Effect of whey concentration on protein recovery in fresh ovine ricotta cheese

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ABSTRACT

Ricotta cheese, particularly the ovine type, is a typical Italian dairy product obtained by heat-coagulation of the proteins in whey. The aim of this work was to investigate the influence of whey protein concentration, obtained by ultrafiltration, on yield of fresh ovine ricotta cheese. Ricotta cheeses were obtained by thermocoagulation of mixtures with protein content of 1.56, 3.10, 4.16, and 7.09 g/100 g from the mixing of skim whey and ultrafiltered skim whey. A fat-to-protein ratio of 1.1 (wt/wt) was obtained for all mixtures by adding fresh cream. The initial mixtures, as well as the final ricotta cheeses, were analyzed for their composition and by SDS-PAGE. Protein bands were quantified by QuantityOne software (Bio-Rad, Hercules, CA) and identified by liquid chromatography-tandem mass spectrometry. Significant differences in the composition of the ricotta cheese were observed depending on protein concentration. Particularly, ricotta cheese resulting from the mixture containing 7.09 g/100 g of protein presented higher moisture (72.88 ± 1.50 g/100 g) and protein (10.18 ± 0.45 g/100 g) contents than that prepared from the mixture with 1.56 g/100 g of protein (69.52 ± 1.75 and 6.70 ± 0.85 g/100 g, respectively), and fat content was lower in this sample (12.20 ± 1.60 g/100 g) compared with the other treatments, with mean values between 15.72 and 20.50 g/100 g. Each protein fraction presented a different behavior during thermocoagulation. In particular, the recovery of β -lactoglobulin and α -lactalbumin in the cheese increased as their content increased in the mixtures. It was concluded that concentrating ovine rennet whey improved the extent of heat-induced protein aggregation during the thermal coagulation process. This resulted in a better recovery of each protein fraction in the product, and in a consequent increase of ricotta cheese yield.

Key words: sheep whey, ultrafiltration, protein recovery, ricotta cheese

INTRODUCTION

Ovine whey cheeses are mainly produced in the Mediterranean countries, such as Italy, Greece, Portugal, and Spain (Kandarakis, 1986). Usually, they are manufactured according to traditional protocols by thermal denaturation of whey proteins. Among them, ricotta cheese is probably the oldest and the best known dairy product obtained from cheese whey (Pizzillo et al., 2005), and in recent decades it has become rather popular in the United States and Canada. In ricotta cheese production, whey represents the raw material, although milk or cream can be added (Farkye, 2004).

Cheese whey is a by-product of the dairy industry, containing mainly lactose, soluble proteins, minerals, and milk fat, which may reach about 50% of the milk TS (Casper et al., 1998). The production of ricotta cheese is considered to be one of the most convenient ways to use whey originating from the cheese-making process (Shukla and Kaur Brar, 1986).

The ovine and caprine types of ricotta cheese are usually manufactured following the traditional protocol described herein. The cheese whey (normally at a pH of about 6.50) without exogenous acidification is heated to a final temperature of 78 to 80°C under constant stirring. The applied heat results in the coagulation of the whey proteins, which is characterized by the appearance of small flakes on the surface as consequence of a multiple-reaction process related to the unfolding and aggregation of the proteins (Morr and Josephson, 1968; Parris et al., 1993). After a resting period of 10 min at 78 to 80°C, the formed coagulum is carefully scooped off and placed in plastic perforated conical molds, where it drains for 8 to 24 h in a cool room (4°C). After the completion of draining, the product can be packed and commercialized.

