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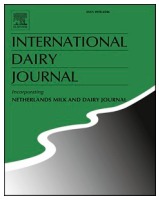

Research paper

Assessment of the impact of pasteurisation on protein denaturation in sheep sweet whey by asymmetrical flow field-flow fractionation according to sampling period

Marie Hennetier [a,*], Clarissa Detomi DE Albuquerque [c], Loubnah Belahcen [a], Inés Martin Saez [a], Romain Valentin [b], Hélène Tormo [a]

[a] Université de Toulouse, Ecole d'Ingénieur de Purpan, 75, voie du TOEC, BP57611, F-31076, Toulouse, Cedex 3, France
[b] Laboratoire de Chimie Agro-industrielle, LCA, Université de Toulouse, INRAE, Toulouse, France
[c] UMR IATE 1208, INRAE, Institut Agro Montpellier, Université de Montpellier. 2 Place Pierre Viala, 34060, Montpellier, Cedex 5, France

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ABSTRACT

Whey recovery is limited in artisanal cheese manufacture. This by-product of cheese manufacture was traditionally considered as waste and is highly polluting. However, interest in the nutritional properties of native whey and its proteins has recently increased. Pretreatment is required to preserve highly perishable whey for processing. Only a few studies have focused on sheep whey, despite its considerable nutritional and technological potential. Here, we investigated the effect of heat treatment on the denaturation and formation of protein aggregates in sweet sheep whey collected in January, April and July. Two pasteurisation protocols were studied (72 °C for 1 min and 80 °C for 15 s). Microbiological quality was assessed by checking for the presence of microbes after five days of storage at 4 °C. Asymmetrical flow field-flow fractionation (AF4) coupled to UV detector, multiangle light scattering (MALS) and refractometer (dRi) is a mild, non-naturing technique for determining the extent of denaturation of whey proteins and the size of whey protein aggregates. Greater whey protein denaturation and larger whey protein aggregates were observed for the 80 °C/15 s protocol than for the 72 °C/1 min protocol, for the January and April samples. A decrease in Immunoglobulin G (IgG) content was observed after heat treatments in the samples from July but not significantly for the other proteins. Moreover, the retention times of the monomeric whey protein peaks on the AF4 fractogram were higher for this period, indicating that the proteins were larger. Microbiological testing showed that both pasteurisation treatments were sufficiently effective to ensure good sanitary quality. The pasteurisation schedule best preserving native proteins was heating at 72 °C for 1 min.

1. Introduction

Whey is the residual fraction of milk remaining after coagulation during cheese manufacture. This fraction accounts for 85–95% of the volume of milk and is rich in whey protein (Guimarães et al., 2010; Nishanthi et al., 2017). Different types of whey, such as sweet whey, acid whey and salted whey, exist and are defined according to the cheese-making technology used. The compositions of these different types of whey differ, particularly in terms of minerals, lactose and lactic acid contents. For example, sweet whey has a pH of about 6, whereas acid whey has a pH of 4.5. Acid whey also contains less lactose and more minerals than sweet whey and is generated during soft cheese manufacture or casein production (Blaschek et al., 2007).

Whey is an important source of proteins and peptides of technological interest. Interest in the nutritional properties of whey and its derivatives is therefore growing, due to their biological activities and beneficial effects on human health (Hernández-Ledesma et al., 2011). Functional whey peptides are derived from both casein and whey proteins. These peptides have multiple activities, including antimicrobial, immunomodulation, mineral binding, and antioxidative functions. (Nielsen et al., 2017). These bioactive peptides can be released during processing (fermentation for example) and during digestion. Industrially, about 70% of whey is processed to generate products such as demineralised whey powder, whey protein concentrate, whey protein

* Corresponding author.
E-mail address: marie.hennetier@purpan.fr (M. Hennetier).

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isolate, lactose, minerals and pure protein fractions (Kaur et al., 2020). The remaining 30% is used to feed pigs or is spread on agricultural land as fertiliser. In artisanal cheese production, whey is used to make other dairy products, such as whey cheeses, which include the famous ricotta cheese of Italy and broccu in France, with its certified protected designation of origin (DOP) in Corsica or Greuil, in the Pyrenees. Nevertheless, whey is still little used in the artisanal cheese sector, with large volumes still treated as waste. Indeed, artisanal cheesemakers produce too little whey for the concentration and molecular separation methods used in industry to be either feasible or affordable. Nevertheless, there is considerable interest in the valorisation of this by-product in this sector, either as an ingredient or as a new product, provided simple technology can be used and production costs kept affordable.

Ewe's milk is mostly transformed into cheese products, which generates large amounts of sheep whey. Sheep whey has a higher protein content than cow or goat whey: 1.71% versus 0.87% for cow whey and 1.0% for goat whey (Giroux et al., 2018). The main proteins present in whey are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), together with smaller amounts of immunoglobulins, serum albumin, lactoferrin, caseinomacropeptide, proteose-peptone and lactoperoxidase. The other important components of whey are lactose, oligosaccharides, minerals and vitamins (Hernández-Ledesma et al., 2011; Setiowati et al., 2020). Sheep whey contains a higher proportion of β-LG than cow or goat whey, and this composition may afford possibilities for whey products with enhanced foaming, gelation, and emulsification properties (Casper et al., 1998).

