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An analytical approach to reveal the addition of heat-denatured whey proteins in lab-scale cheese making

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Abstract

A simple analytical procedure for the detection of self-aggregated heat-denatured whey proteins (HDWP) in model cheeses was developed. The principle of the approach lies in the solubilization of the cheese matrix by a sodium citrate solution (0.2 M, pH 7.0) resulting in the dissociation of the casein micelles and the insolubilization of HDWP aggregates, which are collected in the pellet after a centrifugation step. The reliability of the procedure was tested in lab-scale cheeses from peroxidase-positive pasteurized milk with different protein-based ingredients (microparticulated whey protein concentrate, milk protein concentrate, whey protein isolate and Ricotta cheese) at concentrations ranging from 0.2 to 1.2% protein (w/v on cheese milk). A linear relationship between the amount of the HDWP added to cheese milk and that recovered from model cheeses was observed. Heat-damage indicators, furosine and lysinoalanine, showed levels in the experimental cheese samples not related with added HDWP, but represented a source of information on the ingredients other than liquid milk. Overall, in the model cheeses, the proposed method was an easy-to-apply and reliable tool for the evaluation of the presence of HDWP-based products. Further investigation is required for the application to real cheeses and for the evaluation of possible interferences from proteolysis during ripening.
Keywords: heat-denatured whey proteins; milk cheese; supplementation; extraction; sodium citrate

Abbreviations: CN, casein; CZE, capillary zone electrophoresis; FUR, furosine; HDWP, heat-denatured whey proteins; HTST, high-temperature short-time; HWPI, WPI in-batch heated (100 °C/3 h); LAL, lysinoalanine; MPC, milk protein concentrate; WP, whey proteins; WPC, whey protein concentrate; WPI, whey protein isolate.

1. Introduction

Casein (CN) constitutes the solid protein matrix of cheese, in which different amounts of whey proteins (WP) are entrapped according to raw materials other than liquid milk and the degree of whey draining resulting from the (rennet or acid) coagulation (Fox & McSweeney, 2004). Dairy-based protein ingredients can be added into milk to produce cheese, but the WP/CN ratio should not be modified to the extent of exceeding that of milk. Whey resulting from cheese making is available in large volumes (approximately 80–90% of the volume of milk used) and its disposal represents a major industrial concern. Nonetheless, whey is a source of valuable nutrients which could be recovered and used individually. Traditionally, the recovery of WP, consisting in whey drying, allows obtaining powders which differ in composition and technological properties depending on the pretreatment operations (Jelen, 2011; Sharma, Jana & Chavan, 2012). Instead of heating, the pre-concentration can be performed by membrane technologies that can maintain WP in the native form to satisfy specific properties of the end-product (Foegeding, Luck & Vardhanabhuti, 2011).

The re-integration of WP into cheese matrix has been an issue of utmost importance for the increase of yield, the improvement of nutritional value, the refinement of texture and the increase of profit (Giroux, Lanouette & Britten, 2015). Native WP can be re-integrated in cheese (Lawrence, 1989), although heat-denatured WP (HDWP) are preferred for their high water-binding capacity and better physical retention in the curd during drainage. Therefore, heat-induced association of WP and CN is exploited to increase the
cheese yield. Anyway, the adverse effect of *in situ* denaturation of the WP means the progressive
worsening of the rennet coagulation properties due to the interaction of WP with κ-CN, impairing its
accessibility with the coagulant (Kelly, Huppertz & Sheehan, 2008). Another approach is represented by
ultrafiltration of unheated milk and subsequent coagulation by acidification or renneting of the retentate
enriched in native WP. Combination of heating and membrane technologies to entrap HDWP in the
cheese matrix is the solution which is preferentially adopted for soft cheeses (Guyomarc’h, 2006). The
inclusion of protein-based powders into the cheese milk is obtained by the addition of milk protein
concentrate (MPC) and whey protein concentrate (WPC) (Sharma, Jana & Chavan, 2012; Hunter, Hemar,
Pinder & Anema, 2011). Moreover, a microparticulated whey protein concentrate (MWPC) can be used
in cheese making mainly as a fat replacer. Particulation is a technology by which heated WPC is
submitted to a simultaneous shearing force (Hinrichs, 2001) allowing to aggregate WP into particles the
size of which depends on composition and process conditions (Giroux, Lanouette & Britten, 2015;
Guyomarc’h, 2006). The size of these last aggregates is up to the mm scale and can be reduced by
shearing of the heated whey up to 0.5–10 μm (Giroux, Lanouette & Britten, 2015; Guyomarc’h, 2006).
HDWP can be formed also upon heat treatment of the cheese milk. This process promotes unfolding of
WP and subsequently, *via* disulphide-thiol exchanges and hydrophobic bonds (Kelly, Huppertz &
Sheehan, 2008), the formation of WP-coated casein micelles and/or WP aggregates. Milk pH during heat
treatment plays an important role in the distribution of these two types of interactions (Vasbinder & De
Kruif, 2003). The diameters of complexes between CN and WP is from 30–100 nm (Vasbinder, Alting &
de Kruif, 2003) up to <1 μm (Guyomarc’h, 2006). Finally, the increase in WP content into cheese can be
achieved by addition of Ricotta cheese into cheese milk (Backmann & Schafroth, 2002). The large flocks
of WP in Ricotta remain entrapped into the coagulum and create a soft texture with an increased capacity
to absorb water into the cheese.

