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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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2 **making**

3

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10

11 **Abstract**

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

26

27 **Keywords:** heat-denatured whey proteins; milk cheese; supplementation; extraction; sodium citrate

28

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

33

34 **1. Introduction**

35

36 Casein (CN) constitutes the solid protein matrix of cheese, in which different amounts of whey
37 proteins (WP) are entrapped according to raw materials other than liquid milk and the degree of whey
38 draining resulting from the (rennet or acid) coagulation (Fox & McSweeney, 2004). Dairy-based protein
39 ingredients can be added into milk to produce cheese, but the WP/CN ratio should not be modified to the
40 extent of exceeding that of milk. Whey resulting from cheese making is available in large volumes
41 (approximately 80–90% of the volume of milk used) and its disposal represents a major industrial
42 concern. Nonetheless, whey is a source of valuable nutrients which could be recovered and used
43 individually. Traditionally, the recovery of WP, consisting in whey drying, allows obtaining powders
44 which differ in composition and technological properties depending on the pretreatment operations (Jelen,
45 2011; Sharma, Jana & Chavan, 2012). Instead of heating, the pre-concentration can be performed by
46 membrane technologies that can maintain WP in the native form to satisfy specific properties of the end-
47 product (Foegeding, Luck & Vardhanabhuti, 2011).

48 The re-integration of WP into cheese matrix has been an issue of utmost importance for the increase of
49 yield, the improvement of nutritional value, the refinement of texture and the increase of profit (Giroux,
50 Lanouette & Britten, 2015). Native WP can be re-integrated in cheese (Lawrence, 1989), although heat-
51 denatured WP (HDWP) are preferred for their high water-binding capacity and better physical retention in
52 the curd during drainage. Therefore, heat-induced association of WP and CN is exploited to increase the

53 cheese yield. Anyway, the adverse effect of *in situ* denaturation of the WP means the progressive
54 worsening of the rennet coagulation properties due to the interaction of WP with κ -CN, impairing its
55 accessibility with the coagulant (Kelly, Huppertz & Sheehan, 2008). Another approach is represented by
56 ultrafiltration of unheated milk and subsequent coagulation by acidification or renneting of the retentate
57 enriched in native WP. Combination of heating and membrane technologies to entrap HDWP in the
58 cheese matrix is the solution which is preferentially adopted for soft cheeses (Guyomarc'h, 2006). The
59 inclusion of protein-based powders into the cheese milk is obtained by the addition of milk protein
60 concentrate (MPC) and whey protein concentrate (WPC) (Sharma, Jana & Chavan, 2012; Hunter, Hemar,
61 Pinder & Anema, 2011). Moreover, a microparticulated whey protein concentrate (MWPC) can be used
62 in cheese making mainly as a fat replacer. Particulation is a technology by which heated WPC is
63 submitted to a simultaneous shearing force (Hinrichs, 2001) allowing to aggregate WP into particles the
64 size of which depends on composition and process conditions (Giroux, Lanouette & Britten, 2015;
65 Guyomarc'h, 2006). The size of these last aggregates is up to the mm scale and can be reduced by
66 shearing of the heated whey up to 0.5–10 μm (Giroux, Lanouette & Britten, 2015; Guyomarc'h, 2006).
67 HDWP can be formed also upon heat treatment of the cheese milk. This process promotes unfolding of
68 WP and subsequently, *via* disulphide-thiol exchanges and hydrophobic bonds (Kelly, Huppertz &
69 Sheehan, 2008), the formation of WP-coated casein micelles and/or WP aggregates. Milk pH during heat
70 treatment plays an important role in the distribution of these two types of interactions (Vasbinder & De
71 Kruif, 2003). The diameters of complexes between CN and WP is from 30–100 nm (Vasbinder, Alting &
72 de Kruif, 2003) up to $<1 \mu\text{m}$ (Guyomarc'h, 2006). Finally, the increase in WP content into cheese can be
73 achieved by addition of Ricotta cheese into cheese milk (Backmann & Schafroth, 2002). The large flocks
74 of WP in Ricotta remain entrapped into the coagulum and create a soft texture with an increased capacity
75 to absorb water into the cheese.