The whey is subjected to heat treatment before storage with a view to potential transformation into a product. The choice of time and temperature combination is crucial, to preserve the native structure of the proteins as much as possible. During heat treatment, whey proteins may undergo a structural change known as denaturation, in which the protein unfolds, and its hydrophobic groups are exposed. Whey protein aggregation generally involves the interaction of a free –SH group with the S–S bond of cystine-containing proteins, such as β-LG, κ-casein (κ-Csn), α-LA, and bovine serum albumin (BSA) via –SH/S–S interchange reactions (Wijayanti et al., 2014). When β-LG is denatured, it forms small aggregates, which increase in size at higher temperatures or over longer heating times (Raikos, 2010). When α-LA is denatured, it forms complexes with large denatured β-LG aggregates. In our experiment, before making cheese, the milk was heated at 60 °C for 30s. During heat treatment, some of the κ-casein dissociates from the micelles and is released into the whey (Anema and Li, 2000) which could also participate in whey protein aggregation during heating. During whey heating, proteins aggregate by different way as a function of the pH: whey proteins can aggregate to form fibrils at pH2, fractals at pH7, microgels at pH5.8. The more the heating time and the temperature are increased, the more the size of aggregates increased. (Schmitt, 2011). However, in milk, the heating process form mixed aggregates between casein micelles and whey protein aggregates by disulfide bonds (Li et al., 2021).

Several methods for the analysis of whey protein fractions have been described, the principal methods being based on immunological, chromatographic, and electrophoretic techniques (Mayer et al., 1997). Whey proteins from the milk of various animal species can be analysed with various techniques, such as reverse-phase high-performance liquid chromatography (RP-HPLC), which can be used to analyse the native whey proteins (de Frutos et al., 1992; Thomä et al., 2006). Size-exclusion chromatography coupled to multiangle laser light-scattering techniques (SEC-MALS) can also be used to analyse whey native proteins and whey protein aggregates (de la Fuente et al., 2002; Schokker et al., 2000), but with a risk of the column becoming blocked or of sample denaturation due to the stationary phase. Recent studies have used a new technique, asymmetrical flow field-flow fractionation (AF4), to analyse native and aggregated whey proteins (de Guibert et al., 2020; Kang et al., 2011; Loiseleux et al., 2018). AF4 is a liquid chromatography technique that can separate structures of between 1 nm and a few micrometres in diameter. This technique combines the separating effect of laminar flow and the concentrating effect of unidirectional cross-flow (Messaud et al., 2009). The "soft separation mechanism" of this technique makes it possible to preserve the native structure of the sample, even a casein micelle in milk, much more effectively than is possible by Size-Exclusion Chromatography (Guyomarc'H et al., 2010; Kang et al., 2011).

We report here the first use, to our knowledge, of the AF4-UV-MALS-dRi technique to analyse the proteins present in sheep whey. One of the advantages of this technique is that macromolecules can be injected without pretreatment (e.g. filtration or precipitation of proteins), which is not the case for HPLC analyses. We used the AF4-UV-MALS-dRi technique to study the impact of time-temperature combinations for pasteurisation on native sheep whey proteins and aggregate formation. If whey is to be used as an ingredient or raw material, it must first undergo heat treatment to ensure its preservation during storage/transport. We studied the effect of the pasteurisation process on sheep whey, and its ability to limit protein aggregate formation while preserving the characteristics of the native proteins and ensuring microbiological quality.

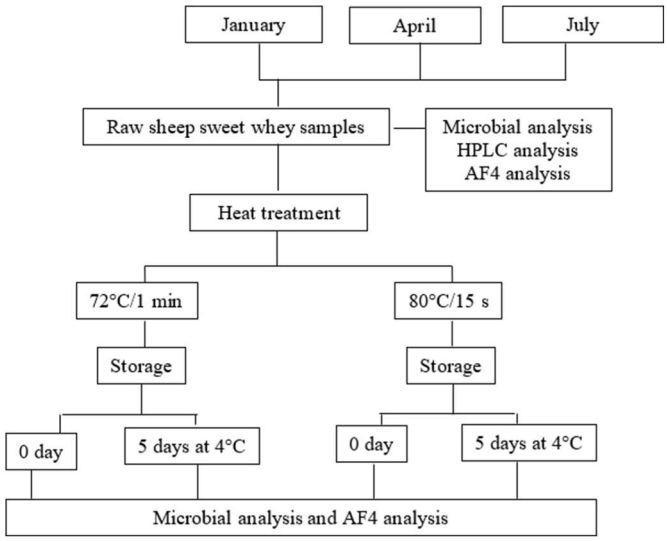

Fig. 1. Experimental design.

2. Materials and methods

2.1. Whey samples

Samples of sheep sweet whey from soft cheese production were collected from the Le Fédou - Hyelzas cheese dairy (Lozère, France). The samples were collected during three periods of the year (January, April and July). Three samples were collected during each of these periods and stored frozen (−20 °C) until analysis.

2.2. Experimental design

Raw sweet whey samples collected during the three periods were frozen for one month and then subjected to heat treatment (72 °C for 1 min or 80 °C for 15 s) (experimental design shown in Fig. 1). For the choice of heat intensity treatments, simulations have been done, and two intensities were selected according to low and higher potential level of denaturation of whey proteins. Raw whey, pasteurised whey without storage and pasteurised whey stored for less than 5 day at 4 °C were characterised to determine their microbiological content, with size analysed by AF4-UV-MALS-dRi.

Table 1
Microbiological methods and detection.

Micro-organisms	Culture medium	Incubation	Inoculation	Inoculum volume	Limit of detection
Total bacterial count (TBC)	Plate Count Agar + 0.1% skimmed milk powder (PCA)	30 °C for 72 h	Surface	100 µl	>10 CFU/ml
Coliform bacteria	Violet-red bile lactose agar (VRBL)	30 °C for 24 h	Double layer	1 ml	>1 CFU/ml
Enterobacteria	Violet-red bile glucose agar (VRBG)	37 °C for 24 h	Double layer	1 ml	>1 CFU/ml
Pseudomonas	Cetrimide agar (CFC) + CFC supplement	25 °C for 48 h	Surface	100 µl	>10 CFU/ml
Moulds/yeast	Oxytetracycline glucose agar (OGA) + OGA supplement	25 °C for 5 days	Surface	100 µl	>10 CFU/ml

Table 2
AF4 analysis method for the characterisation of untreated and heat-treated whey proteins.

