LWT - Food Science and Technology 174 (2023) 114347

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Determination and evaluation of whey protein content in matured cheese via liquid chromatography

Tobias von Oesen [a], Mascha Treblin [b], Alexandra Staudacher [a], Ingrid Clawin-Rädecker [a], Dierk Martin [a], Wolfgang Hoffmann [a], Katrin Schrader [a], Katja Bode [c], Ralf Zink [c], Sascha Rohn [b,d], Jan Fritsche [a,*]

[a] Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Milk and Fish Products, Hermann-Weigmann-Straße 1, 24103, Kiel, Germany
[b] University of Hamburg, Hamburg School of Food Science, Institute of Food Chemistry, Grindelallee 117, 20146, Hamburg, Germany
[c] Center of Expertise Research & Technology (CoE-R&T), DMK Group (Deutsches Milchkontor GmbH), Flughafenallee 17, 28199, Bremen, Germany
[d] Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Department of Food Chemistry and Analysis, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355, Berlin, Germany

ARTICLE INFO

Keywords:
HPLC
Sample pretreatment
Foil-ripened Edam-type cheese
Proteolytic effects
Whey protein determination

ABSTRACT

To date, there is no valid analytical method available to determine the total whey protein (TWP) content in cheese. Therefore, the aim of this study was to develop HPLC methods for whey protein determination in mature cheese. For that purpose, foil-ripened whey protein-enriched model cheese and traditional Edam-type cheese were produced and analyzed. Suitable protein extraction methods and subsequent analytical methods to determine the acid-soluble whey protein (ASWP) and TWP content in cheese were developed. To characterize the influence of proteolysis on native and denatured whey proteins, the ASWP and TWP contents were determined throughout the ripening process. Both chromatographic methods showed that the individual whey proteins (α-lactalbumin and β-lactoglobulin) were not degraded during ripening. However, the analyzed ASWP content increased by up to 25% throughout ripening. Compared to traditional Edam-type cheese, the β-lactoglobulin content in whey protein-enriched cheese (containing 30% high-heat milk) increased by a factor of 3.5. To evaluate the chromatographic results, two different calculation models were used to estimate a reference value for the TWP content in the manufactured cheese. Further studies are required to optimize the quantification of TWP content in hard, semihard, soft, and cream cheeses.

1. Introduction

Currently, whey is a significant byproduct of cheese production. For a long time, it was regarded as an undesirable component (waste) of cheese processing and was either fed to animals or treated as effluent. In recent years, requirements for environmental protection have become more stringent, and costly construction and operation of wastewater treatment plants need to be considered (Mulvihill & Grufferty, 1997). Currently, the high nutritional value of whey and numerous food products containing it as a valuable ingredient are widely recognized. These dairy products play a major role in the food industry (Deeth & Bansal, 2018). Instant whey powder and whey-based beverages are now widespread, and other types of whey-rich/-enriched cheese (e.g., ricotta) are also gaining increased consumer acceptance. Moreover, several technologies are available for incorporating whey proteins into a cheese matrix to increase the nutritional value and resource efficiency in cheese production. According to Masotti, Cattaneo, Stuknytė, and de Noni (2017), whey protein can be included in cheese by pretreating the initial milk ('cheese milk') in various ways such as heating, membrane-based technology, high hydrostatic pressure,

* Corresponding author. Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Milk and Fish Products, Hermann-Weigmann-Straße 1, 24103, Kiel, Germany.

E-mail addresses: tobias.vonoesen@mri.bund.de (T. von Oesen), mascha.treblin@chemie.uni-hamburg.de (M. Treblin), alexandra.staudacher@mri.bund.de (A. Staudacher), ingrid.clawin-raedecker@mri.bund.de (I. Clawin-Rädecker), dierk.martin@mri.bund.de (D. Martin), wolfgang.hoffmann@mri.bund.de (W. Hoffmann), katrin.schrader@mri.bund.de (K. Schrader), katja.bode@dmk.de (K. Bode), ralf.zink@dmk.de (R. Zink), rohn@tu-berlin.de (S. Rohn), jan.fritsche@mri.bund.de (J. Fritsche).

https://doi.org/10.1016/j.lwt.2022.114347
Received 20 May 2022; Received in revised form 16 December 2022; Accepted 18 December 2022
Available online 23 December 2022

ultrahigh-pressure homogenization, transglutaminase treatment, or hybrid variants of the previously described treatments. Furthermore, adding whey protein preparations (e.g., whey protein concentrate/isolate, microparticulated whey proteins) to cheese milk results in the fortification of cheese (Guinee, 2021). However, according to the German food legislation, the fortification of traditional cheese with whey proteins is not yet permitted. Moreover, no validated analytical method to determine whey proteins in the complex cheese matrix is yet available.

With regard to the cheese manufacturing process, numerous biochemical processes occur while cheese ripens. Along with primary casein breakdown (curdling), proteolysis of casein is fundamental to cheese ripening and is widely studied (Grappin, Rank, & Olson, 1985; Sousa, Ardö, & McSweeney, 2001; Upadhyay, McSweeney, Magboul, & Fox, 2004). Proteolysis is primarily responsible for the texture, flavor, and off-flavor (Adda, Gripon, & Vassal, 1982). Oligopeptides, amino acids, and volatile flavor compounds are products of enzymatic hydrolysis caused by a variety of proteases and peptidases (McSweeney, Fox, Cotter, & Everett, 2017). Other minor ingredients, such as carbohydrates and lipids, are also degraded during cheese ripening (Collins, McSweeney, & Wilkinson, 2003; Fox, Lucey, & Cogan, 1990). Therefore, cheese represents a complex matrix consisting of a mixture of native ingredients and their degradation products.