76 Characterization of a food product by objective parameters is a useful and ongoing aim to assure
77 its value and protection on the market, to define classes of quality, to evaluate genuineness and to

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

94 In the early stage of Maillard reaction, the newly formed Amadori compound is converted after
95 acid hydrolysis into furosine (FUR). This molecule, thoroughly investigated, is a marker of ingredients
96 that contain lactose and are prepared under thermal conditions that initiate early stages of the Maillard
97 reaction (Erbersdobler & Somoza, 2007). Fresh cheeses from milk submitted to high-temperature short-
98 time (HTST) treatment without additional heating during processing showed FUR levels similar to those
99 of pasteurized milk. In this case, FUR levels higher than 5–7 mg 100 g⁻¹ protein were a marker of
100 ingredients other than cheese milk, like dairy-based powders (Resmini & Pellegrino, 1991).
101 Lysinoalanine (LAL) is a cross-linked amino acid between two different parts of the protein chain. Its
102 formation is favored by alkali treatment (Friedman, 1999), such as that applied in preparing some milk
103 derivatives. This molecule is a distinguishing parameter between natural and imitation mozzarella cheese

104 and is useful to recognize the addition of caseinate during cheese making (Pellegrino, Resmini, De Noni
105 & Masotti, 1996). It is well known that, the highest concentrations of LAL occur in commercial caseinate
106 (Cuq & Cheftel, 1985), while very small or unquantifiable amounts are measured in natural cheeses
107 (Pellegrino, Resmini, De Noni & Masotti, 1996).

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

112

113 2. Materials and methods

114

115 2.1. Protein-based ingredients

116

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

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

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

127

128 2.2. Experimental cheese makings

129

130 Twice replicated cheese makings were performed at a laboratory-scale using a commercial
131 peroxidase-positive pasteurized (73 °C/16 s, as indicated by the manufacturer) milk (500 mL)
132 supplemented with MWPC, MPC, WPI, HWPI and Ricotta cheese at concentrations ranging from 0.2 to
133 1.2% protein (w/v). The mixture was stirred for 30 min at 25 °C until complete solubilization of flocks.
134 Subsequently, the milk supplemented with sodium chloride (10 g/L) to mimic the salting of cheese, was
135 warmed up to 37 °C and coagulated by calf rennet (140 IMCU/mL, Chr. Hansen, Hørsholm, Denmark) at
136 a rate of 0.2 g/L. After coagulation (about 10 min) the coagulum was let to stand for other 10 min, cut into
137 pieces of 3 x 3 x 3 mm³ with a grid and left for 5 min to allow syneresis. The curd was centrifuged at
138 2,000 g for 3 min at 25 °C. The pellet cheese was grinded and submitted to the targeted chemical analyses
139 as described below.

140

141 2.3. *Extraction of self-aggregated HDWP from experimental cheese samples*

142

143 A cheese amount corresponding to 1.5 g protein was dissolved in 100 mL of 0.2 M sodium citrate
144 at pH 7.0. The mixture was stirred with Ultraturrax® T25 apparatus (IKA-Labortechnik, Staufen,
145 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.
146 The pellet was redissolved in 50 mL of 0.2 M sodium citrate at pH 7.0 stirring with Ultraturrax® and
147 submitted to centrifugation at the same conditions. The washed pellet was freeze-dried and sealed in a
148 glass tube until analyses.

149

150 2.4. *Targeted chemical analyses*

151

152 Protein content (as N x 6.38) was determined by Kjeldahl according to the International standard
153 ISO 8968-1 (2014). Citrate was quantified by high performance liquid chromatography (HPLC)
154 according to Zeppa, Conterno and Gerbi (2001) on a Waters 625 LC System (Waters, Milford, MA,
155 USA). A sample aliquot (5 g) was dispersed in 0.01 N H₂SO₄ with Ultraturrax® T25 apparatus and