Start time (min)	End time (min)	Duration (min)	Mode	Fc[a] start (ml/min)	Fc[a] end (ml/min)	Flow profile
0	1	1	Elution	0	0	Constant
1	2	1	Focus	1.5	1.5	Constant
2	6	4	Focus + Inject	1.5	1.5	Constant
6	32	26	Elution	1.8	1.8	Constant
32	41	9	Elution	1.8	0,06	Exponential
41	81	40	Elution	0.06	0.06	Constant
81	89	8	Elution	0	0	Constant

[a] Cross-flow.

2.3. Heat treatments

Whey samples (40 ml) were thawed overnight at 4 °C before treatment. Pasteurisation was performed in a water bath, with two different temperature/time combinations: 72 °C for 1 min (after the sample reached 72 °C) and 80 °C for 15 s (after the sample reached 80 °C). After heat treatment, the samples were immediately cooled in an ice bath. The pasteurised samples were stored at 4 °C for 5 d.

2.4. Microbiological analysis

Total bacterial counts (TBC) were determined and detection and quantification were performed for total coliforms, *Enterobacteria, Pseudomonas* and moulds/yeasts, in accordance with the experimental plan (Fig. 1) and according to the kit supplier's specifications (Biokar) (Table 1). Peptone water was used for dilution. Microbiological analysis was performed on raw whey samples and pasteurised whey samples that had not been stored or that had been stored for 5 day at 4 °C (Fig. 1).

2.5. Asymmetrical flow field-flow fractionation (AF4) coupled with UV, MALS and dRi detectors

The main component of AF4 systems is the separation channel, in which the sample is carried by an aqueous eluent with a laminar parabolic flow profile. Another flow, perpendicular to the carrier flow and known as the "cross-flow" was used to generate the force field, to separate the macromolecules as a function of their diffusion coefficient, *i.e.*, their hydrodynamic diameter (Dh) during elution.

The AF4 instrument used was a DualTec separation system (Wyatt Technology Europe, Dernbach, Germany). The AF4 channel was trapezoidal in shape, with a length of 19.5 cm, an initial breadth 1.65 cm and a final breadth 0.27 cm. A Mylar spacer with a thickness of 250 µm was placed between the ultrafiltration membrane and the upper glass plate. The accumulation wall was an ultrafiltration membrane of regenerated cellulose with a 10 kDa MWCO (Wyatt Technology, USA). The channel was installed on a ThermosPro oven (Wyatt Technology, USA) regulated at 22 °C. An Agilent 1260 Series isocratic pump (Agilent Technologies, Waldbronn, Germany) with an in-line vacuum degasser and an Agilent 1260 Autosampler delivered the carrier flow and handled sample injection into the AF4 channel. Contamination of the separation system was prevented by placing a filter holder with a 100 nm-pore polyvinylidene fluoride membrane (Millipore Corp, Darmstadt, Germany) after the pump. The eluent was a solution of 50 mM NaCl, 0.02% (w/v) sodium azide in Milli-Q water, passed through a filter with 0.1 µm pores.

The AF4 separation process involved several steps (Table 2), with the detector flow rate kept constant at 1.5 ml/min whereas the cross-flow gradient was varied. Samples were injected during the focusing/injection step at a focus-flow rate of 1.5 ml/min and were subjected to focusing/relaxation for 5 min. The first step in elution was an increase in cross-flow to 1.8 ml/min, with the flow rate subsequently maintained at this level for 35 min. The cross-flow rate was then decreased exponentially for 9 min to 0.06 ml/min, and was then kept constant at 0.06 mL/min for 40 min. At the end of each run, the channel was rinsed with the eluent for 18 min to eliminate any contaminants present before the next injection. The volume of sample solution injected was 200 µl.

The detection system consisted of: (*i*) an 18-angle Dawn Heleos II multi-angle light scattering (MALS) detector (Wyatt Technology, Santa Barbara, CA, USA) operating at a wavelength of 662 nm, (*ii*) an Optilab T-Rex differential refractive index detector (Wyatt Technology, Santa Barbara, CA, USA) operating at a wavelength of 658 nm and (*iii*) a UV detector operating at a wavelength of 280 nm. The MALS unit was calibrated with toluene. Normalization of the MALS unit, interdetector delays and band-broadening calculations were performed with Bovine Serum Albumin (BSA) protein (Mw = 66.4 g/mol).

Raw and pasteurised whey was stored for 5 d at 4 °C. The whey samples were then filtered through a Buchner system with 20 µm-pore filters to remove solids particles visible to the naked eye at the bottom of the vial and were diluted 100-fold with analytical eluent. Standard proteins — α-LA, β-LG, albumin and immunoglobulin (Sigma Aldrich, Sigma, St. Louis, MO) — were injected to obtain the retention time for each compound.

Data were analysed with Astra software (version 7.1.2, Wyatt Technology Corporation). The mean z-average radius of gyration (Rg in nm) and the mean molecular mass (Mw in g/mol) were obtained by multiangle light scattering (MALS) over a scattering angle range of 50–117°, with the Berry method at order 2 (Berry, 1966; Wang & Lucey, 2003) and a refractive index increment of 0.185 ml/g. The fractograms showing protein concentration as a function of elution time corresponded at the differential index signal. The light scattering (LS) signal was plotted under the fractogram of protein content.

