profile. The majority of bulk milk samples are type A, which produces a local maximum between pH 6.6 and 6.8 and a local minimum between pH 6.9 and 7.1 (Aydogdu *et al*. 2023). Milk with higher heat stability typically undergoes less denaturation/aggregation when heated, potentially increasing processing time. Heat-stable milk is also less likely to form sediment during high-temperature storage than non-heat-stable milk Loveday *et al*. 2021 (Huppertz 2016).

Milk composition significantly affects heat stability, and seasonal variations can impact its processability (Pouliot and Boulet 1995; Chen *et al*. 2015). Key factors influencing heat stability include pH, protein and mineral content, with non-protein nitrogen (NPN) playing a notable role due to its high urea content, which stabilises milk during heating (Crowley *et al*. 2014). NPN levels vary with diet, highlighting the importance of cow feeding regimes, especially in grass-based seasonal milk production where weather influences the diet. The molecular size and structure of milk proteins impact their ability to withstand heat treatment (Fox *et al*. 2015).

Calcium is another factor that has a significant influence on milk functionality. Calcium is found in milk in two forms: colloidal calcium phosphate (CCP) and soluble (serum) calcium

Li *et al*. 2019. The CCP is bound to the casein micelle, and the levels vary depending on external factors such as pH, temperature and protein concentration. Heating milk has been shown to increase the levels of CCP in milk (Dumpler *et al*. 2020) due to CCP binding to the casein micelle when the hydrophobic region is exposed during the unfolding of protein. Reduction in colloidal calcium phosphate (CCP) increases heat stability in milk with a pH of <7; however, when >60% CCP is removed at a pH >7.2, destabilisation can occur (Fox and Hoynes 1975).

Most milk and dairy products are heated during processing before consumption. Pasteurisation involves heating milk to at least 72°C for 15 s (Bulletin of the IDF no. 496/2019) for the eradication of pathogenic bacteria and, more recently, to 75°C for 20 s to ensure inactivation of *Mycobacterium avium* subsp. *Para tuberculosis* (FSAI, 2021). Products such as ultra-high temperature (UHT) milk, milk powders and condensed milk require thermal stability to ensure efficient manufacturing processes. Additionally, dairy ingredients produced for use in regulated formulations, such as infant formula, must adhere to stringent specifications for nutrient content. Functionality during processing is a critical criterion to meet these regulatory standards.

A recent review on the heat stability of milk by Dumpler *et al*. (2020) summarises the current literature. Most causes of heat-induced coagulation are due to the denaturation of proteins, mainly whey proteins. Beta-lactoglobulin (β-Lg) begins to denature at 70°C; however, other minor whey proteins, such as bovine serum albumin (BSA) and lactoferrin, have been shown to denature at lower temperatures of around 60°C (Matsarskaia *et al*. 2020). The hydrophobic core of β-Lg is exposed upon heating, forming inter- and intra-molecular aggregates. Increased aggregation can be related to lower heat stability (Li *et al*. 2021), which can influence milk product processing.

FTIR is widely used to measure milk composition through spectral changes associated with the vibration of bonds within protein, fat and lactose components. Indirect measurements are used to a lesser extent; for example, casein is calculated from the protein content (McDermott *et al*. 2016). Studies have used FTIR to identify functionality related to milk processing; however, these prediction models have limited commercial application due to their low accuracy (Manuelian *et al*. 2017; Visentin *et al*. 2017). The models used are primarily based on indirect measurement using the full spectral dataset at a single time point. Thus, while milk composition (protein, fat and lactose) can be directly measured, physicochemical changes in protein denaturation and aggregation are time-dependent and, therefore, more challenging to determine.

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy offers significant benefits and novel applications for measuring structural changes in milk proteins. This technique is non-destructive, rapid and requires minimal sample preparation, making it highly efficient for routine analysis. ATR provides an alternative to traditional transmission infrared, which is often not suitable for liquid samples. In an ATR system, the light is internally reflected, and the sample interacts with the evanescent wave, making it suitable for highly absorbing samples (Grdadolnik, 2002). ATR-FTIR provides detailed molecular fingerprints of milk components, enabling precise identification and quantification of proteins, lipids, carbohydrates and other constituents. Its sensitivity to conformational changes in proteins and interactions among milk components allows for real-time monitoring of structural alterations during simulated thermal processing.

Attenuated total reflectance (ATR) FTIR has been used to investigate secondary structural changes in milk proteins (Markoska *et al*. 2019b). Proteins and polypeptides give rise to nine absorption bands in the infrared region, with amide I and amide II being the most prominent (Kong and Yu 2007). The current study uses time-resolved measurements to ascertain the change in identified peaks related to heat stability, such as the amide I region, peaks relating to β- sheets and α-helix, and the interaction of CCP with the casein micelle. The findings could advance the understanding of milk heat stability, providing a foundation for in-process measuring techniques.

MATERIALS AND METHODS

Experimental design

The study aimed to determine whether structural changes in milk proteins detected by ATR-FTIR can be correlated with heat stability. Milk composition for fat, total protein, lactose and total solids was measured using FT-MIR (Bentley DairySpec FT, Bentley Instruments, Chaska, MN, USA). Milk was collected directly from the bulk tank and was centrifuged to remove fat. All subsequent analyses were conducted on skim milk.

Five replicate trials (S1–S5, where S stands for milk samples from each replicate trial; e.g. S1 = milk samples from trial 1) were conducted between February and May of 2022, and the milk composition for each trial can be found in Table 1. Both skim milk and whey (from the same skim) were tested at three pH values: pH 6.2, native pH at 6.8 and pH 7. Attenuated total reflectance FTIR was carried out on all five trials, with subsequent analysis by HPLC on samples S4 and S5 to validate observed spectral results.

The milk samples used in the study were collected from two research herds at Teagasc Animal and Grassland Research Centre, Moorepark, Cork, Ireland. All cows were fed a predominantly perennial ryegrass (pasture-based system)-based diet supplemented with concentrates, as outlined by Egan *et al*. (2018). The total concentrates fed were between 300 and 500 kg/cow per annum. The mean calving date was 23rd February 2022.

Table 1 Composition of raw milk.

	Date of collection	Protein (%)	Lactose (%)	Fat (%)	pH
S1	03/02/2022	3.70	4.76	4.41	6.78
S2	15/02/2022	3.52	4.80	4.60	6.84
S3	24/3/2022	3.53	4.81	4.10	6.80
S4	25/05/2022	3.62	4.91	3.78	6.75
S5	25/05/2022	3.93	5.05	4.23	6.73

Samples S1–S5 are raw milk samples collected from bulk tanks at Teagasc Moorepark research farms. S1–S3 were collected during early lactation. S4 and S5 were collected in mid lactation.