Traditionally, a variety of analytical methods are used to determine milk proteins (Masci et al., 2022). Electrophoretic techniques (e.g., isoelectric focusing) are well established, especially in combinations of different techniques, for example, two-dimensional gel electrophoresis (Chin & Rosenberg, 1998; Molina, Ramos, & Amigo, 2002). Furthermore, high-performance liquid chromatography (HPLC) offers the ability to separate milk proteins with hydrophobic interactions, ion exchange, or reversed-phase methodologies, thereby providing high resolution and accuracy (Amalfitano et al., 2019, 2020; Bisutti et al., 2022; Bonfatti et al., 2008; Bonfatti et al., 2019; Maurmayr et al., 2018). However, there is currently no officially validated analytical method that enables determination of the whey protein content in cheese.

Consequently, the aim of this study was to develop a cost-effective and easy-to-use HPLC method to determine whey protein in matured cheese. To develop a suitable analytical method, the proteolytic stability of both native and denatured whey proteins needs to be considered to accurately determine the whey protein content in matured cheese. Therefore, the acid-soluble whey proteins (ASWP) and the total whey proteins (TWP, sum of denatured and native whey proteins) were characterized in cheese samples throughout the ripening process. For further evaluation, a TWP reference value was calculated for the manufactured cheese samples based on two calculation models. Finally, an enrichment factor (EF) was calculated to describe the increased TWP content of whey protein-enriched Edam-type cheese compared to traditional Edam-type cheese, enabling an estimation of the whey protein content in the cheese matrix.

2. Materials and methods

2.1. Materials

2.1.1. Cheese production

The production of whey protein-enriched, foil-ripened, semihard Edam-type cheese by adding high-heat milk (HH milk) requires an elevated cooking temperature to improve the firmness of the curd. To evaluate the impact of the higher temperature on the starter culture performance, a preliminary experiment was carried out in the pilot plant at the dairy technical center of the Max Rubner-Institut (MRI), Kiel, Germany. For that purpose, raw bovine milk was obtained from the animal farm of the MRI, Schaedtbek-Dobersdorf, Germany. The starter culture FD-DVS CHN-19 for the preliminary experiment was obtained from Chr. Hansen A/S (Nienburg, Germany) and consisted of *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc* species, *Lactococcus lactis* subsp. *lactis*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. The starter culture DCC-260 for the main experiments consisted of *Lactobacillus helveticus*, *Lactobacillus paracasei*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc* species, and *Streptococcus thermophilus*, and was also purchased from Chr. Hansen A/S (Nienburg, Germany), as well as the rennet (NATUREN® Premium 145 NB) for the preliminary and main experiments. Plastic bags for ripening were purchased from IP Ingredients GmbH (Süderlügum, Germany).

2.1.2. Model proteins and standards

Lyophilized skimmed milk was used as a standard to quantify milk proteins. For this purpose, the lyophilized skimmed milk was reconstituted by stirring in a ratio of 1:10 (w/v) with water, and the concentration of the single proteins was quantified with a certified bovine protein standard (Sigma–Aldrich Corp., St. Louis, MO, USA): α-lactalbumin (α-LA, purity ≥ 85%), lactoferrin (LF, purity ≥ 85%), bovine serum albumin (BSA, purity ≥ 98%), β-lactoglobulin (β-LG, purity ≥ 90%), β-lactoglobulin A (β-LG A, purity ≥ 90%), β-lactoglobulin B (β-LG B, purity ≥ 90%), immunoglobulin G (IgG, purity ≥ 95%), α-casein (α-CN, purity ≥ 70%), β-casein (β-CN, purity ≥ 98%), and κ-casein (κ-CN, purity ≥ 70%).

2.1.3. Reagents

Pierce™ trifluoroacetic acid (purity ≥ 99.5%) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Guanidine hydrochloride (GdnHCl, purity ≥ 99%), Bis-Tris buffer (purity ≥ 98%), and dl-dithiothreitol (DTT, purity ≥ 99%) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Acetonitrile, acetone (all HPLC grade), disodium hydrogen phosphate (purity ≥ 99.99%), sodium dihydrogen phosphate (purity ≥ 99.99%), hydrochloric acid (37%), sulfuric acid (95–97%), sulfuric acid (0.05 mol/L), sodium hydroxide (purity ≥ 99.0%), trisodium citrate dihydrate (purity ≥ 99.0%), boric acid (purity ≥ 99.5%), sodium acetate (purity ≥ 99.0%), trichloroacetic acid (purity ≥ 99.5%), mixed indicator 5, bromophenol blue, and Kjeltabs IB61 were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water (0.055 μS) was obtained using a laboratory water purification system (Sartorius Arium® 611 VF, Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany).

2.2. Methods

2.2.1. Cheese production

Foil-ripened model Edam-type cheese (40% fat in dry matter) was manufactured in a pilot plant at the dairy technical center of the MRI, following the protocols described by Hoffmann et al. (2019). Due to the heat instability of the starter culture used in the preliminary experiment, a more thermophilic starter culture was used for the main experiments. To produce whey protein-enriched semihard cheese, a defined amount of 10%, 20%, or 30% (w/w) high-heat milk (HH milk, 120 s, 95 °C) was added to the pasteurized cheese milk (20 s, 72–73 °C). The cheese loaves were placed between two perforated sheets of stainless steel for continuous contact with circulating brine (17% NaCl (w/w), 0.5% $CaCl_2$ (w/w), pH 5.33, 15 °C). After 5 h of brine retention, the loaves were transferred onto wooden boards and allowed to dry until the next morning. Then, the loaves were packed into plastic foil shrink bags, vacuumed, and sealed. Cheeses were ripened for 6 weeks at a humidity of 80% and temperature of 13 °C. Three independent experiments were conducted with differing amounts of HH milk and were repeated three times. As the experiments were spread over a longer period, a standard model Edam-type cheese (control) was made in parallel with every whey protein-enriched semihard cheese production to evaluate the biological variations in the protein content over the time period. In total, nine standard cheeses (0% (w/w) HH milk) and nine whey protein-enriched cheeses (10%, 20%, and 30% HH milk in cheese milk, three times each) were produced. The added (cheese milk, water) and removed (whey,

cheese) masses were documented for every main experiment (mass balance). The whey drained at three points in the manufacturing process (initial whey in the tank, prepressing, pressing) was pooled to determine its weight ratios and labeled as 'whey mix'.