The decrease in native whey protein levels with the different heat treatments was assessed by determining the monomeric whey protein content for each period from the differential refractive index signal. The native whey protein content of the sample not subjected to heat treatment was the reference and its value for native protein content was set to 100%. After heat treatment, the percentage of monomeric whey proteins remaining was calculated relative to this 100% reference value.

2.6. Statistical analysis

Statistical analyses were performed to assess the impact of the timing of sampling on the microbiological content of raw sheep whey. We then assessed the impact of two heat treatments on the microbiological content of the whey immediately after treatment (day 0) and after 5 d of storage at 4 °C. Microbial data are expressed as means ± standard deviation, in $\log_{10}$ CFU/ml. Significant differences in parameter values

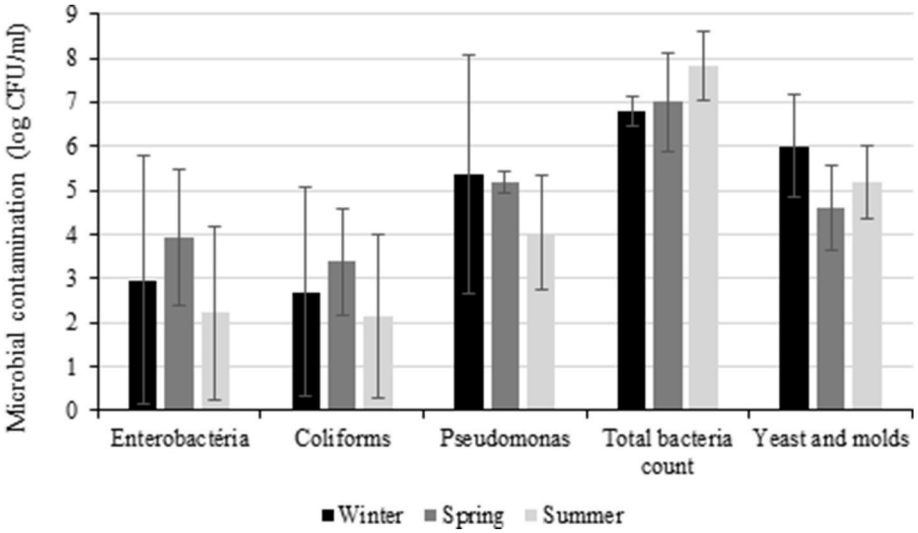

Fig. 2. Microbial concentration for untreated sheep whey samples from three periods: January, April and July.

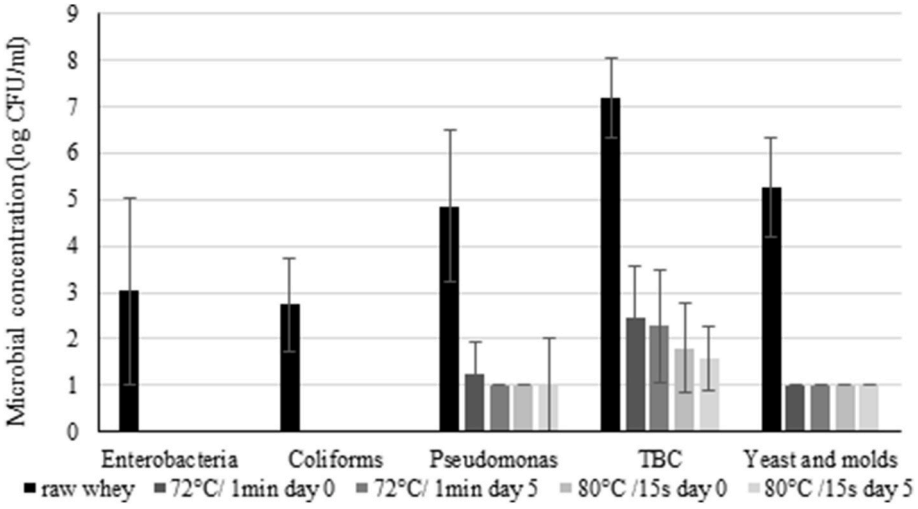

Fig. 3. Microbial concentration (log_{10} CFU/ml) for raw and pasteurised sheep whey after 0 and 5 days of storage at 4 °C.

were evaluated with Kruskal-Wallis tests. Comparisons were performed with Tukey tests, with differences considered significant if $P \leq 0.05$. Data were analysed with XLSTAT 2021.2.1 software (Addinsoft, 2020).

3. Results and discussion

3.1. Microbiological analysis of raw sheep whey

The high total bacterial counts (TBC) and high levels of moulds and yeasts in raw whey highlighted in Fig. 2 resulted from the inoculation of the milk with starters during cheese production. The abundance of *Pseudomonas* was particularly high in January (5.37 ± 2.75 log CFU/ml) and April (5.17 ± 0.25 log CFU/ml). Pseudomonads are a group of spoilage bacteria frequently detected in milk (Demasures et al., 1997). Enterobacteria were detected in whey, with highly variable levels, during all the periods studied (Fig. 2). For Enterobacteriaceae, the means and standard deviation of bacterial counts were 2.97 ± 2.80 log_{10} CFU/ml in January, 3.93 ± 1.53 log CFU/ml in April and 2.23 ± 1.97 log CFU/ml in July. Coliform bacteria had similar levels and variabilities of levels to Enterobacteriaceae. These findings highlight the predominance of coliform bacteria among Enterobacteriaceae.

The levels of the various groups of micro-organisms did differ significantly between time periods.