In situ pH measurement

The pH of milk was measured continually while milk was heated from 20 to 85°C. Skim milk was adjusted to a pH range of 6.2–7 to align with the pH values used in the experimental design. The analysis was carried out as per Aydogdu *et al*. (2023). A combination pH/temperature sensor EasyFerm Bio HB VP 120 Pt1000, supplied by Irish Power and Processing (Co. Kilkenny, Ireland), was used to measure the change in milk pH as it was heated. Milk (20 mL) was gently stirred continuously during heating. An in situ pH probe measured the pH of milk every 60 s until the milk reached 85°C.

Sample preparation

Raw whole milk was collected from a bulk tank. The cream from milk was separated using centrifugation at 4°C at 2688×*g* for 30 min. The pH of the resulting skim milk was altered using 0.1 M HCl to pH 6.2 and 0.5 M NaOH to pH 7. Samples were left to equilibrate for 60 min, and the pH was measured again.

Preparation of whey

Whey was prepared from each skim milk sample (S1–S3). Diluted rennet (1:20) was added to skim milk heated to 32°C for 45 min. The serum phase was filtered using Whattman no 1 filter paper. Whey samples were adjusted to the same pH ranges as the skim.

Attenuated total reflectance Fourier transform infrared spectroscopy

Each sample was analysed using Bruker Invenio S FTIR (Bruker Optik, GmbH, Ettlingen, Germany) with a BioATRcell II attachment. An MCT detector was cooled with liquid nitrogen, and the sample compartment was purged to remove moisture and carbon dioxide. Samples were pre-heated to 50°C in a water bath before being transferred to the ATR crystal, which was pre-heated at 85°C. Each sample was run with 20 repeated scans every 45 s. Each spectrum was an average of 64 scans. The background measurement was taken using a blank ATR crystal. Water spectra were measured and subsequently subtracted from the sample spectra.

Heat coagulation time (HCT)

Heat stability was measured as heat coagulation time (HCT) using the oil bath method on the same day as the FT-MIR spectra were acquired. Each sample was measured in duplicate. The heat stability of the raw skim milk samples was determined using an updated methodology based on that originally described by Davies and White (1966). Aliquots (3.2 g) of raw skim milk samples at the above pH values (6.2, 6.8 and 7) were transferred into 4 mL glass tubes and stoppered. The tubes were placed in a rack which was submerged in a silica gel Elbanton oil bath (Hettich Benelux Laboratory Equipment, Geldermalsen, the Netherlands) at 140°C and oscillated at eight oscillations/min for the duration of the test. The heat stability of the samples was determined as the time taken for visible coagulation to occur, at which point the duration from which the rack was immersed in the oil bath was recorded.

High-performance liquid chromatography (HPLC) analysis

Reverse-phase HPLC was used to quantify the amount of protein denatured in heated samples at each pH level for milk samples S4 and S5. HPLC was conducted as per Mounsey and O'Kennedy (2009). Samples were diluted to a protein content of 0.25% (wt./wt.) using 0.5 M sodium acetate at pH 4.6 to precipitate casein from solution, and samples were centrifuged at 14 000 g×*g* at room temperature for 10 min. The supernatant was subject to HPLC analysis. Samples were analysed in duplicate, and results are presented as the mean of values.

Statistics & spectral data analysis

Spectral analysis was carried out using OPUS 6.5 (Bruker Optik, GmbH, Ettlingen, Germany). All milk samples were treated for atmospheric compensation such as moisture and carbon dioxide. Water subtraction was performed to remove the water band overlapping the protein region. All samples were vector normalised between 900 and 1800 cm^{-1}. Samples were saved as data point tables and exported to Excel. Data analysis was carried out in MATLAB 2020b (The MathWorks, Inc., Natick, MA, USA). Principal Component Analysis (PCA) was employed to segregate samples based on their pH levels. PCA was carried out on spectra between 900 and 1800 cm^{-1} using the PCA function in Unscrambler X (version 10.5.1, CamoSoftware, 2018).

The second derivative of the amide I region (1600–-1700 cm^{-1}) with Savitzky–Golay smoothing (13 smoothing points and polynomial order of 2) was used to detect structural changes in protein. Specific wavelengths associated with secondary structural characteristics of protein (1624, 1656 and 1672 cm^{-1}) were selected, and the rate of change

over time at each peak was measured. Analysis of variance was measured using a one-way ANOVA in Unscrambler to determine if changes over time at the aforementioned wavelengths were significant. ANOVA was also carried out on HPLC data to investigate the differences in native protein between samples of varying pH. Pearson correlations between HPLC results and changes in FTIR spectra at 1624 and 1655 cm^{-1} were carried out to validate the changes seen in the spectra related to whey protein denaturation, thus relating to heat stability.

RESULTS & DISCUSSION

This study examined the effects of pH and heating on time-dependent changes in protein molecular structure in skim milk and whey at 85°C. Composition varied between milk samples, particularly for protein content (Table 1). While milk composition is known to influence heat stability (Kelly *et al*. 1982; Fox *et al*. 2015; Huppertz 2016), the correlation between protein structural change and the onset of thermal instability is less defined. This study's primary aim is to determine the feasibility of using protein structural change as an indicator of heat stability and, thus, processability. Principal component analysis of skim milk samples (pH 6.2–pH 7) observed separate groupings depending on the pH at which they were heated (Figure 1). The effects of pH on milk heat stability are well known, and this result is likely due to the partial destabilisation of casein micelles as the pH of milk decreases towards the isoelectric point (Huppertz 2013; Sutariya *et al*., 2017). Principal component one shows separation over time, particularly for pH 6.8 and pH 7, while for pH 6.2, the change was less, indicating that the changes for pH 6.2 occurred more rapidly. The majority of variance is explained in PC1 (99%), which is mainly related to time, while PC 2 explains the variance in pH. This variance is explained by loading peaks at 1497 and 1643 cm^{-1}. PCA analysis was also carried out using the native whey protein from HPLC as a reference value. The score plot was labelled according to the pH groups, and clear separation was seen for pH 6.2 compared to pH 6.8 and pH 7, which showed a less obvious separation. PC 1 explained 97% variance due to pH, as seen by the separation of pH 6.2 from pH 6.8 and pH 7. The second principal component is most likely related to heating time, as the difference in the concentration of whey protein can be seen along PC2. As expected, pH 6.2 presents the greatest difference, while there was very little separation along PC2 for pH 6.8, which agrees with the spectral data. The loading plots (Figure 2) presented below highlight the main spectral regions associated with the variance in PC1 and PC2. The amide I and II regions are the main source of variation in PC 1, and the region associated with colloidal calcium phosphate explains a lot of the variance in PC2. It is as a result of this exploratory analysis that the amide I region and the colloidal calcium phosphate region were examined in greater detail.