2.2.1.1. Preliminary experiment. Whey protein-enriched cheese was produced from cheese milk containing 30% (w/w) HH milk. As a standard cheese, a traditional Edam-type cheese was made in parallel without adding HH milk. The process steps corresponded to those of the main experiments with the exception of the starter culture composition.

2.2.2. Sampling

Several cheese loaves were obtained per cheese production. One loaf was sampled at each time point (before brining (BB), after brining (AB), and weekly until the sixth week of ripening). Sampling was carried out according to DIN (2008b). In short, a wedge was cut out of the round cheese loaf and cut into small cubes. Subsequently, these cubes were shredded using an electronic grinder (Moulinette electronic Moulinex®, Groupe SEB Deutschland GmbH, Frankfurt am Main, Germany) and stored at −25 °C.

2.2.3. Protein extraction methods

The cheese was defatted and dried prior to protein extraction (cf., Section 2.2.3.1) and then directly used for protein extraction for comparison. A disperser (Ultra-Turrax®, T25 digital, IKA®-Werke GmbH & Co. KG, Staufen, Germany) was used for homogenization. The extraction methods were evaluated by determining the ASWP content using HPLC-UV (cf., Section 2.2.4).

2.2.3.1. Cheese pretreatment prior to protein extraction. To defat and dry the cheese samples, 30 mL of acetone (A) was added to 6.5 g of cheese and suspended using a disperser for 3 min. The supernatant was discarded. Then, 30 mL of acetone was added to the residue, and the procedure was repeated until the supernatant remained clear. Finally, the suspension was filtered. The residue was dried in a desiccator, and the resulting fat-free and anhydrous cheese powder was stored at −25 °C. For comparison, nonpretreated cheese (NP) was directly used for protein extraction.

2.2.3.2. Protein extraction. For homogenization, 1 g of the fat-free anhydrous cheese powder (resulting from the defatting and drying pretreatment) was homogenized in 50 mL of 0.01 mol/L sodium phosphate buffer (pH 6.7) for 3 min at room temperature. For comparison, 2.5 g of cheese was homogenized accordingly. The resulting suspensions were stored at −25 °C.

2.2.4. Determination of the acid-soluble whey protein content via HPLC-UV

After casein precipitation at pH 4.6, the filtrate of the cheese phosphate buffer suspension was used to determine the ASWP content in accordance with DIN (1997) and ISO|IDF (2005). However, the following modifications were made to the protocols. (1) They were extended to α-LA, β-LG, LF, BSA, and IgG (Kahl et al., 2014). (2) They were applied to a cheese matrix at a detection wavelength of 215 nm. The proteins were separated using an UltiMate™ 3000 HPLC system (Dionex™ Thermo Scientific GmbH, Bremen, Germany) equipped with a PLRP-S column (300 Å, 5 µm, 150 mm × 2,1 mm, Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Each sample was prepared in duplicate and measured twice. Due to coelution of LF and BSA with some peptides from cheese ripening, the ASWP content of cheese (w/w) was calculated only as the sum of the main acid-soluble individual whey proteins (ASIWP) α-LA, β-LG, and IgG, while the ASWP content of milk (w/v) was calculated from the sum of ASIWP α-LA, β-LG, IgG, BSA, and LF. Reconstituted lyophilized skimmed milk was used as an external standard for quantification (cf., Section 2.1.2).

2.2.5. Calculation of the (individual) whey protein denaturation in cheese milk

Raw milk was pasteurized for standard cheese production. To obtain cheese samples with increased whey protein content, defined amounts of high-heat raw milk were added to pasteurized milk (cheese samples with 10%, 20%, or 30% HH milk). One should consider that heat treatment of milk results in denaturation of various milk proteins (Lorenzen et al., 2011); otherwise, the composition of cheese milk corresponds to that of raw milk. Consequently, whey protein denaturation (WPD) to determine the sum of α-LA, β-LG, LF, BSA, and IgG in cheese milk was calculated as in Equation (1) (see below). Replacing ASWP with ASIWP yielded the extent of individual whey protein denaturation (IWPD) for α-LA, β-LG, LF, BSA, or IgG.

$$\%\ (\mathrm{I})\mathrm{WPD}_{\text{cheese milk}} = 100\% - \left(\frac{\frac{\text{mg}}{100\ \text{mL}}\mathrm{AS(I)WP}_{\text{cheese milk}}}{\frac{\text{mg}}{100\ \text{mL}}\mathrm{AS(I)WP}_{\text{raw milk}}} \right) \times 100 \qquad \text{(Eq. 1)}$$