3.2. Microbiological analysis of pasteurised sheep whey

The levels of micro-organisms did not differ significantly ($P > 0.05$) between periods of the year. We therefore present the microbiological results as the mean for the three months studied for each micro-organism, by heat treatment and product storage time. Fig. 3 shows the abundance of micro-organisms in sweet sheep whey after pasteurisation in two different sets of conditions: 72 °C for 1 min and 80 °C for 15 s. The microbiological analyses were carried out immediately after heat treatment (Day 0) and after storage for 5 d at 4 °C (Day 5). The two heat treatments significantly decreased the bacterial content of the raw whey ($P < 0.05$). For enterobacteria, coliform bacteria and pseudomonads, counts decreased by about 3 log_{10} CFU/ml. For total bacterial count (TBC) and for yeasts and moulds, counts decreased by about 5 log_{10} CFU/ml and 4 log_{10} CFU/ml, respectively. Furthermore, no bacterial or yeast/mould growth was observed during the five days of storage at 4 °C. Finally, no significant difference in microbe levels was found between the two pasteurisation regimes, regardless of the microbial group considered or the storage time.

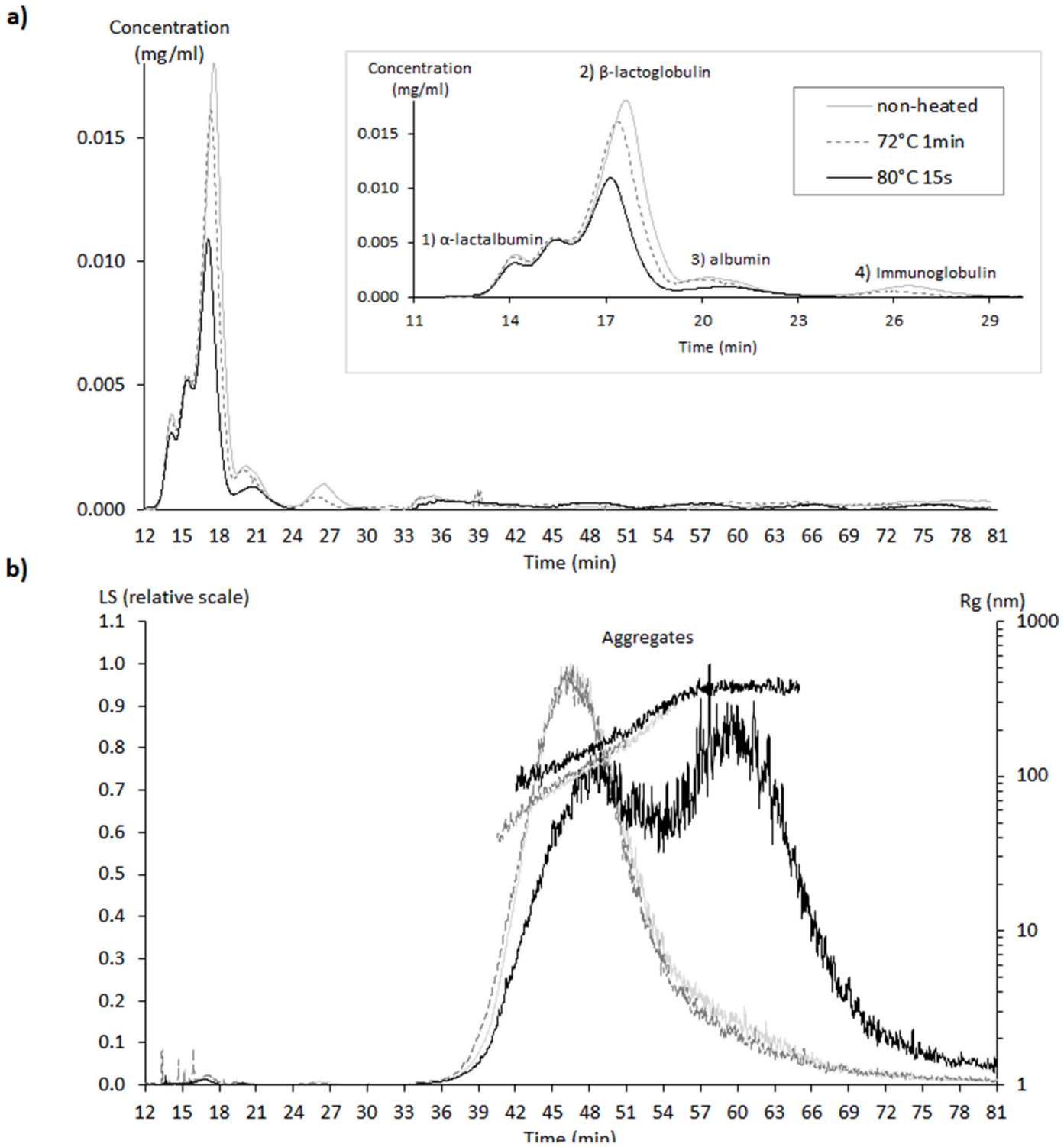

Fig. 4. (***a***) AF4 Fractogram of the protein concentrations (dRI signal) of raw and pasteurised sheep whey (72 °C/1 min and 80 °C/15 s) for the April period, with a magnification of the period between 11 and 29 min; (***b***) Fractogram of the MALS signal and radius of gyration (Rg) distribution of proteins in raw and pasteurised sheep whey (72 °C/1 min and 80 °C/15 s) for the April period.