FTIR results

Skim milk

The structural changes of interest were observed in the region 1700–1600 cm^{-1} (Figure 3), known as the amide I region (Etzion *et al*. 2004) and associated with C=O stretching vibration (Lefèvre and Subirade 2001). Changes in this region are identified with secondary structural characteristics (Grewal *et al*. 2018; Andrade *et al*. 2019), mostly related to

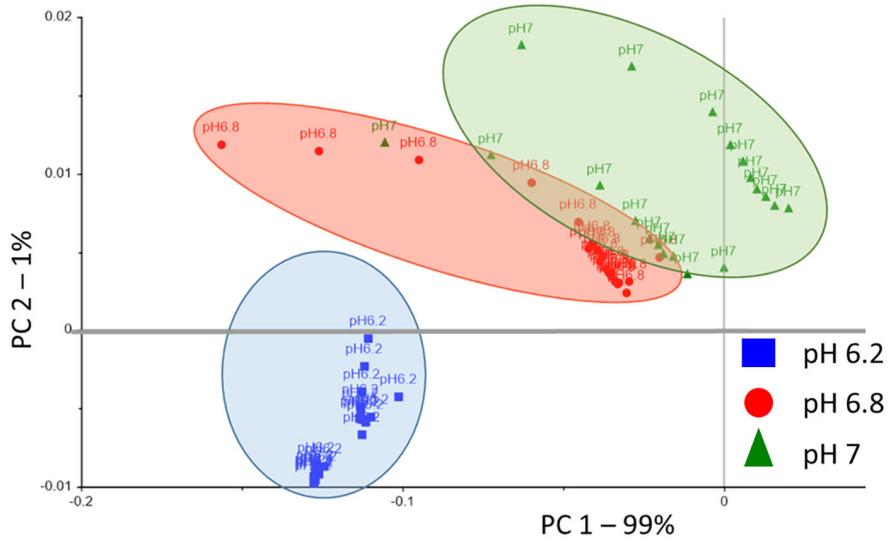

Figure 1 Scores plot from PCA analysis of pH-adjusted milks grouped according to pH (blue = pH 6.2; red = pH 6.8; green = pH 7.0).

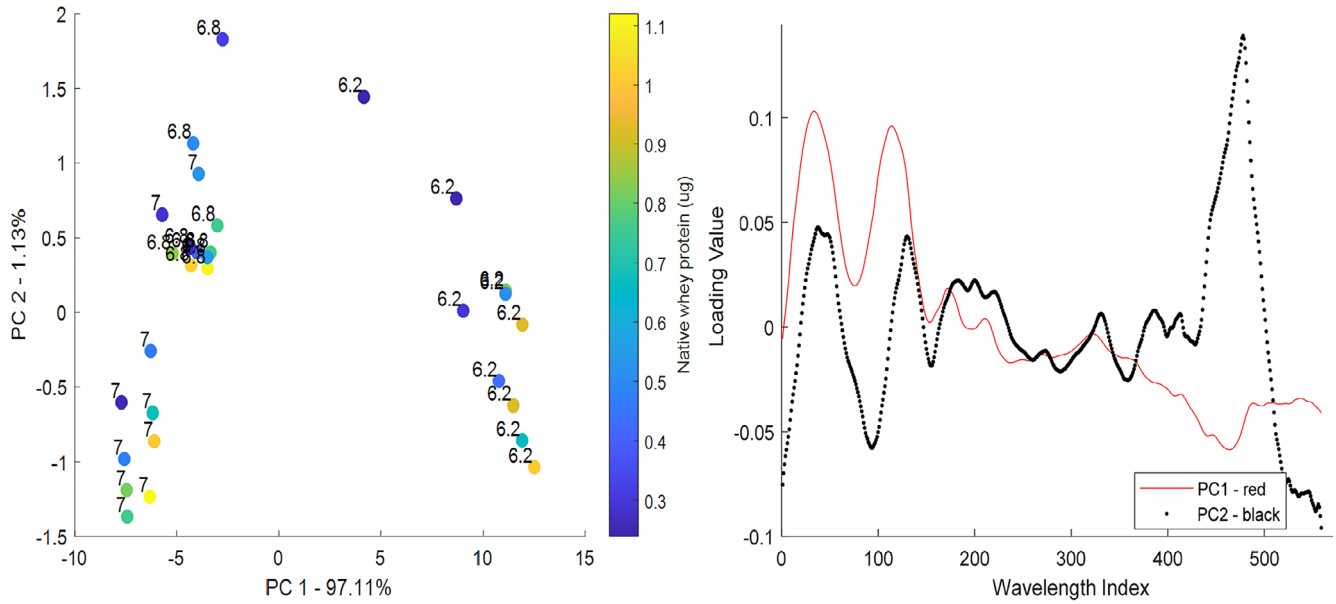

Figure 2 Score plot (left) and loading plot (right) for pH-adjusted samples grouped by concentration of native whey protein according to HPLC.

the whey proteins due to their organised structure and lower heat stability, but can also be attributed to changes in casein (Daniloski *et al*. 2022). Secondary structures such as α helices and β sheets decrease as a function of time and are replaced by intermolecular sheets (Kong and Yu 2007). Multiple studies have related specific wavelengths with secondary structural characteristics of proteins (Lefèvre and Subirade 2001; Carbonaro and Nucara 2010; Mediwaththe *et al*. 2018; Markoska *et al*. 2019a, 2019b). Differences in spectra were observed at 1624 cm^{-1} (β sheets), 1630–-1634 cm^{-1} (intramolecular β sheets), 1645 cm^{-1} (random coil), 1652 cm^{-1} (α helices) and 1672 (β turns). The two main regions examined were 1624 and 1656 cm^{-1}, which showed significant ($P < 0.05$) change during heating. The peak at 1624 cm^{-1} decreased with heating over time for all skim milk samples. Figure 3 shows the amide I region for S3 milk samples, representing the typical graph for all trials. This agrees with studies by Grewal *et al*. (2018) and Markoska *et al*. (2019b), who also observed decreases in milk MIR spectra at 1624 cm^{-1} upon heating. The change in spectra over time can be seen in Figure 3, where for pH 6.2, the peak at 1624 cm^{-1} changes faster than pH 6.8 or 7. There was a positive correlation between the changes in spectra at 1624 cm^{-1} and the decrease in native whey protein measured by HPLC for all pH samples, indicating that the changes observed in the spectra are related to the denaturation of whey protein. There was also a positive correlation between change in quantity of native α-lactalbulmin and change in absorbance at 1655 cm^{-1}, a region associated with α-helix structures mainly.

The rate of change of absorbance was calculated using the equation $y = mx + c$, where y is the absorbance, x is time, m is the slope (rate of change) and c is the x-line intercept. The extent of the decrease varied with pH, and the rate of change in the first 10 min of heating (85°C) was significant for pH 6.2 ($P = 0.023$). The average rate of change between 0 and 10 min for pH 6.2 was -3.7×10^{-5} abs/min compared to an average of -1.5×10^{-5} abs/min for pH 6.8 and -5.32×10^{-6} for pH 7. The change between 10 and 20 min was slower for pH 6.2, indicating that most proteins had already been denatured. In contrast, the rate of change increased for pH 6.8 between 10 and 20 min to 2.28×10^{-5} abs/min, which indicates that the higher pH is more heat stable and requires prolonged heating to denature milk proteins. A similar result can be seen in the HPLC data (Figure 4), where higher levels of denatured protein were evident in pH 6.8 samples after heating for over 10 min.