The typical yield of ricotta cheese is only about 5 to 6%, and this highlights the very low efficiency of the process. In particular, significant losses of proteins with high nutritional and biological value exist. After ricotta cheese production, the residual whey still contains about 1% of proteins (Nudda et al., 2004). It has been demonstrated that parameters, such as heating temperature, protein concentration, pH, and the type and concentration of salts, represent important factors

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affecting the heat-induced aggregation of whey proteins (Mangino, 1984; Taylor et al., 1994; Hollar et al., 1995). Among whey proteins, it has been shown that α -LA has a greater resistance to thermal denaturation compared with the other whey protein fractions (Singh and Havea, 2003). Specifically, the resistance to denaturation decreases from α -LA to β -LG to BSA to Ig (in order). However, when present in a solution containing other proteins, the aggregation behavior is altered. It has been shown, for example, that the interactions between α -LA and β -LG enhance the denaturation of α -LA (Dalglish et al., 1997). The ionic strength of the whey is an important parameter in determining the protein aggregation and consequently the gel-structuring process. In particular, the presence of divalent cations, such as calcium and magnesium, are fundamental to promote the aggregation by partially shielding the negative charges of whey proteins and bridging between protein molecules, reducing the electrostatic repulsion, and favoring the formation of large aggregates.

Although ricotta is a very widespread whey cheese, the literature on this product is scarce and outdated, in particular as it relates to the factors affecting ricotta cheese processing and yield. Protein concentration seems to be an important parameter. Streiff et al. (1979) used condensed whey to produce ricotta cheese and obtained high yields, but minerals were also concentrated in their study. Conversely, very little has been reported on UF of ovine whey for ricotta cheese manufacture. During this process, the protein concentration is increased, whereas the ionic strength is lower than in the case of evaporated whey.

In the present study, we hypothesized that an increased concentration of whey before ricotta cheese-making would change the interactions between the main whey protein fractions. Hence, the aim of the current work was to study the influence of the concentration of the whey by UF on the recovery rate of the different whey proteins and on the resulting ricotta cheese yield.

MATERIALS AND METHODS

Preparation of UF Whey

Ovine whey from protected designation of origin Pecorino Sardo cheese-making was obtained from experimental cheese plant of Agris Sardegna. All thermal treatments described herein occurred in a batch-wise (discontinuous) process. The whey was heated at 63°C (from 40 to 63°C in 8 min) in a stainless steel cheese vat, quickly cooled to 40°C (from 63 to 40°C in 5 min), and skimmed. Cream was kept at 4°C, whereas skim whey was concentrated by UF at 40°C to produce retentate at 6× (based on volume reduction), using a

pilot plant (Mete srl, Membrane Technology, Varese, Italy) equipped with a polyethersulfone membrane (20 kDa; Celgard, Charlotte, NC), with a nominal area of 5 m². Cross-membrane pressure and flow rate were 0.15 MPa and 2.5 m³/h, respectively. Retentate was immediately stored at 4°C until further use.

Preparation of Whey Mixtures and Their Composition

Skim whey, retentate, and cream were used to get 4 different mixtures with different concentrations of fat and protein, but the same fat-to-protein ratio of 1.1. The mixtures had final protein concentrations of 1.56, 3.10, 4.16, and 7.09% (**M1**, **M2**, **M3**, and **M4**, respectively). The amount of each component (skim whey, retentate, and cream), which had to be added to obtain the predetermined mixtures, was calculated by solving the following mathematical system:

$$\begin{cases} d = ax + by + cz / AM \\ d1 = a1x + b1y + c1z / AM, \\ x + y + z = AM \end{cases}$$

where x , y , and z are the amount (kg) of cream, retentate, and skim whey, respectively; AM (kg) is the amount of the whey mixture processed; d and $d1$ are the amount (g/kg) of fat and protein in the mixture, respectively; a , b , and c are the amount of fat (g/kg) in cream, retentate, and skim whey, respectively; $a1$, $b1$, and $c1$ are the amount of protein (g/kg) in cream, retentate, and skim whey, respectively.

Skim whey, retentate, cream, and the whey mixtures were analyzed for pH (Orion model 420A pH meter, Orion Research Inc., Boston, MA), TS (IDF, 1987), fat (Gerber method), total nitrogen (TN), and NPN (Rowland, 1938). The protein content was estimated (nitrogen $\times$ 6.38) by the difference between TN and NPN. Ionic calcium (Ca²⁺) and magnesium (Mg²⁺) contents of whey mixtures were determined by atomic absorption spectrophotometry with a SpectrAA 250 plus (Varian, Palo Alto, CA) using a standard curve and following the procedures recommended by AOAC (1990), with slight modifications.