Characterization of a food product by objective parameters is a useful and ongoing aim to assure
its value and protection on the market, to define classes of quality, to evaluate genuineness and to
establish the correct denomination. This target is achievable if the marker, represented by a newly-formed molecule from a specific process, is easily detectable and quantifiable. Both ingredients and procedures of cheeses protected with Designation of Origin are strictly detailed in the product specification. In particular, the variation of the ratio WP/CN by addition of WP-based products is not foreseen. The availability of an analytical method to verify the presence of these additional ingredients in traditional cheeses would be of great importance to check the agreement with allowed cheese making practices. In the same way, generic cheeses can be obtained by adding different dairy-based products to modify and improve the textural and sensorial attributes in comparison to the cheese prepared only from milk (Dissanayake, Liyanaarachchi & Vasiljevic, 2012). In this case, an objective assay to verify the presence of HDWP, used as ingredient to bring additional functionalities other than native WP, would provide a tool to satisfy consumers’ expectations of a cheese with higher nutritional value. One answer to this analytical topic could be based on the different behavior of CN and the added HDWP in a citrate solution. The first fraction is dissolved by the Ca-chelating action of citrate, while WP, heat-denatured in a whey medium, form large aggregates insoluble in this salt and can be separated by centrifugation. The fate of complexes between CN and WP in presence of a citrate solution should be similar to that of WP aggregates.

In the early stage of Maillard reaction, the newly formed Amadori compound is converted after acid hydrolysis into furosine (FUR). This molecule, thoroughly investigated, is a marker of ingredients that contain lactose and are prepared under thermal conditions that initiate early stages of the Maillard reaction (Erbersdobler & Somoza, 2007). Fresh cheeses from milk submitted to high-temperature short-time (HTST) treatment without additional heating during processing showed FUR levels similar to those of pasteurized milk. In this case, FUR levels higher than 5–7 mg 100 g⁻¹ protein were a marker of ingredients other than cheese milk, like dairy-based powders (Resmini & Pellegrino, 1991). Lysinoalanine (LAL) is a cross-linked amino acid between two different parts of the protein chain. Its formation is favored by alkali treatment (Friedman, 1999), such as that applied in preparing some milk derivatives. This molecule is a distinguishing parameter between natural and imitation mozzarella cheese.
and is useful to recognize the addition of caseinate during cheese making (Pellegrino, Resmini, De Noni & Masotti, 1996). It is well known that, the highest concentrations of LAL occur in commercial caseinate (Cuq & Cheftel, 1985), while very small or unquantifiable amounts are measured in natural cheeses (Pellegrino, Resmini, De Noni & Masotti, 1996).

The aim of the present study was to develop a simple analytical procedure for the **ex post** identification of HDWP in experimental cheeses obtained from peroxidase-positive pasteurized milk supplemented with different protein-based products. A further scope was to evaluate effectiveness of heat damage parameters, FUR and LAL, as markers of the use of these ingredients.

### 2. Materials and methods

#### 2.1. Protein-based ingredients

Three samples of protein-based powders were used for the production of experimental cheeses: MWPC (Nutrilac® CH4560, Arla Foods Ingredients Group, Vibj, Denmark), MPC (Ledor 85T, Hochdorf Swissmilk, Switzerland) and whey protein isolate (WPI) (Fonterra Cooperative Group, Auckland, New Zealand).