While there was a visual change in spectra over time for pH 6.8 and 7, it was not significant. The lower level of denaturation for pH 6.8 and pH 7 can be linked to increased heat stability, that is, no significant change was identified in the spectra (Figure 3). At pH 7, there were slight decreases from 5 to 20 min (Figure 3). While it was not possible to directly correlate results from the oil bath with FTIR due to the difference in temperature, the findings from both show that pH 6.2 was unstable (Table 2), unadjusted milk was the most heat stable and pH 7 was more heat stable than pH 6.2. These changes are most likely associated with β-Lg, one of the most heat-labile proteins in whey. The

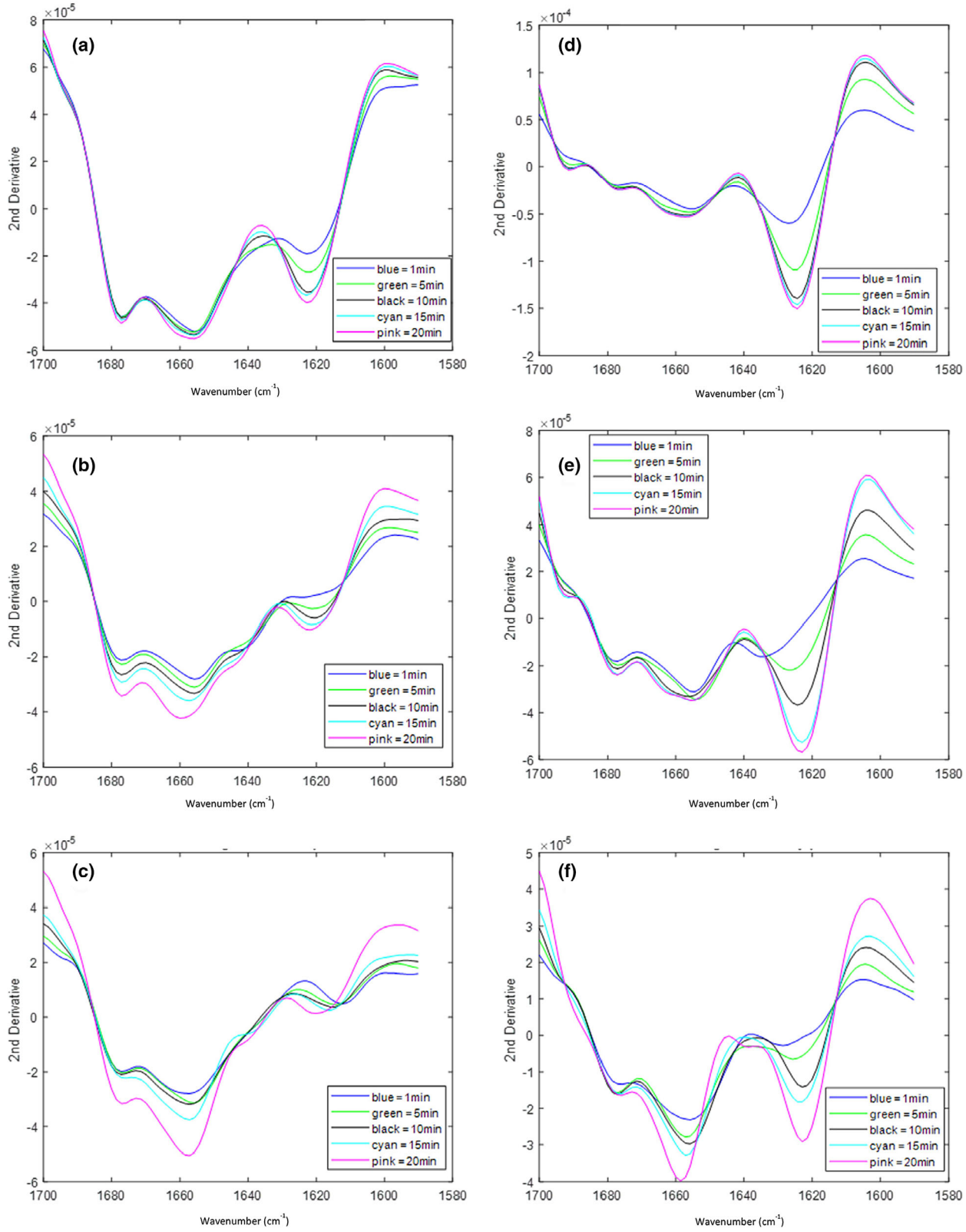

Figure 3 A typical graph of second derivative FTIR amide I spectra for skim milk sample S3 (a–c) and whey (d–f) heated for 20 min to 85°C at three different pH values: pH 6.2 (a, d), pH 6.8 (b, e) and pH 7 (c, f).