2.2.6. Determination of the total (individual) whey protein content via HPLC-FLD

A cheese phosphate buffer suspension was used to determine the total (individual) whey protein (T(I)WP) content according to Bobe, Beitz, Freeman, and Lindberg (1998). TWP describes the sum of native and denatured whey proteins, while TIWP describes the sum of the native and denatured individual whey proteins. An aliquot of the cheese phosphate buffer suspension was added at a ratio of 1:1 (v/v) to a solution (pH 7.0) containing 0.1 mol/L Bis-Tris buffer (pH 6.8), 6 mol/L GdnHCl, 6.12 mmol/L trisodium citrate dihydrate, and 19.5 mmol/L DTT. Each sample was shaken for 10 s, incubated for 1 h at room temperature, and centrifuged for 5 min at 4 °C and 16,000×*g*. The fat layer was removed with a spatula. The remaining supernatant was diluted 1:50 (v/v) with a solution containing 4.5 mol/L GdnHCl, water, acetonitrile, and trifluoroacetic acid at a ratio of 99:1:0.1 (v/v/v). Each sample was subsequently shaken for another 10 s, incubated for 30 min at room temperature, and centrifuged for 5 min at 4 °C and 16,000×*g*. The supernatant was used for the analysis. For the quantification of milk proteins, a standard was prepared that contained certified pure bovine milk proteins in purified water. The standard comprised concentrations of 15.1 mg/mL α-CN, 11.3 mg/mL β-CN, 4.2 mg/mL κ-CN, 1.1 mg/mL α-LA, 1.6 mg/mL β-LG A, and 2.0 mg/mL β-LG B. Standard and cheese phosphate buffer suspensions were treated in the same way except for the second dilution, when only a 1:2 dilution was used for the standard instead of a 1:50 (v/v) dilution. The proteins were separated in a LaChrom Elite® HPLC system (VWR GmbH, Darmstadt, Germany) equipped with a BioBasic™ 4 column (300 Å, 5 µm, 100 × 2.1 mm, Thermo Fisher™ Scientific Inc., Waltham, MA, USA). Gradients were eluted with a mixture of two solvents. Solvent A consisted of water, acetonitrile, and trifluoroacetic acid in a ratio of 99:1:0.1 (v/v/v), and solvent B was acetonitrile, water, and trifluoroacetic acid in a ratio of 99:1:0.1 (v/v/v). The solvent gradient program started at 31.5% of solvent B. A linear gradient was generated immediately after sample injection by increasing the proportion of solvent B by 0.43%/min (22 min), followed by an isocratic elution with 41% B for 12 min. Then, a linear gradient from 41% to 46% B was applied in 12.5 min (0.40% B/min), isocratic elution at 90% B was applied for 12.5 min, followed by a return to the starting conditions in 0.5 min. The column was re-equilibrated under the starting conditions for 12 min. The analysis time per sample was 71.5 min. The injection volume was 10 µL, the flow rate was 0.4 mL/min, the autosampler temperature was set at 4 °C, the column temperature was kept at 30 °C, and the fluorescence detection wavelength was $\lambda_{ex} = 280$ nm, $\lambda_{em} = 340$ nm.

The repeatability, linearity, and recovery of the individual proteins in the cheese matrix were determined using a six-week ripened cheese containing 30% (w/w) HH milk. The repeatability of the method was measured by calculating the coefficient of variation (CV, %). For this, the same cheese sample was injected ten times. The linearity was

determined by means of a dilution series and by calculating the coefficient of determination (R^2). Spiked cheese samples were used for recovery calculations.

2.2.7. Determination of the protein content of milk and cheese samples via Kjeldahl analyses

Raw milk, HH milk, cheese milk, whey mix, and model Edam-type cheese (before brining, after brining, and after three and six weeks of ripening) were measured in triplicate to determine the total nitrogen, noncasein nitrogen (only valid for raw milk), acid-soluble nitrogen, and nonprotein nitrogen, following the method C 30.2–30.4 (VDLUFA, 2000) as described by the *Association of German Agricultural Analytic and Research Institutes*, Speyer, Germany. The standard deviation (SD) of each triplicate was calculated. A matrix-dependent (milk, whey, cheese) SD, averaged over all analyses, was calculated for the various determinations (Table S1). The total protein (TP), casein (CN) (only valid for raw milk), and nonacid-soluble protein (NASP) content (for HH milk, cheese milk, cheese milk containing HH milk, whey mix, cheese) were calculated. The difference between the total nitrogen and nonprotein nitrogen contents was calculated as the pure protein (PP) content. The sum of the ASWP content was calculated by subtracting the sum of the nonprotein nitrogen and CN content or NASP content from the total nitrogen content. The factor 6.38, traditionally used for dairy products, was used to calculate the protein content from the nitrogen content.

2.2.8. Estimation of the total whey protein content of cheese based on the mass balance

The estimation approach was based on the mass balance recorded during the cheese manufacturing process. Considering the weight (W) of raw milk, whey, and cheese, the total whey protein content of cheese was calculated as the difference between the ASWP in the raw milk and the ASWP in the whey mix (Eq. (2)). The calculation was based on the results obtained via Kjeldahl analyses (Supplementary Material, Table S2-S10), which showed that almost no NASP and denatured whey protein (DWP) were found in the whey mix since they remained completely in the cheese matrix (Masotti et al., 2017). As negligible amounts of denatured whey proteins were present in the raw milk and in the whey mix, the ASWP content was assumed to correspond to the TWP content of the raw milk and whey mix. The difference in the mass balance between the educt (raw milk) and product (whey and cheese) corresponds to the added water used to wash the curd. The TWP content was calculated using the ASWP data resulting from the chromatographic analyses (cf., Section 2.2.4) and the ASWP data resulting from the Kjeldahl analyses (cf., Section 2.2.7).

$$\%\,\mathrm{TWP}_{\mathrm{cheese}} = \frac{(\mathrm{kg\,W}_{\mathrm{raw\ milk}} \times \%\,\mathrm{ASWP}_{\mathrm{raw\ milk}}) - (\mathrm{kg\,W}_{\mathrm{whey}} \times \%\,\mathrm{ASWP}_{\mathrm{whey\ mix}})}{\mathrm{kg\,W}_{\mathrm{cheese}}} \quad \text{(Eq. 2)}$$