156 subsequently centrifuged for 5 min (7000 g) at 4 °C. The supernatant was filtered through Whatman paper
157 filter (grade 40; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and analyzed by HPLC. FUR was
158 determined in a sample corresponding to about 50 mg of protein hydrolyzed in presence of 8 M HCl and
159 then submitted to solid phase extraction. The eluate was analyzed by ion-pair reverse-phase HPLC
160 according to the International standard ISO 18329 (2004). LAL analysis consisted of acid hydrolysis
161 (same conditions of FUR) followed by 9-fluorenylmethylchloro-formate derivatization of the amino
162 compounds, selective solid phase extraction of the 9-fluorenylmethylchloro-formate and LAL derivatives
163 and reverse-phase HPLC with fluorescence detection according to Pellegrino, Resmini, De Noni and
164 Masotti (1996). Undenatured WP were determined on the whey, obtained by acidification with HCl up to
165 pH 4.6 by reverse-phase HPLC according to the International standard ISO 13875 (2005). These targeted
166 chemical analyses were run in triplicate.

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

176

177 2.5. *Statistical analysis*

178

179 Analysis of variance (ANOVA) was performed and data visualization was aided with Daniel's
180 XL toolbox addin for Excel, version 6.60, by Daniel Kraus, Würzburg, Germany (available

181 at:<http://xltoolbox.sourceforge.net>). Results were reported as means \pm standard deviations. Student's t test
182 was adopted to examine differences of data.

183

184 3. Results and discussion

185

186 3.1. Characterization of the protein-based ingredients used in the experimental cheese makings

187

188 Protein-based ingredients used for the cheese-milk supplementation consisted of different milk
189 protein powders and Ricotta cheese (Table 1). Targeted chemical analyses were performed on them.
190 Among powders, MWPC was characterized by a strong WP denaturation (94% on total WP) and severe
191 heat damage as revealed by the high FUR content (328.2 mg 100 g⁻¹ protein). In this powder, the level of
192 Amadori compound, expressed as FUR, was attributable to the abundant occurrence of both proteins and
193 lactose (44.01 and 38.24%, respectively), which under the processing conditions favored the Maillard
194 reaction. Indeed, the protein crosslinking was poorly enhanced, as demonstrated by the LAL content of
195 MWPC (< 0.2 mg 100 g⁻¹ protein). The MPC showed higher protein content (82.08%) in comparison to
196 MWPC, and 56% of its WP fraction was in native (undenatured) form. Both FUR and LAL levels (172.5
197 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
198 protein, respectively) reported by Cattaneo, Masotti and Pellegrino (2012) for similar powders. Among
199 the tested protein-based ingredients, WPI presented the highest protein content (92.71%). In this powder,
200 WP were almost soluble (> 99%) thus suggesting a mild heat treatment in processing, as revealed by the
201 low FUR and LAL levels (Table 1). This powder, further submitted to in-batch heating (100 °C/3 h) to
202 denature WP (by obtaining HWPI), was also used as an ingredient in lab-scale cheese makings. The
203 heating step promoted a partial degradation of the Amadori compound as the FUR level decreased from
204 18.8 mg to 11.7 mg 100 g⁻¹ protein. In the same time, an extensive formation of LAL occurred: its amount
205 increased from 1.5 mg 100 g⁻¹ protein in WPI to 26.2 mg 100 g⁻¹ protein in HWPI. Such behavior was
206 attributable to the low content of lactose in WPI, likely because of a diafiltration step adopted in

207 processing. Another source of HDWP used in lab-scale cheese makings was a commercial sample of
208 Ricotta cheese. This product, obtained from rennet whey by acid-heat coagulation, contained only HDWP
209 (> 99%), which occurred as self-aggregated flocks. The commercial Ricotta was characterized by a FUR
210 level of 182.0 mg 100 g⁻¹ protein, while the degree of protein crosslinking was negligible (LAL < 0.2 mg
211 100 g⁻¹ protein) (Table 1).