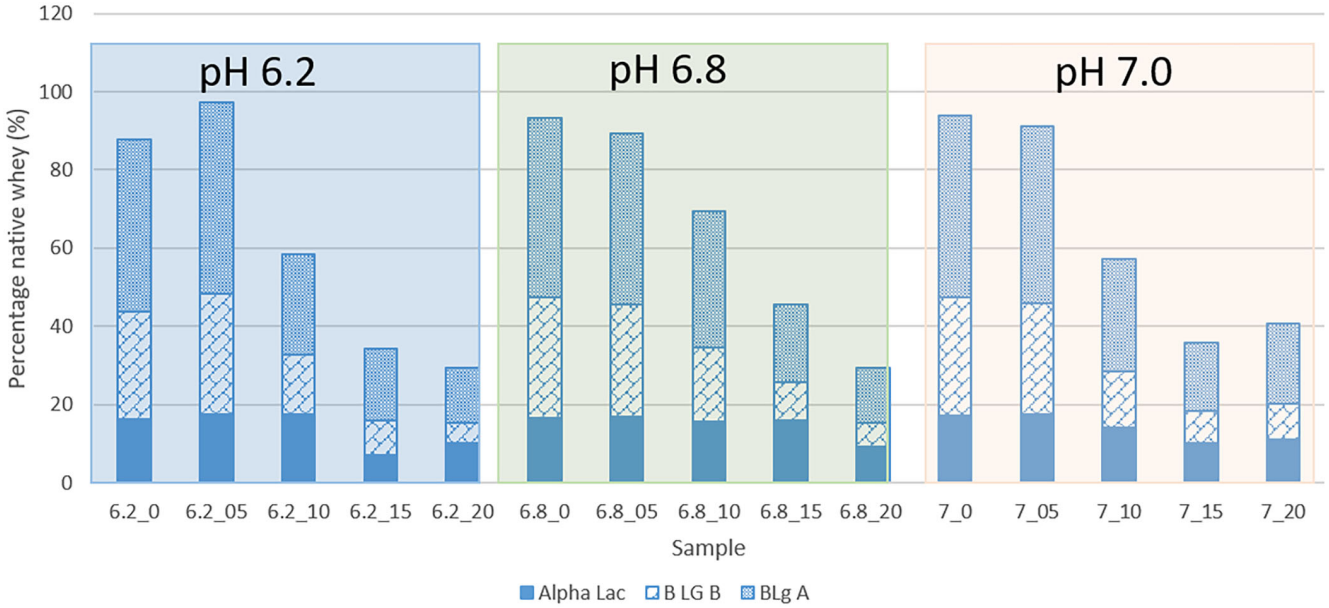

Figure 4 HPLC results based on skim milk for each pH milk based on combined results from S4 and S5. Results show levels (%) of three whey proteins after being heated to 85°C for 5, 10, 15 or 20 min at each pH. Alpha lactalbumin (solid blue), beta lactoglobulin B (brick pattern) and beta lactoglobulin A (shaded).

Table 2 Heat coagulation time using the oil bath method.

Time (mins)							
pH	S1	S2	S3	S4	S5	Mean	SD
6.2	0.47	0.55	0.5	0.65	0.45	0.524	0.07144
7	5.35	4.28	8.18	6.56	8.35	6.544	1.58048
Native pH	16.25	18.13	14.51	15.15	14.90	15.78	1.30593

The results consist of 5 replicates (S1–S5). Each sample was analysed in duplicate.

results also agree partly with those obtained from HPLC (Figure 4), as discussed below. The lack of such obvious spectral changes with milk adjusted to pH 7 could likely be due to the increased electrostatic charge on whey proteins, reducing aggregation (Nicolai *et al*. 2011).

The region from 1634 to 1640 cm^{-1} related to β sheet structures also changed over time when held at 85°C, with the greatest change observed for pH 6.2. Figure 3(a) shows a shift from 1630 to 1638 cm^{-1}, which is not as evident either in Figure 3(b or c). This region is associated with aggregated β sheets, indicating the milk at pH 6.2 had a higher rate of aggregation than milk with a higher pH, which agrees with the HPLC analysis (Figure 4). Buggy *et al*. (2018) examined the effect of pH on whey protein isolate and found that whey protein isolate at pH 6.2 was more extensively denatured compared to WPI at pH 6.7 and pH 7.2. At lower pH, whey proteins associate with casein micelles, whereas at high pH (>pH 6.9), almost all the denatured whey proteins remain in the serum phase of milk (Anema and Li 2003; Mediwaththe *et al*. 2024).

These results are similar to a study by Grewal *et al*. (2018), who also reported increases in this region relating to aggregated β sheets. At 1656 cm^{-1}, which is generally associated with α- helices, no change was observed for skim pH 6.2; however, the rate of change increased for unadjusted pH and skim pH 7, albeit only after 10 min (Figure 3). It is possible that this region could be related to hydrophobic interactions between α-lactalbumin, which, at temperatures below 90°C, form aggregates through hydrophobic interactions and not through covalent bonds (Oldfield *et al*. 1998). This rate of change was significant (P = 0.04) for pH 7; however, it was not significant for either pH 6.2 or pH 6.8 (P = 0.94 and P = 0.4, respectively). It is possible that this may be related to an increased casein micelle size at the higher pH (Sinaga *et al*. 2016). It is also possible that this region was related to aggregated β-Lg found in milk serum due to the high pH (Anema and Li 2003). There was a positive correlation between the change in peak intensity over time at 1620 and 1652 cm^{-1}. Both regions decrease over time when heated. Finally, aggregated β-turns are associated with the region 1672 cm^{-1} (Grewal *et al*. 2017), and peak increase can be seen to occur more at pH 6.8 and pH 7 compared to pH 6.2 (Figure 3a–c). Changes in pH can influence β-turns as they are stabilised by hydrogen bonding. This could explain the lack of a peak for skim pH 6.2.

Whey

The changes over time in spectra for whey at the same pH values mentioned above followed similar trends to the skim milk samples. The spectral region at 1624 cm^{-1} had a greater peak intensity and changed to a greater extent over time than skim samples. This was attributed to β-Lg as the main protein present. There was a slight increase at 1636 cm^{-1} for all three whey samples (Figure 3d–f). Such shifts are also due to the aggregation of β-sheets in this region. There was an increase at 1645 cm^{-1} for whey pH 7, which was not seen in the other whey samples. One reason could be the increased random coil formation due to the higher pH (Markoska *et al*. 2021). It could also result from increased levels of whey protein in the serum phase due to a higher pH. The decrease between 1660 and 1680 cm^{-1} was more pronounced at pH 7 in the skim milk and whey spectra. Previous studies mention that β-turns can transform into polyproline II helices as temperature increases (Farrell *et al*. 2001).

The difference observed at 1652 and 1672 cm^{-1} in skim milk (Figure 3a–c), but not in whey (Figure 3d–f), is potentially due to casein interactions. It is well known that casein can form complexes with β-Lg due to heat-induced aggregation, and casein peaks overlap with whey in the amide I region. Markoska *et al*. (2021) showed that β-casein structure was altered at pH 7 with a reduction in α-helices, which may also explain this difference.

Colloidal calcium phosphate (CCP)

The influence of calcium is known to affect the heat stability of milk. Over 60% of total calcium is found as CCP in unconcentrated milk at its native pH (Dumpler *et al*. 2020). Calcium moves to the serum phase with a reduction in pH, while increases in temperature cause calcium to move out of the serum phase to form CCP (Huppertz 2013). Previous studies have associated the spectral region 1070 cm^{-1} with CCP binding to the casein micelle (Boiani *et al*. 2018; Markoska *et al*. 2019a). Wang and Ma (2020) observed a distinct increase at 1073 cm^{-1} with heating, with a notable difference seen in milk at pH 7.1. This is consistent with the results of the current study, where the greatest increase was found at 1072 cm^{-1} from pH 7 milk (Figure 5). The lower pH samples showed little change in the 1072 cm^{-1} region, while the higher pH (7.2) samples significantly increased over time ($P = 0.017$). This could be due to ionic calcium increasing as pH decreased. The peak began to decrease again with cooling, confirming that the CCP added to the micelle when heated to <95°C is reversible due to calcium phosphate being restored to the serum phase. These changes became irreversible when heated above 95°C, indicating that calcium phosphate remains attached to the casein micelle (*data not shown*). The reversible nature of the CaP formation during heating to 80°C was also reported by Boiani *et al*. (2018) and Pouliot *et al*. (1989). Using NMR to study mineral equilibria, Boiani *et al*. (2018) showed that CCP increased during heating milk between 25°C and 80°C. They found that increasing temperature in this range reduced the solubility of calcium (Ca) and inorganic phosphate (Pi) in solution, resulting in precipitation.