Ricotta Cheese Production and Analysis

Ricotta cheeses were manufactured starting from each corresponding whey mixture (**R1**, **R2**, **R3**, and **R4** from **M1**, **M2**, **M3**, **M4**, respectively) according to a traditional protocol. Each whey mixture was heated (in a pilot-scale cheese coagulation vat) to 78 to 80°C under stirring without further acidification (see Table

Table 1. Composition of whey mixtures¹

Item	M1	M2	M3	M4
pH	6.51 ± 0.02	6.53 ± 0.02	6.53 ± 0.03	6.50 ± 0.02
TS (g/100 g)	8.76 ± 0.22 ^d	11.62 ± 0.31 ^c	13.70 ± 0.47 ^b	19.42 ± 0.45 ^a
Fat (g/100 g)	1.61 ± 0.10 ^d	3.44 ± 0.06 ^c	4.50 ± 0.04 ^b	7.81 ± 0.27 ^a
Protein ² (g/100 g)	1.56 ± 0.08 ^d	3.10 ± 0.27 ^c	4.16 ± 0.41 ^b	7.09 ± 0.48 ^a
Fat-to-protein ratio (wt/wt)	1.03 ± 0.06	1.12 ± 0.08	1.09 ± 0.12	1.11 ± 0.09
Ca ²⁺ per protein (mg/g)	22.94 ± 0.50 ^a	13.43 ± 0.77 ^b	10.98 ± 0.74 ^c	7.31 ± 0.53 ^d
Mg ²⁺ per protein (mg/g)	4.34 ± 0.30 ^a	2.83 ± 0.38 ^b	2.18 ± 0.39 ^{bc}	1.46 ± 0.13 ^c

^{a-d}Values in the same row with different superscript letters differ ($P < 0.05$).

¹Values are means and SD of 3 replicates of whey mixtures with different protein concentrations: M1 (1.56%), M2 (3.10%), M3 (4.16%), and M4 (7.09%). All mixtures had the same fat to protein ratio (1.1).

²Protein = total N × 6.38.

1 for pH and mineral content of each mixture). When proteins started to flocculate, stirring was stopped to facilitate the formation of large aggregates. The hot mixture was left to rest (about 10 min) to allow rising of the curd on the surface. The curd was then scooped, moved into perforate molds, and then kept overnight in a cool room (4°C) for draining. Three replicates (with 3 separate UF whey batches) were carried out for each treatment level.

Ricotta cheese was sampled for analysis after 1 d of production. On each sampling day, the samples were analyzed for pH (Orion model 420A pH meter, Orion Research Inc.), DM (IDF, 1982), fat (Soxhlet method), and TN and NPN (Gripon et al., 1975).

The calculations of ricotta cheese yields and recoveries were performed as

$$\text{Actual yield} = (\text{AR}/\text{AW}) \cdot 100,$$

$$\text{Adjusted yield} = (\text{AR}/\text{AW} \cdot 100) \cdot \text{Mc}/\text{M70},$$

$$\begin{aligned} \text{Recoveries (fat or protein)} = \\ (\text{SR} \cdot \text{AR})/(\text{SM} \cdot \text{AM}) \cdot 100, \end{aligned}$$

where AR is the amount of ricotta cheese [to compare yields between samples it is necessary to relate the amount of ricotta cheese (kg) to the amount of initial whey (kg) used in the entire production process]; AW (kg) is the amount of initial whey used to obtain the mixtures (sum of each component of the initial whey used in the preparation of the mixture; retentate was multiplied by the volume concentration ratio); Mc is the experimentally determined moisture of ricotta cheese (g/100 g); M70 is the moisture representing the reference mean value for the product (70 g/100 g); SR is the amount of solids (fat or protein) contained in ricotta cheese (g/100 g); SM is the amount of solids (fat or protein) contained in whey mixtures (g/100 g); and AM (kg) is the amount of the whey mixture processed (sum of each component used in the preparation of the

mixture; in this case retentate was not multiplied by the volume concentration ratio).