In addition, heated WPI (HWPI) was prepared as follows: the WPI powder was dissolved at a concentration of 4.5% (w/v) in milliQ® treated water. The dispersion (pH 6.80) was heat-treated in a sealed glass tube in an oven at 100 °C for 3 h, cooled at 25 °C and freeze-dried. The obtained powder of HWPI was subsequently used as an ingredient for lab-scale cheese makings.

A commercial sample of Ricotta (9.32% protein, 14.02% fat and 3.82% lactose; w/w; data provided by the manufacturer) was used.

#### 2.2. Experimental cheese makings
Twice replicated cheese makings were performed at a laboratory-scale using a commercial peroxidase-positive pasteurized (73 °C/16 s, as indicated by the manufacturer) milk (500 mL) supplemented with MWPC, MPC, WPI, HWPI and Ricotta cheese at concentrations ranging from 0.2 to 1.2% protein (w/v). The mixture was stirred for 30 min at 25 °C until complete solubilization of flocks. Subsequently, the milk supplemented with sodium chloride (10 g/L) to mimic the salting of cheese, was warmed up to 37 °C and coagulated by calf rennet (140 IMCU/mL, Chr. Hansen, Hørsholm, Denmark) at a rate of 0.2 g/L. After coagulation (about 10 min) the coagulum was let to stand for other 10 min, cut into pieces of 3 x 3 x 3 mm³ with a grid and left for 5 min to allow syneresis. The curd was centrifuged at 2,000 g for 3 min at 25 °C. The pellet cheese was grinded and submitted to the targeted chemical analyses as described below.

2.3. *Extraction of self-aggregated HDWP from experimental cheese samples*

A cheese amount corresponding to 1.5 g protein was dissolved in 100 mL of 0.2 M sodium citrate at pH 7.0. The mixture was stirred with Ultraturrax® T25 apparatus (IKA-Labortechnik, Staufen, Germany) at 8,000 rpm for 20 min, left to set for 5 min and centrifuged at 12,000 g for 10 min at 25 °C. The pellet was redissolved in 50 mL of 0.2 M sodium citrate at pH 7.0 stirring with Ultraturrax® and submitted to centrifugation at the same conditions. The washed pellet was freeze-dried and sealed in a glass tube until analyses.

2.4. *Targeted chemical analyses*

Protein content (as N x 6.38) was determined by Kjeldahl according to the International standard ISO 8968-1 (2014). Citrate was quantified by high performance liquid chromatography (HPLC) according to Zeppa, Conterno and Gerbi (2001) on a Waters 625 LC System (Waters, Milford, MA, USA). A sample aliquot (5 g) was dispersed in 0.01 N H₂SO₄ with Ultraturrax® T25 apparatus and
subsequently centrifuged for 5 min (7000 g) at 4 °C. The supernatant was filtered through Whatman paper filter (grade 40; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and analyzed by HPLC. FUR was determined in a sample corresponding to about 50 mg of protein hydrolyzed in presence of 8 M HCl and then submitted to solid phase extraction. The eluate was analyzed by ion-pair reverse-phase HPLC according to the International standard ISO 18329 (2004). LAL analysis consisted of acid hydrolysis (same conditions of FUR) followed by 9-fluorenylmethylchloro-formate derivatization of the amino compounds, selective solid phase extraction of the 9-fluorenylmethylchloro-formate and LAL derivatives and reverse-phase HPLC with fluorescence detection according to Pellegrino, Resmini, De Noni and Masotti (1996). Undenatured WP were determined on the whey, obtained by acidification with HCl up to pH 4.6 by reverse-phase HPLC according to the International standard ISO 13875 (2005). These targeted chemical analyses were run in triplicate.