212

213 3.2. *Assessment of protein supplementation by extraction of HDWP*

214

215 The pasteurized milk sample supplemented with different protein-based ingredients was used for
216 lab-scale cheese making. The milk fortification ranged from 0.2% to 1.2% in protein (w/v) depending on
217 the added ingredient. The obtained cheese samples were assessed for gross composition and protein
218 supplementation of self-aggregated HDWP. The main chemical characteristics of the experimental cheese
219 samples were determined and reported in Table 2. The cheese supplemented with MWPC (sample E) had
220 a lower dry matter content in comparison to the control sample (sample D) due to the high water binding
221 properties of the HDWP. Being the size distribution of the MWPC particles similar to that of fat globules,
222 a competition in their entrapment into the coagulum network was expected. This fact explained both the
223 lower fat content and the slightly higher amount of protein in the cheese. Milk supplementation with MPC
224 (sample F) did not influence the cheese fat content, while protein level increased, being the main
225 contribution exerted by CN. The addition of WPI to milk (sample G) increased the protein content in
226 cheese as a result of the higher concentration of the added soluble WP in the water phase. The fat content
227 was slightly higher than that of the control sample (sample D). A similar trend was recorded also by
228 Hinrichs (2001) who observed an average increase of 20% in the protein content of a soft cheese when
229 milk was supplemented with a MPC containing only undenatured WP. In comparison to WPI addition,
230 the cheese obtained from milk and HWPI (sample H) was characterized by an increase in protein content
231 ($p < 0.05$), while fat remained unaffected. This result was in accordance with literature data (Hinrichs,
232 2001) reporting an increased retention of the added WP in parallel with their degree of denaturation.

233 Finally, in the case of milk fortification with Ricotta (sample I), the obtained experimental cheese was
234 characterized by an increase of both water and protein contents.

235 Self-aggregated HDWP were extracted from the experimental cheeses. The freeze-dried pellets
236 were characterized for weight, protein content (Table 3) and protein-type (by CZE). The weight of the
237 protein pellet of the cheese from pasteurized milk (Table 3, sample D control) was negligible (1.2 mg)
238 and, with the exception of the cheese containing WPI (sample G), was significantly ($p < 0.05$) lower than
239 that of all the other experimental cheeses. The proteins of sample D consisted only of CN. The lab-scale
240 cheese containing MWPC (sample E) showed a pellet (86.5 mg) heavier than control ($p < 0.05$) and it was
241 made of proteins (40.3 mg) and citrate salts (41.5 mg). Among all cheese makings, the higher amount of
242 protein added to milk (1.2%) was reflected in a heavier pellet (185.5 mg) in the model cheese from milk
243 and HWPI (sample H). Differently, traces of the pellet (3.1 mg) were recovered when 1.2% WPI was
244 added to cheese milk (sample G), and, as expected, only CN fractions were revealed in the corresponding
245 CZE pattern. This behavior could be explained by the fact that the undenatured WP retained in the cheese
246 were discarded in the supernatant after the centrifugation step of the extraction procedure. The analytical
247 approach applied to cheese obtained from milk and MPC (sample F) provided 3.7 mg of protein in the
248 pellet, an amount higher than that of control and significantly different ($p < 0.05$). As previously
249 underlined, the protein matrix of MPC consisted of CN and WP in the same ratio as in milk, besides 56%
250 of WP were soluble at pH 4.6, whereas the remaining were supposed to interact with κ -CN through thiol-
251 disulphide exchange reactions (Anema & Li, 2003). These data explain why only traces of WP ($< 1\%$
252 total peak area) were measured by CZE. The milk supplementation with Ricotta cheese (sample I) was
253 low in terms of added protein (0.2% protein), but the recovered pellet (44.1 mg) was significantly ($p <$
254 0.05) heavier than that of control (sample D), as the protein fraction of Ricotta was represented mainly by
255 HDWP.

256 Although the protein ingredients added to cheese milk differed in composition, the procedure to
257 extract HDWP was efficient as confirmed by the relationship between cheese WP insoluble in citrate and
258 self-aggregated HDWP added to cheese milk (Figure 2). These data also underlined that when the milk

259 was submitted to only HTST treatment (i.e. at 73 °C/16 s) the level of WP insoluble in sodium citrate was
260 far below 1% of total protein content after rennet coagulation.