The whey spectra showed minimal change in this region (1072 cm^{-1}), further indicating that the changes occurring were due to increased binding of CCP to the casein micelle. The high heat stability of casein is associated with the formation of calcium bridges within the micelle. The lack of secondary structures in casein micelles contributes to high heat stability. The findings demonstrate the potential of using changes in the FTIR spectral region 1070 cm^{-1}, associated with CCP, as a parameter in models for the prediction of milk heat stability.

HPLC results

The amount of remaining native protein in heated skim milk samples after 5, 10, 15 and 20 min at pH 6.2, pH 6.8 and pH 7 was quantified using reverse-phase HPLC. Figure 4 shows the quantity (%) of native α-lac, β-Lg A and β-Lg B for each heat treatment at pH 6.2, 6.8 and 7. There was a significant difference in the amount of denatured protein between heated (20 min) and unheated samples for all pH values. Skim milk at pH 6.2 had a higher level of denaturation after 10 min compared to the other samples. This agrees with the FTIR results shown in Figure 3, where the rate of change increased at pH 6.2 after 5 min, while changes in the region of 1624 cm^{-1} were not seen until after 10 min for pH 6.8 and pH 7, indicating that the samples at these pHs are more heat stable than the sample at pH 6.2. As mentioned previously, this region is related to β-Lg denaturation, one of the most heat-labile dairy proteins. Bouvier *et al*. (2014) investigated whey protein denaturation at temperatures ranging from 70 to 95°C. The study examined β-Lg denaturation in a heat exchanger from outlet temperatures, resulting in denaturation rates of whey protein >85% depending on product flow rate (L/h).

Heat coagulation time (HCT) and changes in milk pH with temperature

The time taken to coagulate at 140°C was measured using the traditional oil bath method (Table 2). As expected, the lowest pH was the least heat stable (average HCT = 0.52 min), and the native pH was the most stable (average HCT = 15.78 min). The average HCT for pH 7 was 6.54 min. This follows the typical heat stability curve of type A milk, as discussed by Rose (1962). The pH of milk has a significant influence on functionality during heating (Oldfield *et al*. 1998). Figure 6 shows the change in milk pH as it is heated from 25 to 85°C. The pH of skim milk was measured at 1-min intervals from 25 to 85°C to investigate the effect of temperature on pH. There was an average of 0.54 (±0.02) unit drop in milk pH when heated

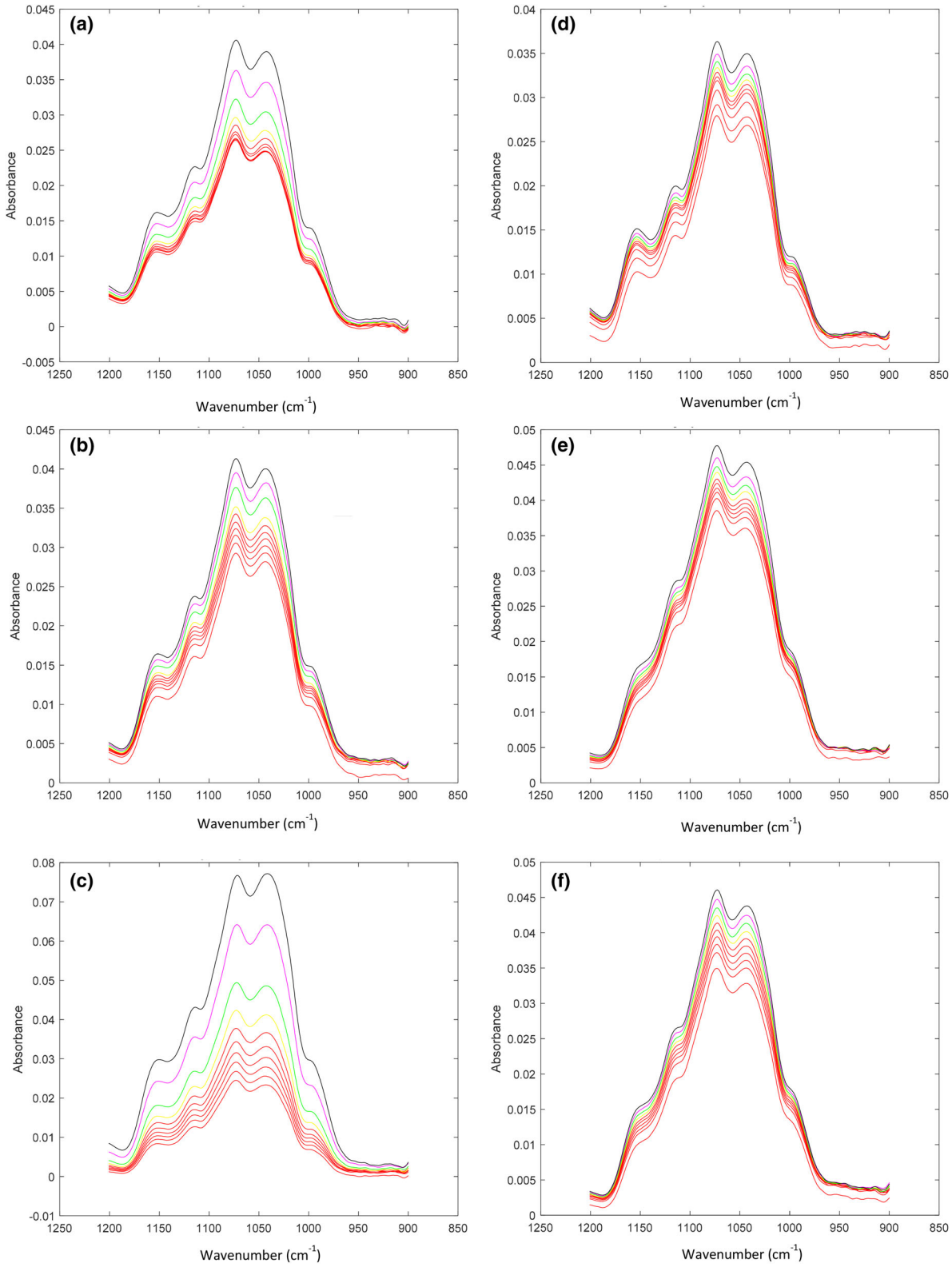

Figure 5 Vector normalised FTIR spectra for skim milk (a–c) and whey (d–f) heated for 20 min to 85°C at three different pH values: pH 6.2 (a, d), pH 6.8 (b, e) and pH 7 (c, f). (Red = 0-10 min, yellow = 12 min, green = 14 min, pink = 16 min, black = 18 min).