2.2.9. Estimation of the total whey protein content of cheese based on a constant ratio of nonacid-soluble protein to denatured whey protein

Heat induces the interaction and formation of disulfide bonds of denatured whey proteins with κ-CN in milk, as reported by Anema (2021). However, how subsequent cheese manufacturing affects quantitative whey protein transfer into cheese matrices remains open. Therefore, the second estimation approach was calculated based on the assumption that the ratio of NASP to DWP as present in the cheese milk is also valid for the (final) cheese. Therefore, the DWP content of cheese milk was calculated according to Equation (3). For this approach, only ASWP data resulting from the chromatographic analyses were used (cf., Section 2.2.4 and Supplementary Material, Tables S11–S19).

$$\%\mathrm{DWP}_{\mathrm{cheese\ milk}} = \frac{\%\,\mathrm{ASWP}_{\mathrm{raw\ milk}} \times \%\,\mathrm{PP}_{\mathrm{cheese\ milk}}}{\%\mathrm{PP}_{\mathrm{raw\ milk}}} - \%\,\mathrm{ASWP}_{\mathrm{cheese\ milk}} \quad \text{(Eq. 3)}$$

The ratio of NASP to DWP present in the cheese milk was transferred to the cheese according to Equation (4).

$$\%\,\mathrm{DWP}_{\mathrm{cheese}} = \frac{\%\,\mathrm{NASP}_{\mathrm{cheese}} \times \%\,\mathrm{DWP}_{\mathrm{cheese\ milk}}}{\%\,\mathrm{NASP}_{\mathrm{cheese\ milk}}} \quad \text{(Eq. 4)}$$

The TWP content of the cheese was then calculated as shown in Equation (5). It was calculated as a mean value (MV) for cheese before brining, after brining, and after three and six weeks of ripening.

$$\%\,\mathrm{TWP}_{\mathrm{cheese}} = \%\,\mathrm{DWP}_{\mathrm{cheese}} + \%\,\mathrm{ASWP}_{\mathrm{cheese}} \quad \text{(Eq. 5)}$$

Replacing the ASWP with the ASIWP yielded the extent of individual whey protein denaturation (DIWP) of cheese milk (Eq. (3)) and cheese (Eq. (4)). The TIWP in cheese was calculated according to Equation (5) by replacing the DWP with the DIWP and the ASWP with the ASIWP.

2.2.10. Calculation of whey protein enrichment

An enrichment factor (EF) was calculated (Eq. (6)) to describe the enrichment of TWPs compared to that of the standard cheese (control). Unless otherwise specified, for the standard cheese, the MV of the TWPs was used from nine standard cheese productions. For each cheese sample using the same amount of HH milk, the MV of the TWPs was calculated from three productions.

$$\mathrm{EF}_{\mathrm{TWP}} = \frac{\%\,\mathrm{TWP}_{\mathrm{enriched\ cheese}}}{\%\,\mathrm{TWP}_{\mathrm{standard\ cheese}}} \quad \text{(Eq. 6)}$$

Replacing the TWP with the TIWP revealed the EF for the TIWP.

2.2.11. Statistical analysis

2.2.11.1. Levene's test. Levene's test, an F statistic for comparing two variances from independent datasets, was used for statistical analysis of the chromatographic results. Here, the hypotheses were as follows: the null hypothesis was that the variances were equal, and the alternate hypothesis was that the variances were not equal. The data were analyzed (two-sample F test) with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The calculated F value was compared to the critical F value (F table, $p < 0.05$). The null hypothesis was rejected when the calculated F value was higher than the table value. Therefore, the result was statistically significant ($p < 0.05$) (Fahrmeir, Heumann, Künstler, Pigeot, & Tutz, 2016).

2.2.11.2. Two-sample t-test (Student's t-test). A two-sample *t*-test (two sided) was used to determine if a significant difference between the means of two datasets was present. Here, the hypotheses were as follows: the null hypothesis was that the means were equal; the alternate hypothesis was that the means were not equal. A two-sample *t*-test was performed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The calculated t value was compared to the critical t value (t table, $p < 0.05$). The null hypothesis was rejected if the calculated t value was higher than the table value. Thus, the result was statistically significant ($p < 0.05$) (Rasch, Friese, Hofmann, & Naumann, 2014).

2.2.11.3. Neumann's trend test. Neumann's trend test was used to determine if a trend may be present for at least four consecutive characteristics. This test is used to check whether neighboring values are more similar than values farther away. The Q value was calculated according to Equation (7) and compared to a table value (Neumann table, $p < 0.05$). Here, x_i and x_{i+1} are the measured values in chronological order, n represents the number of measured values, and s is the standard deviation. If Q was smaller than the table value, a trend was present. Therefore, the result was statistically significant ($p < 0.05$) (Kromidas, 2011).

$$\mathrm{Q} = \frac{\sum_{i=1}^{n-1} (x_i - x_{i+1})^2}{(n-1)s^2} \quad \text{(Eq. 7)}$$

3. Results and discussion

3.1. Appropriate protein extraction method

The aim of this study was to develop liquid chromatographic methods to determine the whey protein content of semihard cheese. Primarily, cheese from the preliminary experiment (standard cheese and cheese containing 30% (w/w) HH milk) was used to establish a valid protein extraction method for chromatographic determination. Cheese samples were pretreated with acetone to optimize protein extraction from the fatty cheese matrix. In parallel, cheese samples were used for protein extraction without any pretreatment. Both extraction methods were evaluated by determining the acid-soluble whey protein (ASWP) content using HPLC-UV. The acid-soluble individual whey proteins (ASIWP) α-LA, β-LG, and IgG were determined and summed to calculate the final ASWP content. The ASIWP LF and ASIWP BSA could not be determined in the cheese, as casein peptides coeluted with these proteins.