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

266

267 3.3. *Assessment of HDWP supplementation by heat damage evaluation*

268

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

280 The LAL level was monitored both in the protein-based ingredients and in the experimental
281 cheeses as well. Except in HWPI (26.2 mg 100 g⁻¹ protein), the levels of LAL were low in the protein
282 ingredients, ranging from 0.2 mg to 2.3 mg 100 g⁻¹ protein (Table 1). The lack of additional heat
283 treatments in the experimental cheese makings combined with the low amount of added proteins justified
284 the trace amounts (< 0.2 mg/100 g protein) of LAL found in the final cheeses. The presence of ingredients

285 other than liquid milk could be inferred by LAL level only in the sample obtained from milk and HWPI
286 (6.5 mg 100 g⁻¹ protein). This last analytical parameter confirmed to be a sensitive marker of added WP in
287 cheese making only in the case of the addition of products containing highly HDWP and processed in
288 absence of lactose.

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

292

293 4. Conclusions

294

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

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

302

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304

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386 **Figure captions**

387

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

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

393

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

397

398 **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.

Ingredient	Protein content (%)	Soluble WP (%)		Furosine (mg 100 g ⁻¹ protein)	Lysinoalanine
		α -La	β -Lg		
MWPC	44.01 \pm 0.28	1.0 \pm 0.1	1.1 \pm 0.1	328.2 \pm 12.6	< 0.2
MPC	82.08 \pm 0.34	1.3 \pm 0.1	6.7 \pm 0.3	172.5 \pm 10.3	2.3 \pm 0.2
WPI	92.71 \pm 0.28	15.8 \pm 0.2	74.8 \pm 0.5	18.8 \pm 0.9	1.5 \pm 0.2
HWPI	92.71 \pm 0.21	< 0.1	< 0.1	11.7 \pm 0.4	26.2 \pm 0.3
Ricotta	9.15 \pm 0.12	< 0.1	< 0.1	182.0 \pm 9.8	< 0.2

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 \pm standard deviation (n = 3).

Table 2

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

Sample code	Ingredients	Protein added to milk (%)	Dry matter (%)	Fat (%)		Protein (%)	
				on cheese	on dry matter	on cheese	on dry matter
D	Milk	0	28.06 ± 1.4	12.01 ± 0.7	42.80 ± 0.5	9.06 ± 0.9	32.29 ± 1.8
E	Milk + MWPC	0.5	25.65 ± 1.8	9.79 ± 1.0	38.17 ± 1.1	9.34 ± 0.8	36.42 ± 0.3
F	Milk + MPC	1.1	27.21 ± 0.5	11.72 ± 0.9	43.08 ± 2.5	11.20 ± 0.5	41.16 ± 1.3
G	Milk + WPI	1.2	30.47 ± 0.6	12.85 ± 0.5	42.17 ± 0.9	10.54 ± 0.4	34.59 ± 0.8
H	Milk + HWPI	1.2	28.84 ± 0.5	11.22 ± 0.3	38.90 ± 0.2	13.37 ± 0.4	46.36 ± 0.6
I	Milk + Ricotta	0.2	26.11 ± 1.1	11.49 ± 1.2	44.02 ± 2.6	9.70 ± 0.8	37.15 ± 1.6