3.3. AF4 analysis of sheep whey proteins

3.3.1. Example of heat treatment on a sample collected in April

Raw and pasteurised whey samples collected during three sampling periods were treated by pasteurisation at two different time/temperature combinations and were then analysed by AF4-UV-MALS-dRi. A typical fractogram is presented in Fig. 4, to illustrate the monomeric proteins and aggregates present in the heat-treated and untreated whey samples collected in April. Fig. 4a shows the fractogram with the protein concentration (dRi signal) and a magnification of the part of the fractogram corresponding to between 11 and 29 min. Fig. 4b shows the fractogram with the light scattering signal (LS) and the radius of gyration (Rg).

In Fig. 4a, the first peaks between 13 and 30 min correspond to the

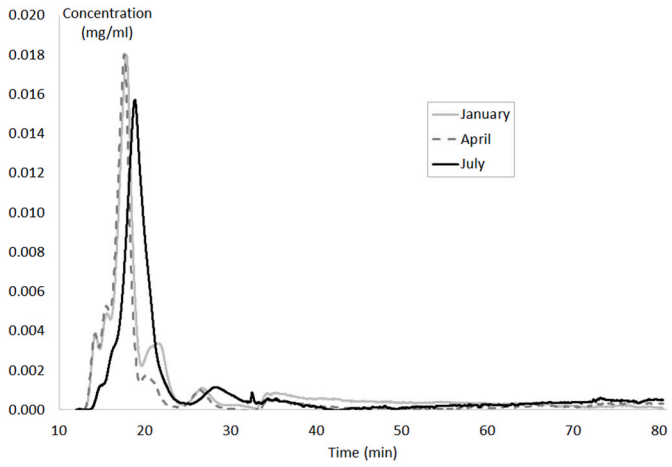

Fig. 5. AF4 fractogram of the protein concentration of untreated sheep whey for the three periods: January, April and July.

monomeric whey proteins α-LA (1), β-LG (2), albumin (3) and igG (4). To identify these peaks, standard of each protein were analysed by AF4-UV-MALS-dRi. The concentrations of the monomeric whey proteins decreased after heat treatment (magnification of Fig. 4a), particularly after heating at 80 °C for 15 s. The whey proteins must, therefore, have formed aggregates. The second peak at 15.5 min between α-LA and β-LG in Fig. 4a was not identifiable. This peak did not correspond to a protein, as no absorbance was observed at 280 nm. This peak was not detected in the AF4 analyses of Halabi et al. (2020) on heat-induced protein aggregates in model infant milk formulas. This peak may therefore correspond to a complex of fatty acid or lactose/sugar, which would account for its lack of absorption at 280 nm. Whey protein aggregates were eluted between 33 and 45 min, albeit at very low concentrations (Fig. 4a). These aggregates were detected in the MALS signal (Fig. 4b) between 37 and 80 min. The untreated whey protein sample contained a few aggregates even before heat treatment, as a strong signal was detected between 36 and 72 min on MALS. The MALS signal for the Rg of protein aggregates was similar between raw whey samples and whey samples treated at 72 °C for 1 min, with a Rg between 40 and 296 nm. Following treatment at 80 °C for 15 s, this population between 36 min and 55 min was larger as Rg values were larger, at 86–296 nm. A new population of larger aggregates was detected by MALS, between 55 and 80 min, for treatment at 80 °C for 15 s. These new aggregates had an Rg of between 296 and 366 nm.

The concentration of aggregates was low between 33 and 45 min (Fig. 4a). Previous AF4 studies by de Guibert et al. (2020) and Loiseleux et al. (2018) used bovine whey samples (whey protein isolate powder dissolved at concentrations of 47 and 50 g/l in 10 and 45 mM NaCl, respectively). A pilot heat treatment at 70 °C to 90 °C, with different flow regimes and heating residence times, was used in the study by de Guibert et al. (2020). By contrast, Loiseleux et al. (2018) heated whey proteins for 2 h in a water bath at 80 °C. Both these studies reported higher bovine whey protein aggregate peaks on AF4 fractogram than were obtained for sheep whey in our study; this difference probably reflects differences in heating time, which was shorter in our study than in these two previous studies (15 s versus 2 h in the study by Loiseleux et al., 2018). The concentration of protein was also higher in these previous studies, at 5%. Guibert et al. (2020) showed that their pilot system promoted protein aggregation because of the dynamic regime. Conversely, Giroux et al. (2018) showed that heating-induced whey protein aggregates were larger for sheep whey protein than for cow or goat whey.

3.3.2. *Impact of heat treatment on samples from each of the sampling periods*

We investigated the effect of sampling period on the protein structure of sweet sheep whey by plotting protein concentration as a function of elution time for unheated whey samples collected in the three study periods (Fig. 5). The whey proteins in the July period differed from those in the other periods in having longer retention times, implying that the objects detected had a greater hydrodynamic radius. The coelution of peaks for the July samples makes it very difficult to identify individual proteins in the AF4 fractogram. The data generated by Agrolabs (an approved laboratory for milk analyses) for milk samples revealed that the fat content was 25.8% higher in July compared with January (Figure in supplementary data). In whey, fatty acid content was higher in July. Abilleira et al. (2010) also reported 27% higher in the fat content of sheep milk between February and June. Le Maux et al. (2014) showed that β-LG can interact with fatty acids. The greater fatty acid levels in milk in July may lead to higher fatty acid concentrations in whey and, therefore, greater interaction with proteins. This might account for the retention times and larger sizes of the peaks and for peak coelution.

Finally, we investigated the influence of heat treatment for each sampling period, by dividing the amount of non-aggregated protein in the pasteurised sample by that in the unpasteurised sample, which was set to 100% and used as the reference (Table 3). The amount of non-aggregated protein was determined from the dRI signal. Data for the July period are not presented in the table for any of the proteins considered due to poor peak resolution. The fractograms of the heated and unheated whey samples for July are shown in Fig. 6 to provide an overview.