Extraction and Electrophoresis of Whey and Ricotta Cheese Proteins

The ricotta cheese, relative whey mixtures, and residual whey were analyzed in triplicate by SDS-PAGE and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Ricotta samples were prepared by homogenizing approximately 50 g of raw product and resuspending 100 mg of the homogenate in 10 mL of Milli-Q ultrapure water (Millipore, Billerica, MA). Whey did not require any previous treatment. Protein concentrations in whey and ricotta samples were determined with the bicinchoninic acid method (Thermo Scientific, Rockford, IL). All samples were subjected to SDS-PAGE, according to Laemmli (1970), by loading in each well the similar amount of total protein per sample. After electrophoresis, gels were stained with brilliant blue G250, as described by Westermeier (2006), and digitally acquired with an ImageScanner (GE Healthcare, Little Chalfont, UK). Protein patterns were analyzed with QuantityOne (Bio-Rad, Hercules, CA) software, using the following parameters for band detection: sensitivity, 10.000; lane width, 2.400 mm; minimum density, 0.00%; noise filter, 4.00; shoulder sensitivity, 1.00; and size scale: 5. To compensate for minimal loading differences due to experimental variation, band volumes were normalized to the total protein amount loaded in each electrophoresis run by conversion into percentage of the total band volume.

LC-MS/MS Analysis

Seven protein bands for each sample, indicated by QuantityOne software analysis as more abundant in whey and ricotta cheese, were excised from the gel, destained by multiple washings with 50 mM, pH 8.0 ammonium bicarbonate and acetonitrile, reduced with 10 mM dithiothreitol at 56°C, carbamidomethylated

with 55 mM iodoacetamide at room temperature, and, finally, digested overnight with 60 to 100 ng of trypsin (according to band intensity) at 37°C. Then, LC-MS/MS analysis was performed as previously described (Addis et al., 2009; Pisanu et al., 2011) on a Q-TOF hybrid mass spectrometer with a nano-lock Z-spray source, coupled on-line with a capillary chromatography system CapLC (Waters, Manchester, UK). Briefly, after loading, peptides were concentrated and washed onto a reverse-phase precolumn (Symmetry 300, C18, 5 μ m, NanoEase, Waters), and then fractionated onto a C18 reverse-phase column (Symmetry, 75 μ m $\times$ 15 mm, Waters). The mass spectrometer was set up in a data-dependent MS/MS mode, where a full scan spectrum was followed by tandem mass spectra, and peptide ions were selected as the 3 most intense peaks of the previous scan. Suitable collision energy was applied, depending on the mass and charge of the precursor ion, and argon was used as the collision gas. Raw MS and MS/MS spectra were analyzed with the ProteinLynx software to generate a peak list, which was introduced in the in-house Mascot MS/MS ion search software (Matrix Science, Boston, MA) for protein identification.

Statistical Analyses

Statistical treatment of the data was performed using the SPSS statistical package, release 11.5 (SPSS, Chicago, IL). The results of the whey mixtures and ricotta cheese composition, ricotta cheese yield parameters, and the recovery rate of the single whey proteins were examined using a mono-factorial ANOVA model with protein concentration in whey as fixed effect. Tukey's test for multiple comparisons was used to separate treatment means.

RESULTS AND DISCUSSION

Chemical Composition

The chemical composition of the whey mixtures is reported in Table 1. Total solids, fat, and protein increased with the concentrated samples, whereas the fat-to-protein ratio was kept constant (1.1) to be able to compare the final yields. The pH values were not significantly different (6.50–6.53; $P > 0.05$) between samples, and this was rather important because the aggregate formation in ricotta cheese production is affected, among other factors, by the pH variation in cheese whey.

The values of Mg^{2+} and Ca^{2+} of whey mixture M1 were 6.77 ± 0.46 and 35.78 ± 0.80 mg/100 g, respectively. These values were in agreement with those reported in literature for Mg^{2+} and slightly lower for Ca^{2+}

(Mg^{2+} content in sheep milk is 18 mg/100 g, soluble fraction 56%; Ca^{2+} content in sheep milk is 193 mg/100 g, soluble fraction 21%; Park et al., 2007). This discrepancy is possibly due to the changes in the processing parameters and the contribution of calcium bridges to the rennet-induced gel formation (Crabbe, 2004) during protected designation of origin Pecorino Sardo cheese-making. During UF, the soluble salts were transmitted through the membrane, so their total amount remained similar with concentration (Salvatore et al., 2011).