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

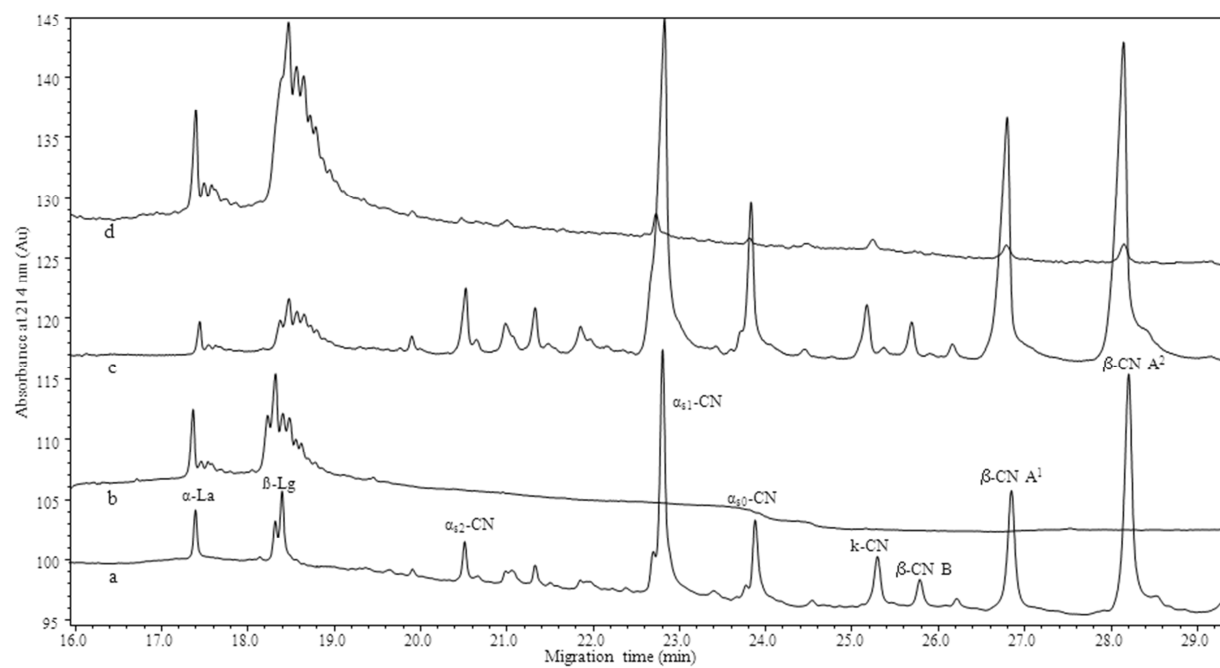
Values are presented as means ± standard deviation (n = 3).

Table 3

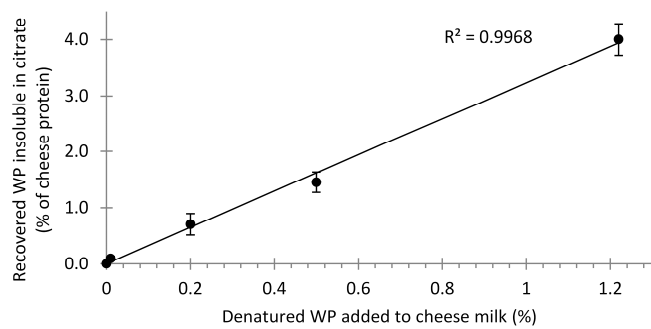
Characteristics of the freeze-dried pellet extracted from the lab-scale cheese samples dispersed in sodium citrate (0.2 M, pH 7.0).

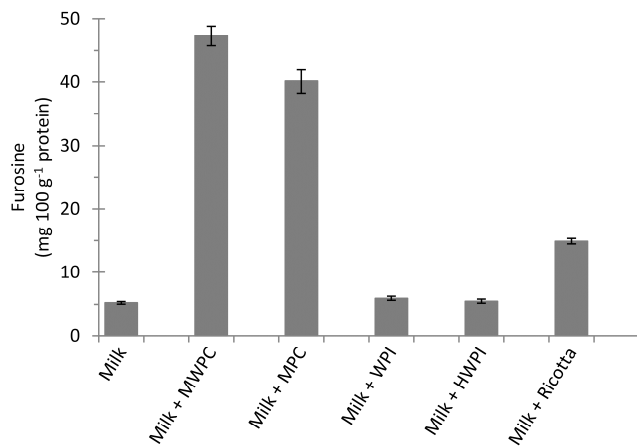
Sample code	Cheese Milk	Protein added to cheese milk (%)	HDWP added to cheese milk (%)	Cheese solubilized in Na citrate (g)	Freeze-dried pellet		
					Weight (mg)	Protein (mg)	Sodium citrate (mg)
D	Milk	0.0	0.0	16.6	3.2 ± 1.4 a	1.2 ± 1.0 a	N.d.
E	Milk + MWPC	0.5	0.5	16.1	86.5 ± 18.2 b	40.3 ± 3.5 b	41.5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

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 ($p < 0.05$).



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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.