In untreated whey samples, the content of the native α-LA and β-LG proteins were higher between the months of January and April, whereas the albumin and IgG contents of the whey samples were lower (Table 3). Casper et al. (1998) showed that the concentration of each protein as a function of season depends on the type of whey: IgG levels decrease after May for goat's cheese whey but remain constant for Cheddar-type whey. No variation in β-LG content and a decrease in α-LA content after September were reported for the cheddar-type whey.

Heat treatment had an effect on total monomeric protein content by sampling period (Table 3). Following pasteurisation at 72 °C for 1 min, the levels of monomeric whey proteins decreased, and this decrease was particularly marked for the July samples: 17.7% lower in July (summer), 11.8% in April (spring) and 8.6% in January (winter) than non-heated sample. Following pasteurisation at 80 °C for 15 s, the decrease in monomeric whey protein levels was greater (about 40%) than that observed for the 72 °C/1 min pasteurisation procedure for the samples

Table 3
Undenatured protein concentrations obtained by AF4-MALS-UV-dRi in sheep whey (%) following the different heat treatments, by sampling period.[a].

Concentration of sheep whey proteins (%)											
Undenatured proteins	Total protein			α-LA		β-LG		Albumin		IgG	
Heat treatment/period	January	April	July	January	April	January	April	January	April	January	April
Raw whey	100	100	100	7.8	9.1	66.6	75.6	19.4	9.3	6.2	6.0
72 °C/1 min	91.4	88.2	82.3	7.7	8.4	66.3	68.1	10.6	9.0	6.8	2.7
80 °C/15 s	58.7	60.3	89.3	9.2	7.7	46.1	46.3	3.4	6.3	0	0

[a] Abbreviations: α-LA, α-lactalbumin; β-LG: β-lactoglobulin; IgG: Immunoglobulin. Values represent undenatured proteins (monomeric whey proteins) calculated with area of dRi signal between 12 and 33min in AF4 fractogram. The approximation was made that the raw whey only contains undenatured proteins.

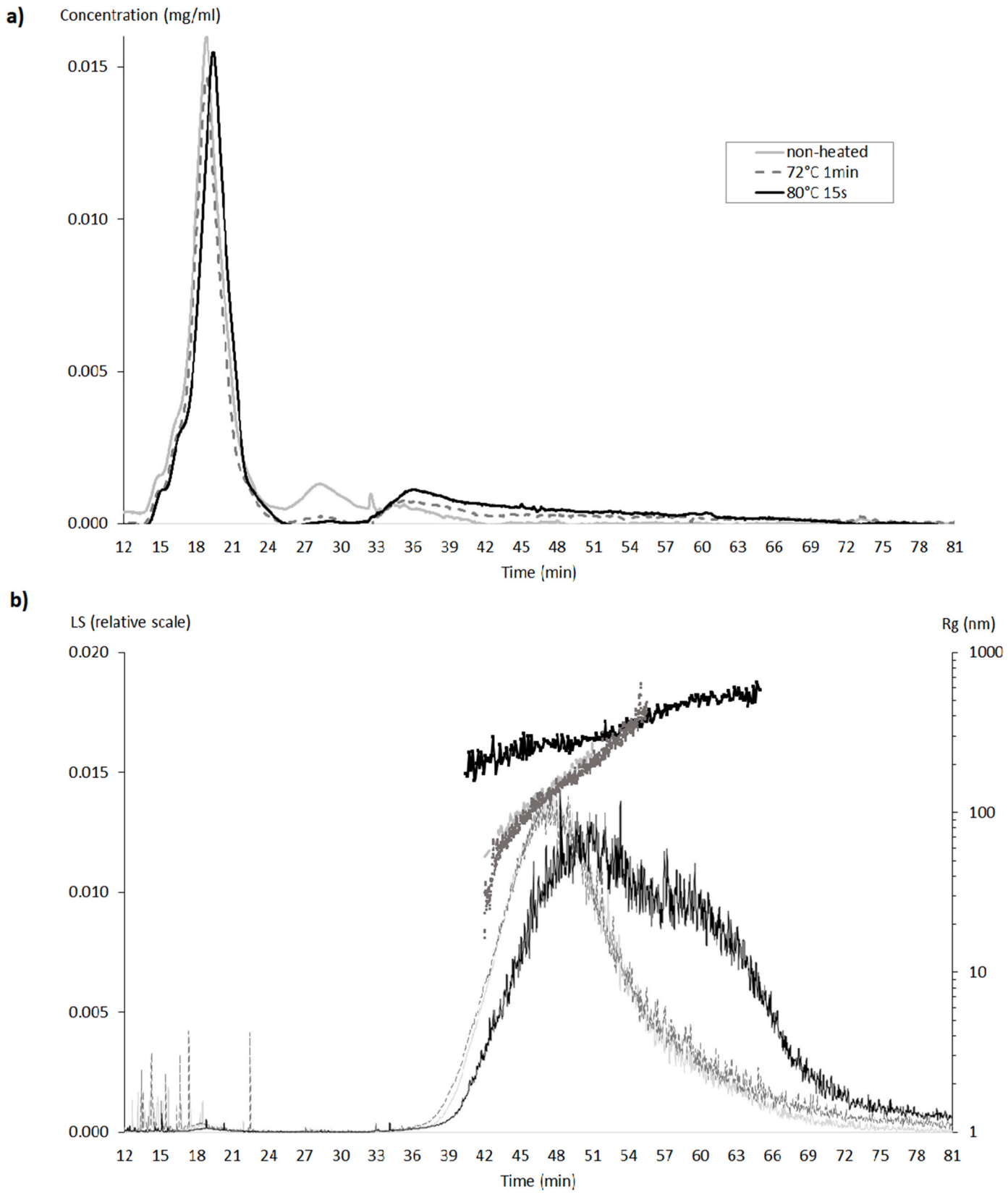

Fig. 6. (***a***) AF4 Fractogram of the protein concentrations (dRI signal) of raw and pasteurised sheep whey (72 °C/1 min and 80 °C/15 s) for the July period; (***b***) Fractogram of the MALS signal and radius of gyration (Rg) distribution of proteins in raw and pasteurised sheep whey (72 °C/1 min and 80 °C/15 s) for the July period.