Before ricotta cheese production, calcium or other ion sources are usually added to whey with the aim of improving the dehydration and destabilization of whey proteins (Farkye, 2004). Furthermore, ionic strength influences the structure and rheological characteristics of protein aggregates, because cations, by neutralizing the negative charges, determine a reduction of charge repulsion between the protein particles. However, it is important to note (see Table 1) that the amount of ions (available per gram of protein) in the whey mixture decreased significantly with concentration, and this will have important consequences to the whey protein denaturation and the type of aggregates formed (as discussed further).

Table 2 summarizes the composition of ricotta cheese. The samples did not present differences in terms of pH, whereas they were statistically different from each other for moisture, fat, and protein. The composition of R1 (protein content: 6.70 ± 0.85 g/100 g; fat content: 20.50 ± 2.19 g/100 g) agrees somewhat with values reported by Pintado et al. (2001) for ricotta cheese made from sheep whey with a traditional manufacture technique. Those authors reported that the content of protein and fat ranged from 6.1 to 8.7 and 10.2 to 24.5% (wt/wt), respectively. This highlighted a very low efficiency of the process in terms of protein recovery with a high fat-to-protein ratio. Ricotta cheese (R4) manufactured from the whey with the highest protein content (M4) contained less fat compared with the other samples. In general, it was evident that increasing the protein content of the whey mixtures (while maintaining the same fat-to-protein ratio) resulted in products with a reduced content of fat and an increased amount of protein. Maubois and Kosikowski (1978) noted that the concentration of milk proteins by UF improved their precipitation by heat. The authors attributed this effect to the close proximity between the proteins because of the high-volume fraction. A statistically significant higher value of moisture content was observed for R4 compared with the lower protein concentration ricotta cheese, and this was probably due to the water-binding capacity of denatured whey proteins (Mangino, 1984), which were more abundant in this product.

Table 2. Composition of ricotta cheese and yields and solid recoveries^{1,2}

Item	R1	R2	R3	R4
pH	6.69 ± 0.01	6.79 ± 0.09	6.72 ± 0.15	6.63 ± 0.06
Moisture (g/100 g)	69.52 ± 1.75 ^b	69.36 ± 2.28 ^b	70.34 ± 2.72 ^{ab}	72.88 ± 1.50 ^a
Fat (g/100 g)	20.50 ± 2.19 ^a	17.34 ± 2.47 ^{ab}	15.72 ± 2.92 ^b	12.20 ± 1.60 ^c
Protein ³ (g/100 g)	6.70 ± 0.85 ^b	9.61 ± 0.36 ^a	10.12 ± 0.40 ^a	10.18 ± 0.45 ^a
Fat/TS (wt/wt)	67.18 ± 5.03 ^a	56.42 ± 3.75 ^b	52.67 ± 5.03 ^b	44.84 ± 3.53 ^c
Protein/TS (wt/wt)	22.03 ± 2.74 ^c	31.46 ± 2.06 ^b	34.32 ± 3.58 ^{ab}	37.64 ± 2.29 ^a
Actual yield ⁴ (kg/100 kg)	6.14 ± 0.66 ^c	7.25 ± 0.71 ^{bc}	7.77 ± 0.99 ^b	10.74 ± 1.24 ^a
Adjusted yield ⁵ (kg/100 kg)	6.23 ± 0.74 ^c	7.39 ± 0.66 ^{bc}	7.66 ± 1.02 ^b	9.66 ± 0.79 ^a
Fat recovery ⁶ (%)	78.84 ± 9.65	78.38 ± 7.48	79.27 ± 12.11	85.20 ± 5.99
Protein recovery ⁶ (%)	26.85 ± 5.64 ^c	48.55 ± 2.68 ^b	55.72 ± 4.67 ^b	81.50 ± 6.95 ^a

^{a-c}The data in the same row with different superscript letters differ ($P < 0.05$).