Fig. 1 shows the ASWP content during ripening of the standard cheese and that containing 30% HH milk. The results include samples that were exposed to acetone prior to protein extraction (A) and a sample preparation method without any pretreatment (NP). Regarding the ASWP content, the variance in the course of ripening for standard cheese was 5758 $(mg/100\ g)^2$ when using sample preparation with acetone, whereas the variance for sample preparation without any pretreatment for standard cheese was 1401 $(mg/100\ g)^2$. The variance during ripening of cheese containing 30% HH milk was 7562 $(mg/100\ g)^2$ when using sample preparation with acetone, whereas the variance for sample preparation without any pretreatment for cheese containing 30% HH milk was 27 $(mg/100\ g)^2$. Therefore, sample preparation with acetone showed higher variance regarding the ASWP content during ripening for both datasets. Moreover, Levene's test revealed that the null hypothesis of equal variances had to be rejected ($p < 0.05$). Both protein extraction methods were compared to identify an appropriate protein extraction method for cheese throughout the ripening process. Therefore, statistical analysis was more focused on the ripening process as a whole (before brining *vs.* six weeks of ripening). The mean values of the ASWP content in the standard cheese and in the cheese containing 30% HH milk before brining and after six weeks of ripening were compared via two-sample *t*-test for both sample preparation methods. For pretreatment with acetone, the mean values for the ASWP content were significantly different before brining and after six weeks of ripening ($p < 0.05$). However, for sample preparation without any pretreatment, no significant difference was found in the mean values of the ASWP content before brining and after six weeks of ripening ($p < 0.05$). Further statistical analysis (two-sample *t*-test) was performed with regard to the different ripening times. The quite complex results are shown in the Supplementary Material (Tables S20–S23). Furthermore, Neumann's trend test showed a trend regarding the ASWP content in standard cheese and cheese containing 30% HH milk during ripening when using sample preparation with acetone ($p < 0.05$). For sample preparation without any pretreatment, Neumann's trend test did not show any trend in the ASWP content within the SD ($p < 0.05$). This was also valid for the ASIWP contents of α-LA, β-LG, and IgG (data not shown). Acetone could have caused a partial denaturation of the ASWP, leading to precipitation of the whey proteins during acidic sample preparation (cf., Section 2.2.4). This would explain why a lower ASWP content was found in the first two weeks when using acetone for pretreatment. However, this does not explain the increase in the ASWP content during the later ripening process. If proteolytic degradation of the ASWP would have taken place during ripening, a decreasing trend for sample preparation without any pretreatment would have been observed. The reason for the observed increase in ASWP content during ripening cannot be conclusively clarified yet. As acetone had a negative influence on the protein extraction compared to the sample preparation without any pretreatment (higher variances and increasing ASWP content during ripening), sample preparation without any pretreatment was applied to cheese in the main experiments.

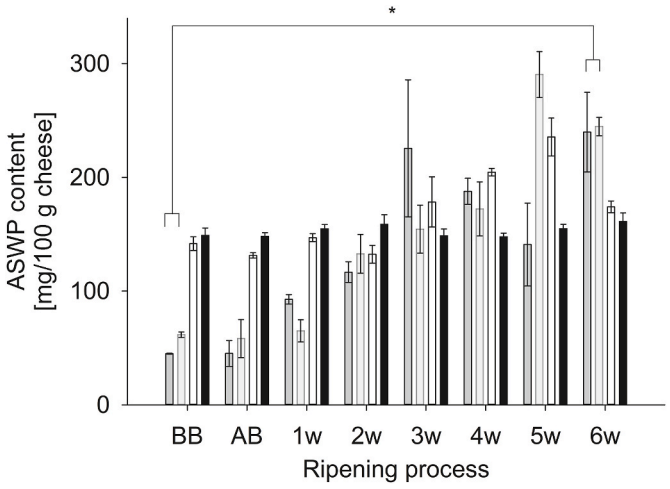

Fig. 1. Mean and SD of the ASWP content during cheese ripening (BB: before brining, AF: after brining, and weekly until the sixth week of ripening) of cheese in the preliminary experiment (standard cheese (n = 1) and cheese containing 30% (w/w) HH milk (n = 1)) using two different types of pretreatments (A and NP). Sample preparation was performed in duplicate and measured twice. (▒) ASWP content in standard cheese using sample preparation A; (▒) ASWP content in cheese containing 30% (w/w) HH milk using sample preparation A; (□) ASWP content in standard cheese using sample preparation NP; (■) ASWP content in cheese containing 30% (w/w) HH milk using sample preparation NP; *Significance ($p < 0.05$) between the mean ASWP content BB and that after six weeks of ripening for standard cheese and cheese containing 30% (w/w) HH milk with sample preparation A; the ASWP content showed a significant trend ($p < 0.05$) during ripening.