The relative abundance of CN and WP in the recovered pellet of protein-based ingredients and experimental cheese samples was determined by capillary zone electrophoresis (CZE) according to Masotti, Hogenboom, Rosi, De Noni & Pellegrino (2010) using the conditions described by Recio and Olieman (1996) on a Beckman MDQ (Beckman, Fullerton, CA, USA) equipment. Both the pasteurized milk (Figure 1a) and the pellet of a dispersion of MWPC after extraction with citrate (Figure 1b) were analyzed as protein standards. Peaks were identified by comparison with literature data (Jones, Tier & Wilkins, 1998; Recio & Olieman, 1996). To show the selective role exerted by sodium citrate, the pellets of pasteurized milk supplemented with 0.3% MWPC without (Figure 1c) or with (Figure 1d) preliminary citrate extraction were analyzed by CZE.

2.5. Statistical analysis

Analysis of variance (ANOVA) was performed and data visualization was aided with Daniel’s XL toolbox addin for Excel, version 6.60, by Daniel Kraus, Würzburg, Germany (available
at: http://xltoolbox.sourceforge.net). Results were reported as means ± standard deviations. Student’s t test was adopted to examine differences of data.

3. Results and discussion

3.1. Characterization of the protein-based ingredients used in the experimental cheese makings

Protein-based ingredients used for the cheese-milk supplementation consisted of different milk protein powders and Ricotta cheese (Table 1). Targeted chemical analyses were performed on them. Among powders, MWPC was characterized by a strong WP denaturation (94% on total WP) and severe heat damage as revealed by the high FUR content (328.2 mg 100 g⁻¹ protein). In this powder, the level of Amadori compound, expressed as FUR, was attributable to the abundant occurrence of both proteins and lactose (44.01 and 38.24%, respectively), which under the processing conditions favored the Maillard reaction. Indeed, the protein crosslinking was poorly enhanced, as demonstrated by the LAL content of MWPC (< 0.2 mg 100 g⁻¹ protein). The MPC showed higher protein content (82.08%) in comparison to MWPC, and 56% of its WP fraction was in native (undenatured) form. Both FUR and LAL levels (172.5 mg and 2.3 mg 100 g⁻¹ protein, respectively) were in the range (41–241 mg and 0.5–5.6 mg 100 g⁻¹ protein, respectively) reported by Cattaneo, Masotti and Pellegrino (2012) for similar powders. Among the tested protein-based ingredients, WPI presented the highest protein content (92.71%). In this powder, WP were almost soluble (> 99%) thus suggesting a mild heat treatment in processing, as revealed by the low FUR and LAL levels (Table 1). This powder, further submitted to in-batch heating (100 °C/3 h) to denature WP (by obtaining HWPI), was also used as an ingredient in lab-scale cheese makings. The heating step promoted a partial degradation of the Amadori compound as the FUR level decreased from 18.8 mg to 11.7 mg 100 g⁻¹ protein. In the same time, an extensive formation of LAL occurred: its amount increased from 1.5 mg 100 g⁻¹ protein in WPI to 26.2 mg 100 g⁻¹ protein in HWPI. Such behavior was attributable to the low content of lactose in WPI, likely because of a diafiltration step adopted in
processing. Another source of HDWP used in lab-scale cheese makings was a commercial sample of Ricotta cheese. This product, obtained from rennet whey by acid-heat coagulation, contained only HDWP (> 99%), which occurred as self-aggregated flocks. The commercial Ricotta was characterized by a FUR level of 182.0 mg 100 g\(^{-1}\) protein, while the degree of protein crosslinking was negligible (LAL < 0.2 mg 100 g\(^{-1}\) protein) (Table 1).

3.2. Assessment of protein supplementation by extraction of HDWP

The pasteurized milk sample supplemented with different protein-based ingredients was used for lab-scale cheese making. The milk fortification ranged from 0.2% to 1.2% in protein (w/v) depending on the added ingredient. The obtained cheese samples were assessed for gross composition and protein supplementation of self-aggregated HDWP. The main chemical characteristics of the experimental cheese samples were determined and reported in Table 2. The cheese supplemented with MWPC (sample E) had a lower dry matter content in comparison to the control sample (sample D) due to the high water binding properties of the HDWP. Being the size distribution of the MWPC particles similar to that of fat globules, a competition in their entrapment into the coagulum network was expected. This fact explained both the lower fat content and the slightly higher amount of protein in the cheese. Milk supplementation with MPC (sample F) did not influence the cheese fat content, while protein level increased, being the main contribution exerted by CN. The addition of WPI to milk (sample G) increased the protein content in cheese as a result of the higher concentration of the added soluble WP in the water phase. The fat content was slightly higher than that of the control sample (sample D). A similar trend was recorded also by Hinrichs (2001) who observed an average increase of 20% in the protein content of a soft cheese when milk was supplemented with a MPC containing only undenatured WP. In comparison to WPI addition, the cheese obtained from milk and HWPI (sample H) was characterized by an increase in protein content (\(p < 0.05\)), while fat remained unaffected. This result was in accordance with literature data (Hinrichs, 2001) reporting an increased retention of the added WP in parallel with their degree of denaturation.
Finally, in the case of milk fortification with Ricotta (sample I), the obtained experimental cheese was characterized by an increase of both water and protein contents.