¹Values are means and SD of 3 replicates.

²R1, R2, R3, and R4 are the ricotta cheeses obtained from whey mixtures with different protein concentrations: M1 (1.56%), M2 (3.10%), M3 (4.16%), and M4 (7.09%). All mixtures had the same fat-to-protein ratio (1.1).

³Protein = total N × 6.38.

⁴Actual yield = amount of ricotta cheese (kg) obtained per 100 kg of initial whey.

⁵Adjusted yield = actual yield adjusted to 70% moisture content.

⁶For the calculation of fat and protein recovery, the mean amount of whey mixture used (see equations in text) was about 100 (M1), 46.2 (M2), 34.2 (M3), and 19.6 kg (M4), respectively.

These results indicate that, by increasing the protein and decreasing the mineral-to-protein ratio (with a constant fat-to-protein ratio) in the original mixture, it is possible to increase the protein recovery in ricotta cheese. Indeed, the results suggest that the higher fat concentration needed to maintain the fat-to-protein ratio in the mixtures resulted in higher fat losses during the formation of the network.

Proteomic Analysis of Whey Mixtures and Ricotta Cheese

The 4 starting mixtures and the corresponding ricotta cheese products were subjected to proteomic analysis by SDS-PAGE followed by LC-MS/MS. Upon loading of an equivalent amount of total proteins per lane for each of the 4 whey mixtures and of the resulting ricotta products, all samples showed reproducible and comparable electrophoretic profiles (Figure 1A), similar to those previously reported for milk whey or whey protein concentrates (Su and Chiang, 2003; Veith and Reynolds, 2004). After digitalization, 7 more intense protein bands were detected by QuantityOne software. Then these bands were excised and subjected to tandem mass spectrometry identification, with results summarized in Table 3. Each band corresponded to one or more proteins contained in the whey mixtures and, consequently, in ricotta cheese. To provide quantification of the proteins and to enable comparison of their relative abundances among all samples, densitometric profiles were generated and subjected to differential analysis. Figure 1B shows the superimposition of the lane densitograms in a representative experiment. Differential analysis revealed that the relative percentages

of whey proteins were constant within the 4 whey mixtures, indicating that all proteins were concentrated to the same extent during UF. The percentages of band 6 (β -LG; average value of 45.1%) and 7 (α -LA; average value of 15.65%), corresponding to the major protein fractions of the whey, were slightly lower when compared with literature values for cow milk (54% for β -LG, and 21% for α -LA; Kinsella, 1984). Bands 1 (xanthine dehydrogenase/oxidase), 2 (lactotransferrin; polymeric immunoglobulin receptor; lactoperoxidase), 3 (serum albumin; butyrophilin subfamily 1 member A1; complement C3), 4 (lactadherin), and 5 (Ig lambda chain; glyCAM 1) presented mean values (within the 4 mixtures) of 1.37, 7.29, 9.78, 12.00, and 8.79%, respectively. It should be noted that some proteins, such as those identified in bands 1 (xanthine dehydrogenase/oxidase), 3 (butyrophilin), and 4 (lactadherin), derived from the milk fat globule membrane (Pisanu et al., 2011). This is probably due to the mechanical treatments undergone by the whey (skimming in particular), which may have caused the transfer of these proteins from the milk fat globule membrane to whey, as well as their presence in very small fat globules not fully separated by centrifugation (Argov-Argaman et al., 2010).

As for ricotta cheese samples, relative differences in abundance ($P < 0.05$) could be observed only for bands 4 and 7 (Figure 2). In particular lactadherin (band 4) showed a progressive reduction (14.64% for R1 and 11.42% for R4) with increasing protein concentration of whey mixtures. As previously mentioned, lactadherin is associated with the milk fat globule membrane; hence, these results are in full agreement with the decrease in fat content in the high-concentration ricotta cheeses. Conversely, α -LA (band 7) showed a significant increase