3.2. Method validation for the determination of acid-soluble whey protein content of cheese via HPLC-UV

Based on the calibration curve methodology, the limit of detection (LOD) and limit of quantification (LOQ) were determined according to DIN (2008a). As there is no cheese matrix completely devoid of whey proteins, the LOD and LOQ were determined by using skimmed milk as the standard matrix. The lowest LOD was determined for α-LA and IgG (0.05 g/100 mL), whereas BSA showed the highest LOD (0.20 mg/100 mL). Regarding the LOQ, BSA showed the lowest value (0.07 mg/100 mL), and β-LG showed the highest value (0.49 mg/100 mL). The recovery rate, repeatability, and method stability were calculated as described by Kromidas (2011) and reported in Table 1. For the recovery experiments, lyophilized skimmed milk was reconstituted in water at a ratio of 1:10 (w/v), diluted by a factor of 20 with 0.01 mol/L sodium phosphate buffer, and added at a 1:1 ratio to a cheese phosphate buffer suspension. The recovery rate for whey proteins in the cheese matrix ranged between 95% (IgG) and 115% (α-LA). To test for comparability, a cheese sample was measured ten times in series. The method stability was determined by performing eight independent sample preparations of a commercial Edam-type cheese. To evaluate the repeatability conditions and method stability, the coefficients of variation (CVs) were determined for each protein. The CVs of the repeatability in the cheese matrix ranged between 1.2% (β-LG) and 3.1% (α-LA). Regarding β-LG and α-LA, the CVs of the method stability were in the same range (1.3% and 2.2%, respectively), whereas that of IgG was slightly higher at 9.8%. The CVs of the repeatability and method stability for determining the ASIWP of β-LG in cheese matrix and in milk samples were under 5% and 14% relative standard deviation, respectively (ISO|IDF, 2005).

Table 1
Validation parameters for determination of the acid-soluble whey protein (ASWP) content in cheese.

		Individual whey protein (IWP)				
		α-LA	β-LG	LF	BSA	IgG
Validation parameters	LOD[a] [mg/100 mL]	0.05	0.13	0.07	0.02	0.05
	LOQ[a] [mg/100 mL]	0.18	0.49	0.23	0.07	0.17
	Recovery rate[b] [%]	115 ± 1.5	105 ± 0.5	[e]	[e]	96 ± 1.8
	CV Repeatability[c] [%]	3.1	1.2	[e]	[e]	1.5
	CV Method stability[d] [%]	2.2	1.3	[e]	[e]	9.8

[a] Determined in reconstituted lyophilized skimmed milk; chemometric model: calibration curve method; number m of calibration samples: 10; number n of measurements of the analytical sample: 2; significance level α = 5%, reciprocal relative result uncertainty k = 3.
[b] Sample preparation in duplicate, measured twice.
[c] One sample preparation, measured ten times.
[d] Eight sample preparations of one sample, each measured twice.
[e] Evaluation not possible due to coelutions with peptides from cheese ripening.

3.3. Acid-soluble whey protein content of whey protein-enriched cheese

To date, HPLC methods to determine the acid-soluble whey protein (ASWP) content of milk and milk products are commonly used to evaluate heat treatment (Dumitraşcu et al., 2013; Haque, Aldred, Chen, Barrow, & Adhikari, 2013) or assess the authenticity of milk/cheese from different animal species (Ferreira & Caçote, 2003; Romero, Perez-Andujar, Olmedo, & Jimenez, 1996). González de Llano and Santa-María (1997) used HPLC analysis to describe the proteolytic stability of acid-soluble α-LA, β-LG, and BSA during ripening of Afuega'l Pitu cheese (soft cheese). Pellegrino, Rosi, D'Incecco, Stroppa, and Hogenboom (2015) showed a proteolytic decrease in α-LA during whey fermentation in PDO Grana Padano cheese-making. However, the proteolytic stability of ASWP in semihard cheese has not been studied thus far.

With regard to the samples, previous studies revealed that a cheese to phosphate buffer ratio of 1:4 (w/v) is more appropriate for extraction of the soluble nitrogen fraction of cheese than a ratio of 1:20 (cf., Section 2.2.3.2) (Kuchroo & Fox, 1982). To improve the signal-to-noise ratio, the cheese used in the main experiments was extracted accordingly. Fig. 2 shows the content of ASWP (sum of the ASIWP β-LG, α-LA, IgG) during ripening for cheeses containing 30%, 20%, and 10% (w/w) HH milk and the corresponding standard cheeses. As the main experiments were spread over a longer period, milk from different seasons was used. However, it is well known that the protein composition of milk varies throughout the year (Heck, van Valenberg, Dijkstra, & van Hooijdonk, 2009; Töpel, 2004). In this study, this also led to a variation in the ASWP content of raw milk (653.82 ± 30.74 mg/100 mL) and, consequently, to a variation in the ASWP content of differently processed cheese. After six weeks of ripening, standard cheese (n = 9) contained on average the most ASWP (186.30 ± 21.41 mg/100 g). This was followed by cheese containing 10% (w/w) HH milk (n = 3) at 175.18 ± 19.60 mg/100 g, cheese containing 20% (w/w) HH milk (n = 3) at 159.56 ± 16.53 mg/100 g, and cheese containing 30% (w/w) HH milk (n = 3) at 147.76 ± 14.52 mg/100 g. A 10% increase in the amount of HH milk in cheese milk resulted in a decrease of approximately 10% in the ASWP content of cheese. As high-temperature treatment of milk results in partial denaturation of whey proteins (Dannenberg & Kessler, 1988), higher amounts of high-heat milk in cheese milk resulted in lower ASWP contents in the cheese matrix in the present study. Fig. 2 shows the ASWP content during ripening. The ASWP content of standard cheese increased by 23% ± 7% from before brining to the sixth week of ripening. For