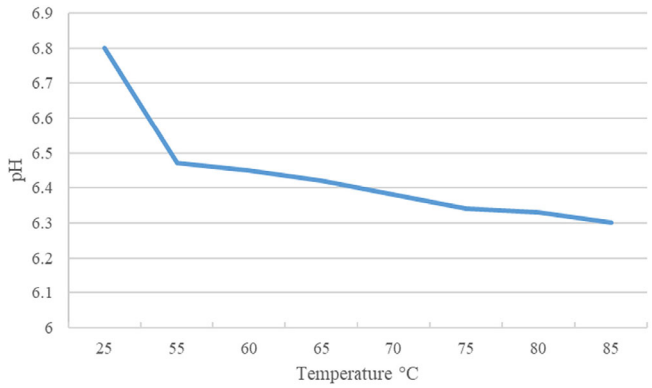

Figure 6 In situ pH probe measurement of change in skim milk pH during continuous heating from 25 to 85°C.

from 25 to 85°C for all pH values. The initial pH appeared to have no significant impact on the extent of the final pH reduction. Lactose influences the pH of milk through the production of Maillard reactions when heated. Further heating will decrease pH through the formation of formic and acetic acids (Aydogdu *et al*. 2023). Increasing temperature reduces calcium phosphate solubility, and the FTIR spectra show this at 1072 cm^{-1} (Figure 5), i.e., the region associated with colloidal calcium phosphate. Milk processing treatments such as spray drying, evaporation and pasteurisation occur at much lower temperatures than 140°C, and thus, with the oil bath standard method, it is difficult to interpret how milk will behave at lower temperature/time combinations; use of ATR-FTIR may overcome this limitation.

CONCLUSION

Structural changes in milk proteins were determined during continuous measurement at 85 °C using ATR-FTIR spectroscopy. Changes in β-sheet structure at 1624 cm^{-1} within the spectra related to the denaturation of β-Lg were measured over 20 min and confirmed using HPLC analysis. The spectral region from 1655 to 1672 cm^{-1} also changed during heating and is likely related to casein interactions with whey. A significantly faster rate of change was observed at 85°C over a 20-min interval in these spectral regions for skim milk at pH 6.2 compared to milk at either pH 6.8 or 7. Time-dependent changes in colloidal calcium phosphate were correlated with heat by measuring the change at 1072 cm^{-1}, and the highest rate of change was seen in milk samples heated to 85°C at pH 7. This was confirmed in whey taken from the same samples, whereby the extent of change in CCP over time was absent when casein was not present. Direct measurement of changes in protein structure and CCP demonstrate the potential of ATR-FTIR as an analytical tool for in situ testing of the thermal stability of milk, dairy concentrates and formulations in which they are used. It has potential uses for process optimisation, reducing product waste and meeting consumer demands for high-quality dairy products.

AUTHOR CONTRIBUTIONS

Elena Hayes: Data curation; methodology; main writer of manuscript; editing. **Norah O'Shea:** Supervision; review and editing. **Colm O'Donnell:** Supervision, review and editing.

ACKNOWLEDGEMENTS

This study has emanated from research conducted with the financial support of Science Foundation Ireland (SFI) and the Department of Agriculture, Food and Marine on behalf of the Government of Ireland under Grant Number [16/RC/3835] – VistaMilk. The authors would like to acknowledge the work of the Technical services laboratory at Teagasc Moorepark for providing access to the milk composition data.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Liam Kelly: Data curation; methodology; resources; writing – review and editing. **Derek Greene:** Funding acquisition; resources; supervision; writing – review and editing. **Mark A Fenelon:** Conceptualization; formal analysis; funding acquisition; investigation; supervision; validation; writing – review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

Andrade J, Pereira C G, Almeida Junior J C D, Viana C C R, Neves L N D O, Silva P H F D, Bell M J V and Anjos V D C D (2019) FTIR-ATR determination of protein content to evaluate whey protein concentrate adulteration. *LWT* **99** 166–172.

Anema S G and Li Y (2003) Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *Journal of Dairy Research* **70** 73–83. https://doi.org/10.1017/S0022029902005903.

Aydogdu T, O'Mahony J A, Huppertz T, Magan J B and McCarthy N A (2023) Measuring pH of skim milk and milk permeate at ultra-high temperatures at laboratory and pilot scale. *International Dairy Journal* **139** 105565.

Boiani M, Fenelon M, Fitzgerald R J and Kelly P M (2018) Use of 31P NMR and FTIR to investigate key milk mineral equilibria and their interactions with micellar casein during heat treatment. *International Dairy Journal* **81** 12–18.

Bouvier L, Moreau A, Ronse G, Six T, Petit J and Delaplace G (2014) A CFD model as a tool to simulate β-lactoglobulin heat-induced denaturation and aggregation in a plate heat exchanger. *Journal of Food Engineering* **136** 56–63.

Buggy A K, McManus J J, Brodkorb A, Hogan S A and Fenelon M A (2018) Pilot-scale formation of whey protein aggregates determine the stability of heat-treated whey protein solutions-effect of pH and protein concentration. *Journal of Dairy Science* **101** 10819–10830. https://doi.org/10.3168/jds.2017-14177.

Carbonaro M and Nucara A (2010) Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region. *Amino Acids* **38** 679–690. https://doi.org/10.1007/s00726-009-0274-3.

Chen B, Grandison A S and Lewis M J (2015) Effect of seasonal variation on some physical properties and heat stability of milk subjected to ultra-high temperature and in-container sterilisation. *Food Chemistry* **181** 227–234.

Crowley S V, Megemont M, Gazi I, Kelly A L, Huppertz T and O'Mahony J A (2014) Heat stability of reconstituted milk protein concentrate powders. *International Dairy Journal* **37** 104–110.

Daniloski D, McCarthy N A, O'Callaghan T F and Vasiljevic T (2022) Authentication of β-casein milk phenotypes using FTIR spectroscopy. *International Dairy Journal* **129** 105350.

Davies D and White J (1966) The stability of milk protein to heat: I. Subjective measurement of heat stability of milk. *Journal of Dairy Research* **33** 67–81.

Dumpler J, Huppertz T and Kulozik U (2020) Invited review: Heat stability of milk and concentrated milk: Past, present, and future research objectives. *Journal of Dairy Science* **103** 10986–11007.

Egan M, Galvin N and Hennessy D (2018) Incorporating white clover (*Trifolium repens* L.) into perennial ryegrass (*Lolium perenne* L.) swards receiving varying levels of nitrogen fertiliser: Effects on milk and herbage production. *Journal of Dairy Science* **101** 3412–3427.

Etzion Y, Linker R, Cogan U and Shmulevich I (2004) Determination of protein concentration in raw Milk by mid-infrared Fourier transform infrared/attenuated Total reflectance spectroscopy. *Journal of Dairy Science* **87** 2779–2788.

Farrell H M, Wickham E D, Unruh J J, Qi P X and Hoagland P D (2001) Secondary structural studies of bovine caseins: Temperature dependence of β-casein structure as analysed by circular dichroism and FTIR spectroscopy and correlation with micellisation. *Food Hydrocolloids* **15** 341–354.