Self-aggregated HDWP were extracted from the experimental cheeses. The freeze-dried pellets were characterized for weight, protein content (Table 3) and protein-type (by CZE). The weight of the protein pellet of the cheese from pasteurized milk (Table 3, sample D control) was negligible (1.2 mg) and, with the exception of the cheese containing WPI (sample G), was significantly ($p < 0.05$) lower than that of all the other experimental cheeses. The proteins of sample D consisted only of CN. The lab-scale cheese containing MWPC (sample E) showed a pellet (86.5 mg) heavier than control ($p < 0.05$) and it was made of proteins (40.3 mg) and citrate salts (41.5 mg). Among all cheese makings, the higher amount of protein added to milk (1.2%) was reflected in a heavier pellet (185.5 mg) in the model cheese from milk and HWPI (sample H). Differently, traces of the pellet (3.1 mg) were recovered when 1.2% WPI was added to cheese milk (sample G), and, as expected, only CN fractions were revealed in the corresponding CZE pattern. This behavior could be explained by the fact that the undenatured WP retained in the cheese were discarded in the supernatant after the centrifugation step of the extraction procedure. The analytical approach applied to cheese obtained from milk and MPC (sample F) provided 3.7 mg of protein in the pellet, an amount higher than that of control and significantly different ($p < 0.05$). As previously underlined, the protein matrix of MPC consisted of CN and WP in the same ratio as in milk, besides 56% of WP were soluble at pH 4.6, whereas the remaining were supposed to interact with $\kappa$-CN through thiol-disulphide exchange reactions (Anema & Li, 2003). These data explain why only traces of WP ($< 1\%$ total peak area) were measured by CZE. The milk supplementation with Ricotta cheese (sample I) was low in terms of added protein (0.2% protein), but the recovered pellet (44.1 mg) was significantly ($p < 0.05$) heavier than that of control (sample D), as the protein fraction of Ricotta was represented mainly by HDWP.

Although the protein ingredients added to cheese milk differed in composition, the procedure to extract HDWP was efficient as confirmed by the relationship between cheese WP insoluble in citrate and self-aggregated HDWP added to cheese milk (Figure 2). These data also underlined that when the milk
was submitted to only HTST treatment (i.e. at 73 °C/16 s) the level of WP insoluble in sodium citrate was far below 1% of total protein content after rennet coagulation.

Supplementation of HDWP is preferably adopted in soft or semi-hard cheeses mainly to favor the yield increase by water retention. This practice in long ripened cheeses can promote some consequences like the formation of bitter peptides during ripening (Lemieux & Simard, 1991). In any case, the reliability of the extraction procedure should be evaluated for each cheese variety to verify the possible interferences of newly-formed peptides during ripening.

3.3. Assessment of HDWP supplementation by heat damage evaluation

It is well known that the heat processing applied to obtain powdered dairy products promotes, among the others, the Maillard reaction and the crosslinking of proteins giving rise to newly-formed molecules potentially useful as markers of these products when included in cheese formulation. Upon heating, WP and CN undergo diverse degree of protein lactosylation and crosslinking. Therefore, the effectiveness of FUR and LAL indicators in revealing the cheese milk supplementation with self-aggregated HDWP relies on the assessment of the diverse heat damage of WP retained in the cheese matrix. The cheese sample obtained from the pasteurized milk (control) showed a mean FUR level of 5.3 mg 100 g protein⁻¹ (Figure 3), in agreement with that reported in literature for not thermally stabilized fresh cheeses (Resmini & Pellegrino, 1991). Samples prepared from milk supplemented with MWPC, MPC and Ricotta showed FUR values higher than the control (p < 0.05). In contrary, FUR was ineffective to trace back the incorporation of WPI and HWPI into the cheese milk.