A

B

Figure 1. (A) Representative SDS-PAGE profile of whey mixtures and ricotta cheese samples. The whey mixtures had the following protein concentrations: 1.56% (M1), 3.10% (M2), 4.16% (M3), and 7.09% (M4) with the same fat-to-protein ratio (1.1); R1, R2, R3, and R4 are the ricotta cheeses obtained from M1, M2, M3, and M4, respectively; MWM = the marker lane with molecular weights (kDa) shown on the left of the gels. The 7 bands subjected to differential analysis and liquid chromatography-tandem mass spectrometry identification are indicated in the schematic drawing on the right of the gels. (B) Example of the densitometric profiles of SDS-PAGE analysis of whey mixtures and ricotta cheese samples.

in its relative percentage compared with the other proteins (7.50% for R1 and 15.83% for R4), whereas the relative percentage of β -LG was rather high (51% on average for all ricotta cheeses) and did not vary significantly with increasing the protein concentration. It has been shown that α -LA has a greater resistance to thermal denaturation when compared with β -LG (Law et al., 1994), and the rate of denaturation of α -LA is more affected than that of β -LG by changes in whey protein concentration (Oldfield et al., 2005). Furthermore, some authors (Calvo et al., 1993; Law and Leaver, 1997) reported that denaturation and aggregation of α -LA, which are dependent on the availability of free thiol groups, are strongly influenced by the increase of β -LG and BSA concentration because these proteins, with

their thiol groups, permit the formation of intermolecular disulphide bonds between the whey proteins. In general, the resistance of the whey proteins to thermal denaturation (in milk) decreases from α -LA to β -LG to BSA to Ig (in order; Singh and Havea, 2003). Therefore, it seems reasonable to conclude that the variations observed in relative abundances among whey proteins are linked to a change in the association behavior of α -LA with increasing concentration of whey proteins and a decrease in the ratio of calcium per protein. Indeed, α -LA is able to bind Ca^{2+} or other metal ions; the removal of Ca^{2+} ions destabilizes its conformation (Relkin and Mulvihill, 1996) and increases its sensitivity to heat denaturation. It has been previously reported that a decrease in calcium concentration increases the

Table 3. Protein identities obtained by SDS-PAGE followed by liquid chromatography-tandem mass spectrometry

Band ¹	Acc. No. ²	Protein name	Organism	Molecular weight (Da)	Score	Peptide
1	P80457	Xanthine dehydrogenase/oxidase	<i>Bos taurus</i>	148,863	250	16
2	Q29477	Lactotransferrin	<i>Capra hircus</i>	79,361	436	17
2	P81265	Polymeric immunoglobulin receptor	<i>Bos taurus</i>	83,695	234	12
2	P80025	Lactoperoxidase	<i>Bos taurus</i>	81,504	228	12
3	P14639	Serum albumin	<i>Ovis aries</i>	71,139	2,987	97
3	P18892	Butyrophilin subfamily 1 member A1 ³	<i>Bos taurus</i>	59,923	173	6
3	Q2UVX4	Complement C3 ³	<i>Bos taurus</i>	188,675	69	4
4	Q95114	Lactadherin	<i>Bos taurus</i>	48,520	93	4
5	Q1RMN8	Immunoglobulin lambda chain	<i>Bos taurus</i>	24,536	38	2
5	P81447	GlyCAM 1 ³	<i>Capra hircus</i>	17,102	37	11
6	P67975	Beta-lactoglobulin	<i>Ovis orientalis musimon</i>	18,425	788	40
7	P00712	Alpha-lactalbumin	<i>Capra hircus</i>	16,700	141	6

¹The band number refers to the 7 bands shown in Figure 1A. Identifications were performed in triplicate gels for each sample (whey and ricotta cheese). The best results are reported for each replicate identification.

²Uniprot accession number.

³Protein identifications not falling in the predicted molecular weight range of the stained band.

rate of formation of α -LA protein aggregates (Liu et al., 2011). As can be seen in Table 1, the availability of divalent cations (Ca^{2+} and Mg^{2+}) per protein was significantly reduced when the protein concentration in whey mixtures increased.