collected in January and April, but smaller for the samples collected in July (10.7%). It is possible that the higher levels of fatty acids probably present in the samples collected in July protected the proteins against the effects of heat treatment. Native β-LG has been demonstrated to bind numerous hydrophobic ligands such as retinol, vitamin D, cholesterol, curcumin, fatty acids and their derivatives underlined in the review of (Le Maux et al., 2014). Binding of fatty acids, for exemple, palmitate to β-LG also protects the protein against heat-induced and chaotrope-induced denaturation (Barbiroli et al., 2011). Fatty acids act by stabilizing the calyx at the hydrophobic interface between the barrel itself and the long helix, were the thiol group of Cys121 is buried. Furthermore, the heating of the whey for a longer period, even at a lower temperature, may have resulted in less efficient protection of these proteins than heat treatments at higher temperatures but for shorter periods.

For the effect of season on each protein type (Table 3), we observed no denaturation of α-LA, β-LG or IgG in whey samples collected in January and subjected to pasteurisation at 72 °C for 1 min. Only albumin displayed denaturation and a decrease in levels (45% those in the unheated sample). By contrast, for samples collected in April, all proteins, including β-LG and immunoglobulins, were denatured to a similar extent (protein levels 45% those in the unheated sample) following heating at 72 °C for 1 min. For the heat treatment at 80 °C for 15 s, a complete aggregation of immunoglobulins (heat-sensitive proteins) was observed for all the sampling periods studied (Table 3). β-LG also denatured with this heat treatment (80 °C/15 s). The pasteurised sample thus had β-LG levels 31% and 38% lower than those in untreated whey for samples collected in January and April respectively. The corresponding rates of denaturation and aggregation for albumin were 82% in January and 32% in April. These aggregation rates are twice those observed for the 72 °C/1 min treatment on samples collected in January and 10 times those for samples collected in April and subjected to the same treatment. Finally, α-LA was the protein for which aggregation rates were lowest (the least heat-sensitive protein), even for the 80 °C/15 s treatment.

Our aggregation results for each whey protein as a function of temperature are consistent with published findings: Law (1995) showed that sheep, goat and cow whey proteins heated at temperatures between 70 and 90 °C could be ranked in descending order of irreversible denaturation rates as follows: immunoglobulins, albumin, β-LG and, finally, α-LA. Law and Leaver (1997) also showed that sheep whey proteins were less strongly denatured than cow whey proteins during heat treatment at 70 °C, whereas the opposite pattern was observed following heating at 80 or 90 °C. This phenomenon may be explained by the higher β-LG/α-LA ratio of sheep whey than of cow whey (Giroux et al., 2018), together with a higher overall serum protein concentration and differences in primary structure between species (Law, 1995).

For samples collected in July and subjected to heat treatment at 80 °C for 15 s, 10.7% of the total proteins aggregated (Table 3). According to the protein concentration fractogram (Fig. 6a), the amount of immunoglobulin decreased after pasteurisation (peak at 28 min), but the other proteins did not aggregate, regardless of the heat treatment administered. In summary, with the exception of IgG, proteins appear to be more heat resistant in samples collected in July, a period during which fat content increases in both sheep milk (Abilleira et al., 2010), and sheep whey.

Finally, Fig. 6b shows our findings for aggregate formation for pasteurised samples collected in July. A new population of larger particles with a retention time greater than 56 min emerged following treatment at 80 °C, as previously described. All the aggregates in the July samples had an Rg of 200–579 nm. This Rg is greater than that for untreated samples and samples subjected to heating at 72 °C, for which the Rg values obtained were between 50 and 435 nm. The Rg of the population of aggregated whey proteins was greater for the July period than for the January and April periods (Fig. 6b).

4. Conclusion

The aim of this study was to define an effective heat treatment for the preservation of sheep whey for storage before its use in manufacturing. The challenge was to retain the highest possible levels of the native forms of proteins and to ensure good microbial quality. We took the season into account because sheep milk and the whey produced from it undergo biochemical changes during the course of the year, particularly at the end of lactation. For both heat treatments tested, the rates of decrease in microorganism levels were sufficient to ensure the safety of a product stored for 5 day at 4 °C. The rates of protein denaturation for the treatment at a higher temperature (80 °C for 15 s) depended on the season. Protein aggregation rates were higher in January and April than in July (40% vs. 11%). Aggregation rates were lowest (13%) for the gentle heat treatment (72 °C for 1 min). This treatment was thus identified as the best treatment for whey preservation before use in manufacturing.

Our results indicate that AF4 is a good technique for analysing the changes in whey proteins due to heat treatment and with the seasons. This study provides new insight into the combined effects of season and heat treatment on the denaturation of sheep whey proteins.

CRediT authorship contribution statement

Marie Hennetier: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Clarissa Detomi DE Albuquerque:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology. **Loubnah Belahcen:** Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Inés Martin Saez:** Writing – original draft, Investigation, Formal analysis, Data curation. **Romain Valentin:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Hélène Tormo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

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