Food Safety Authority of Ireland (2021) Annual report 2021. [Internet document] URL https://www.fsai.ie.

Fox P F and Hoynes M C T (1975) Heat stability of milk: Influence of colloidal calcium phosphate and β-lactoglobulin. *Journal of Dairy Research* **42** 427–435. https://doi.org/10.1017/S0022029900015466.

Fox P F, Uniacke-Lowe T, Mcsweeney P L H and O'mahony J A (2015) Heat-induced changes in Milk. *Dairy Chemistry and Biochemistry* **20** 345–375.

Grewal M K, Chandrapala J, Donkor O, Apostolopoulos V, Stojanovska L and Vasiljevic T (2017) Fourier transform infrared spectroscopy analysis of physicochemical changes in UHT milk during accelerated storage. *International Dairy Journal* **66** 99–107.

Grdadolnik J (2002) An attenuated total reflection infrared spectroscopy of water solutions. *International Journal of Vibrational Spectrosocpy* **6** 2–6.

Grewal M K, Huppertz T and Vasiljevic T (2018) FTIR fingerprinting of structural changes of milk proteins induced by heat treatment, deamidation and dephosphorylation. *Food Hydrocolloids* **80** 160–167.

Huppertz T (2013) Chemistry of the caseins. In *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects*, 4th edn. Mcsweeney P L H and Fox P F, eds. Boston, MA: Springer.

Huppertz T (2016) Heat stability of Milk. In *Advanced Dairy Chemistry: Volume 1B: Proteins: Applied Aspects*, Mcsweeney P L H and O'Mahony J A, eds. New York, NY: Springer.

Kelly P M, O'Keeffe A M, Keogh M K and Phelan J A (1982) Studies of Milk composition and its relationship to some processing criteria: III: Seasonal variation in heat stability of Milk. *Irish Journal of Food Science and Technology* **6** 29–38.

Kong J and Yu S (2007) Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica* **39** 549–559. https://doi.org/10.1111/j.1745-7270.2007.00320.x.

Lefèvre T and Subirade M (2001) Molecular structure and interaction of biopolymers as viewed by Fourier transform infrared spectroscopy: Model studies on β-lactoglobulin. *Food Hydrocolloids* **15** 365–376.

Li S, Ye A and Singh H (2019) Seasonal variations in composition, properties, and heat-induced changes in bovine milk in a seasonal calving system. *Journal of Dairy Science* **102** 7747–7759.

Li H, Zhao T, Li H and Yu J (2021) Effect of heat treatment on the property, structure, and aggregation of skim Milk proteins. *Frontiers in Nutrition* **8** 2120.

Loveday S M, Weeks M, Luo D and Cakebread J (2021) Type a and B bovine milks: Heat stability is driven by different physicochemical parameters. *Journal of Dairy Science* **104** 11413–11421.

Manuelian C L, Visentin G, Boselli C, Giangolini G, Cassandro M and De Marchi M (2017) Short communication: Prediction of milk coagulation and acidity traits in Mediterranean buffalo milk using Fourier-transform mid-infrared spectroscopy. *Journal of Dairy Science* **100** 7083–7087.

Markoska T, Huppertz T, Grewal M K and Vasiljevic T (2019a) FTIR analysis of physiochemical changes in raw skim milk upon concentration. *LWT* **102** 64–70.

Markoska T, Huppertz T, Grewal M K and Vasiljevic T (2019b) Structural changes of milk proteins during heating of concentrated skim milk determined using FTIR. *International Dairy Journal* **89** 21–30.

Markoska T, Daniloski D, Vasiljevic T and Huppertz T (2021) Structural changes of β-casein induced by temperature and pH analysed by nuclear magnetic resonance, Fourier-transform infrared spectroscopy, and Chemometrics. *Molecules* **26** 1022.

Matsarskaia O, Bühl L, Beck C, Grimaldo M, Schweins R, Zhang F, Seydel T, Schreiber F and Roosen-Runge F (2020) Evolution of the structure and dynamics of bovine serum albumin induced by thermal denaturation. *Physical Chemistry Chemical Physics* **22** 18507–18517.

McDermott A, Visentin G, De Marchi M, Berry D P, Fenelon M A, O'Connor P M, Kenny O A and McParland S (2016) Prediction of individual milk proteins including free amino acids in bovine milk using mid-infrared spectroscopy and their correlations with milk processing characteristics. *Journal of Dairy Science* **99** 3171–3182.

Mediwaththe A, Bogahawaththa D, Grewal M K, Chandrapala J and Vasiljevic T (2018) Structural changes of native milk proteins

subjected to controlled shearing and heating. *Food Research International* **114** 151–158.

Mediwaththe A, Huppertz T, Chandrapala J and Vasiljevic T (2024) Effect of pH and shear on heat-induced changes in Milk protein concentrate suspensions. *Food* **13** 1517. https://doi.org/10.3390/foods13101517.

Mounsey J S and O'Kennedy B T (2009) Stability of β-lactoglobulin/micellar casein mixtures on heating in simulated milk ultrafiltrate at pH 6.0. *International Journal of Dairy Technology* **62** 493–499.

Nicolai T, Britten M and Schmitt C (2011) β-Lactoglobulin and WPI aggregates: Formation, structure and applications. *Food Hydrocolloids* **25** 1945–1962.

Oldfield D J, Singh H, Taylor M W and Pearce K N (1998) Kinetics of denaturation and aggregation of whey proteins in skim Milk heated in an ultra-high temperature (UHT) pilot plant. *International Dairy Journal* **8** 311–318.

Pouliot Y and Boulet M (1995) Observations on the seasonal variations in the salt balance of concentrated milk. *International Dairy Journal* **5** 75–85.

Pouliot Y, Boulet M and Paquin P (1989) Observations on the heat-induced salt balance changes in milk II. Reversibility on cooling. *Journal of Dairy Research* **56** 193–199. https://doi.org/10.1017/S0022029900026418.

Rose D (1962) Factors affecting the heat stability of Milk1. *Journal of Dairy Science* **45** 1305–1311.

Sutariya S G, Huppertz T and Patel H A (2017) Influence of milk preheating conditions on casein–whey protein interactions and skim milk concentrate viscosity. *International Dairy Journal* **69** 19–22. https://doi.org/10.1016/j.idairyj.2017.01.007.

Sinaga H, Bansal N and Bhandari B (2016) Effects of milk pH alteration on casein micelle size and gelation properties of milk. *International Journal of Food Properties* **20** 179–197.

Visentin G, De Marchi M, Berry D P, McDermott A, Fenelon M A, Penasa M and McParland S (2017) Factors associated with milk processing characteristics predicted by mid-infrared spectroscopy in a large database of dairy cows. *Journal of Dairy Science* **100** 3293–3304.

Wang Q and Ma Y (2020) Effect of temperature and pH on salts equilibria and calcium phosphate in bovine milk. *International Dairy Journal* **110** 104713.