Yields and Solids Recoveries

Yields and solids recoveries are reported in Table 2. Actual yield, as well as the yield adjusted to 70%

moisture content, significantly ($P < 0.05$) increased with the concentration of fat and protein in the whey mixtures. The actual yield increased from 6.14 (R1) to 10.74% (R4), representing a 75% increase. Adjusting for moisture (adjusted yield), the percentage was about 55% (6.23% for R1 and 9.66% for R4), indicating that the increase in yield was partially due to the moisture and not only because of the protein recovery. The fat recovery did not differ significantly among samples

Figure 2. Percent distribution of proteins in ricotta cheese. The whey mixtures had the following protein concentration: 1.56% (M1), 3.10% (M2), 4.16% (M3), and 7.09% (M4) with the same fat-to-protein ratio (1:1); R1, R2, R3, and R4 are the ricotta cheeses obtained from whey mixtures M1, M2, M3, and M4, respectively. The numbers from 1 to 7 represent the bands detected by QuantityOne software (Bio-Rad, Hercules, CA) with protein identification reported in Table 3. Error bars indicate standard deviation of 3 replicates. Different letters (a–c) indicate significant differences ($P < 0.05$) relating to a specific band.

and was rather high in all treatments, 78.84% for R1, whereas protein recovery values ($P < 0.05$) passed from 26.85% in R1 to 81.50% in R4. With the aim of verifying which proteins were more responsible for these recovery values, the protein recovery rate of ricotta cheese was calculated for each protein band (Figure 3). These values were obtained by dividing the amount of proteins contained in each band of the ricotta cheese samples (expressed as the relative percentage obtained for each band multiplied by grams of proteins), with the corresponding values obtained for whey mixtures. After statistical analysis, a significant increase ($P < 0.05$) in protein recovery was observed for all bands (except for band 1), revealing the percent increase in the content of all proteins in ricotta cheese obtained from mixtures with increasing protein concentrations (Figure 3). The highest protein recovery rates (with increasing concentration) were obtained for α -LA (band 7), which changed from 15.68% in R1 to 65.01% in R4, followed by β -LG (38.65% in R1 and 83.62% in R4), consistent with what was discussed previously concerning the association behavior of α -LA with an increasing concentration of whey proteins and a decreasing calcium-to-protein ratio.

When the protein content in the cheese whey is low, gel formation represents some challenges. These aspects explain the lower capacity of flocculation of cow whey (less rich in protein) compared with sheep whey. With increasing protein concentrations, the number of linkag-

es increases during heating, resulting in a more compact protein gel characterized by an improved water-holding capacity, as reported in the literature (Mangino, 1984; see the moisture values between ricotta cheese samples in Table 2).

Although not within the scope of this work, the ricotta cheeses were also observed visually and taste-tested by trained sensory judges, who found some differences in texture between them. In particular, ricotta cheese with the highest protein content was harder than other samples. Further studies could use a microstructural approach with the aim of modifying the texture of ricotta cheese to obtain a product with high yields that could be better appreciated by consumers.

CONCLUSIONS

Concentrating ovine rennet whey improved the extent of heat-induced protein aggregation during the thermal coagulation process. This resulted in a better recovery of proteins in the product, particularly of α -LA, and in a consequent increase of ricotta cheese yield. In contrast, the fat recovery in the product did not show an increase with concentration of the whey mixtures. The lower availability of Ca^{2+} and Mg^{2+} per gram of protein in samples with increased protein concentration demonstrates that the close proximity between molecules due to the protein concentration has a major effect on protein recovery and ricotta cheese

Figure 3. Protein recovery rate in ricotta cheese. The whey mixtures had the following protein concentration: 1.56% (M1), 3.10% (M2), 4.16% (M3), and 7.09% (M4) with the same fat-to-protein ratio (1.1); R1, R2, R3, and R4 are the ricotta cheeses obtained from whey mixtures M1, M2, M3, and M4, respectively. The numbers from 1 to 7 represent the bands detected by QuantityOne software (Bio-Rad, Hercules, CA) with protein identification reported in Table 3. Error bars indicate standard deviation of 3 replicates. Different letters (a,b) indicate significant differences ($P < 0.05$) relating to a specific band.

yield compared with the parameters linked to the ionic strength of the medium.

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