The LAL level was monitored both in the protein-based ingredients and in the experimental cheeses as well. Except in HWPI (26.2 mg 100 g⁻¹ protein), the levels of LAL were low in the protein ingredients, ranging from 0.2 mg to 2.3 mg 100 g⁻¹ protein (Table 1). The lack of additional heat treatments in the experimental cheese makings combined with the low amount of added proteins justified the trace amounts (< 0.2 mg/100 g protein) of LAL found in the final cheeses. The presence of ingredients
other than liquid milk could be inferred by LAL level only in the sample obtained from milk and HWPI (6.5 mg 100 g\(^{-1}\) protein). This last analytical parameter confirmed to be a sensitive marker of added WP in cheese making only in the case of the addition of products containing highly HDWP and processed in absence of lactose.

Overall, no relationship was observed among FUR, LAL and HDWP in lab-scale cheeses. However, these indicators represent a source of information on ingredients other than liquid milk provided that no thermal stabilization during cheese making is applied.

4. Conclusions

In this study, we developed a simple analytical procedure for the identification of self-aggregated HDWP in model cheeses. In principle, this procedure is a useful tool to evaluate \textit{ex post} the presence of HDWP-based ingredients in commercial cheeses and to assess the compliance to allowed technological processes. Coupling of this approach with the levels of targeted indicators of heat damage, FUR and LAL, supplied additional information on the nature of HDWP supplemented into cheese milk.

Further research is necessary to verify the application of this assay on real cheese varieties, evaluating the presence of possible interfering peptides formed during ripening.

References


**Figure captions**

**Fig. 1** Electropherograms of the pasteurized milk (a), the pellet of microparticulated whey protein concentrate (MWPC) after extraction with 0.2 M sodium citrate (pH 7.0) (b), the pellets of pasteurized milk added with microparticulated whey protein concentrate (MWPC, 0.3% w/v) and extracted without (c) or with (d) sodium citrate.

α-La: α-Lactalbumin; β-Lg: β-Lactoglobulin; CN: casein.

**Fig. 2** Relationship between denatured whey proteins (WP) added to cheese milk and WP insoluble in sodium citrate (0.2 M, pH 7.0) recovered from the lab-scale cheese samples reported in Table 3. Error bars represent 1 standard deviation.

**Fig. 3** Furosine level recorded in the lab-scale cheese samples. Error bars represent 1 standard deviation.
Table 1

Targeted chemical features of the protein-based ingredients used for milk supplementation in experimental cheese makings.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Protein content (%)</th>
<th>Soluble WP (%)</th>
<th>Furosine (mg 100 g⁻¹ protein)</th>
<th>Lysinoalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-La</td>
<td>β-Lg</td>
<td></td>
</tr>
<tr>
<td>MWPC</td>
<td>44.01 ± 0.28</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>328.2 ± 12.6</td>
</tr>
<tr>
<td>MPC</td>
<td>82.08 ± 0.34</td>
<td>1.3 ± 0.1</td>
<td>6.7 ± 0.3</td>
<td>172.5 ± 10.3</td>
</tr>
<tr>
<td>WPI</td>
<td>92.71 ± 0.28</td>
<td>15.8 ± 0.2</td>
<td>74.8 ± 0.5</td>
<td>18.8 ± 0.9</td>
</tr>
<tr>
<td>HWPI</td>
<td>92.71 ± 0.21</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>Ricotta</td>
<td>9.15 ± 0.12</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>182.0 ± 9.8</td>
</tr>
</tbody>
</table>

WP: whey proteins; MWPC: microparticulated whey protein concentrate; MPC: milk protein concentrate; WPI: whey protein isolate; HWPI: WPI in-batch heated (100 °C/3 h); α-La: α-Lactalbumin. β-Lg: β-Lactoglobulin.

Values are presented as means ± standard deviation (n = 3).
### Table 2

Dry matter, fat and protein content of the lab-scale cheese samples.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Ingredients</th>
<th>Protein added to milk (%)</th>
<th>Dry matter (%)</th>
<th>Fat (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>on cheese</td>
<td>on dry matter</td>
<td>on cheese</td>
</tr>
<tr>
<td>D</td>
<td>Milk</td>
<td>0</td>
<td>28.06 ± 1.4</td>
<td>12.01 ± 0.7</td>
<td>42.80 ± 0.5</td>
</tr>
<tr>
<td>E</td>
<td>Milk + MWPC</td>
<td>0.5</td>
<td>25.65 ± 1.8</td>
<td>9.79 ± 1.0</td>
<td>38.17 ± 1.1</td>
</tr>
<tr>
<td>F</td>
<td>Milk + MPC</td>
<td>1.1</td>
<td>27.21 ± 0.5</td>
<td>11.72 ± 0.9</td>
<td>43.08 ± 2.5</td>
</tr>
<tr>
<td>G</td>
<td>Milk + WPI</td>
<td>1.2</td>
<td>30.47 ± 0.6</td>
<td>12.85 ± 0.5</td>
<td>42.17 ± 0.9</td>
</tr>
<tr>
<td>H</td>
<td>Milk + HWPI</td>
<td>1.2</td>
<td>28.84 ± 0.5</td>
<td>11.22 ± 0.3</td>
<td>38.90 ± 0.2</td>
</tr>
<tr>
<td>I</td>
<td>Milk + Ricotta</td>
<td>0.2</td>
<td>26.11 ± 1.1</td>
<td>11.49 ± 1.2</td>
<td>44.02 ± 2.6</td>
</tr>
</tbody>
</table>

MWPC: microparticulated whey protein concentrate; MPC: milk protein concentrate; WPI: whey protein isolate; HWPI: WPI in-batch heated (100 °C/3 h).

Values are presented as means ± standard deviation (n = 3).
Table 3

Characteristics of the freeze-dried pellet extracted from the lab-scale cheese samples dispersed in sodium citrate (0.2 M, pH 7.0).

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Cheese Milk</th>
<th>Protein added to cheese milk (%)</th>
<th>HDWP added to cheese milk (%)</th>
<th>Cheese solubilized in Na citrate (g)</th>
<th>Weight (mg)</th>
<th>Protein (mg)</th>
<th>Sodium citrate (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Milk</td>
<td>0.0</td>
<td>0.0</td>
<td>16.6</td>
<td>3.2 ± 1.4 a</td>
<td>1.2 ± 1.0 a</td>
<td>N.d.</td>
</tr>
<tr>
<td>E</td>
<td>Milk + MWPC</td>
<td>0.5</td>
<td>0.5</td>
<td>16.1</td>
<td>86.5 ± 18.2 b</td>
<td>40.3 ± 3.5 b</td>
<td>41.5 ± 4.1</td>
</tr>
<tr>
<td>F</td>
<td>Milk + MPC</td>
<td>1.1</td>
<td>0.1</td>
<td>13.4</td>
<td>6.7 ± 1.8 c</td>
<td>3.7 ± 0.8 c</td>
<td>N.d.</td>
</tr>
<tr>
<td>G</td>
<td>Milk + WPI</td>
<td>1.2</td>
<td>0.0</td>
<td>12.9</td>
<td>3.1 ± 1.2 a, c</td>
<td>1.6 ± 0.8 a</td>
<td>N.d.</td>
</tr>
<tr>
<td>H</td>
<td>Milk + HWPI</td>
<td>1.2</td>
<td>1.2</td>
<td>11.2</td>
<td>185.5 ± 15.1 d</td>
<td>87.8 ± 6.5 d</td>
<td>N.d.</td>
</tr>
<tr>
<td>I</td>
<td>Milk + Ricotta</td>
<td>0.2</td>
<td>0.2</td>
<td>15.5</td>
<td>44.1 ± 3.9 e</td>
<td>18.2 ± 1.9 e</td>
<td>N.d.</td>
</tr>
</tbody>
</table>

HDWP: heat-denatured whey proteins; MWPC: microparticulated whey protein concentrate; MPC: milk protein concentrate; WPI: whey protein isolate; HWPI: WPI in-batch heated (100 °C/3 h). N.d.: not determined. Values are presented as means ± standard deviation (n = 4). Values with different lower-case letters (a–e) within the same column differ significantly (p < 0.05).
Highlights

- Heat-denatured whey proteins (HDWP) used as cheese ingredients
- Analytical procedure to reveal the addition of HDWP in cheese making
- Extraction of HDWP in experimental cheeses supplemented with protein-based products.