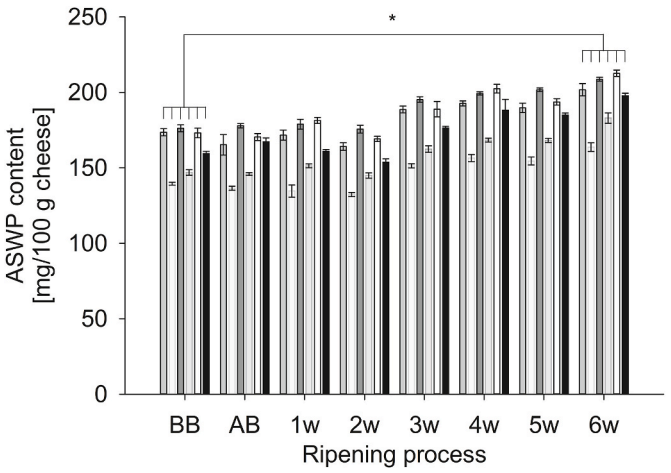

Fig. 2. Mean and SD of the ASWP content during cheese ripening (BB: before brining, AF: after brining, and weekly until the sixth week of ripening) for cheese containing 30% (■), 20% (▒), and 10% (□) (w/w) HH milk (n = 3 each) and the respective standard cheese (▒, ■, □; n = 3 each) in the main experiments with no pretreatment prior to protein extraction (NP). Sample preparation was performed in duplicate and measured twice. *Significance ($p < 0.05$) between the mean ASWP content BB and that after six weeks of ripening for all cheese productions; the ASWP content showed a significant trend ($p < 0.05$) during ripening.

cheese containing 10% (w/w) HH milk, the ASWP content increased by 24% ± 8%, while it increased by 25% ± 5% for cheese containing 20% (w/w) HH milk. For cheese containing 30% (w/w) HH milk, the ASWP content increased by 18% ± 13%. Quite low standard deviations indicate good reproducibility of both the production of cheese and the analytical method used to determine the ASWP content. However, the change in the cheese matrix during ripening affects the quantitative determination of the ASWP content. Since this study aimed to evaluate the stability of AS(I)WP during cheese ripening, the statistical analysis was more focused on the ripening process as a whole (before brining *vs.* six weeks of ripening). A two-sample *t*-test was used to determine if the mean values for the ASWP content before brining and after six weeks of ripening were different for cheese with the same amount of HH milk (n = 9 for standard cheese, n = 3 for cheeses containing 10%, 20%, 30% HH milk). The *t*-test revealed that there was a significant difference ($p < 0.05$) in the ASWP content between all cheese samples before brining and after six weeks of ripening. Further statistical analysis (two-sample *t*-test) was performed with regard to the different ripening times. The quite complex results are shown in the Supplementary Material (Tables S24–S29). Generally, proteolysis during cheese ripening results in the formation of peptides. In this study, some of these peptides appeared to coelute with ASIWP. In addition, Neumann's trend test confirmed a trend regarding an increase in the ASWP content in all cheese samples during ripening ($p < 0.05$).

Importantly, an increase in the ASIWP content was also observed. Depending on the individual whey proteins (α-LA, β-LG, and IgG), the increase in the ASIWP content differed. The ASIWP α-LA increased by 33% ± 5%, ASIWP β-LG showed a 12% ± 3% increase, and ASIWP IgG increased by 52% ± 5% on average for all products. However, the ASWP content of cheese from the preliminary experiment did not show such an increase (Fig. 1). The use of a different starter culture could have caused other non-coeluting peptides to form. Another possible explanation could be the degradation of residual κ-CN, which is often associated with cheese ripening. In short, the whey proteins, which were previously not detected during the acidic sample preparation (cf., Section 2.2.4), could have redissolved and reorganized during the degradation of κ-CN during ripening to result in detection.

Fig. 3a and b shows the individual whey protein denaturation (IWPD) in cheese milk with and without HH milk. The higher the slope

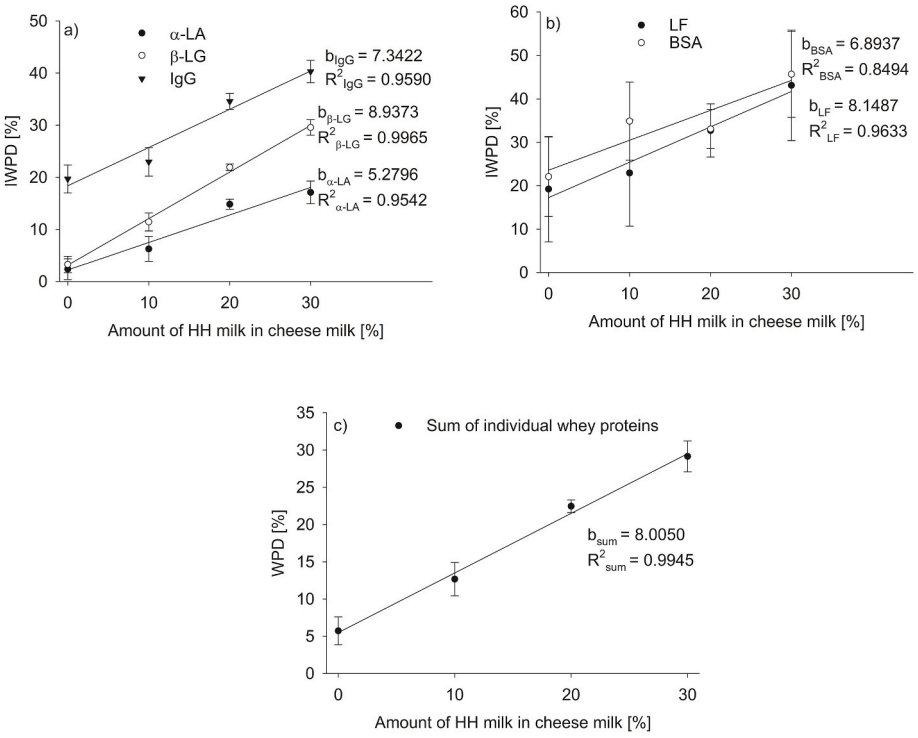

Fig. 3. Mean, SD, slope (b), and coefficient of determination (R^2) for a) the IWPD of the IWP α-LA, β-LG, IgG, b) the IWPD of the IWP LF and BSA, and c) the WPD of the sum of the previously mentioned IWPs in cheese milk. For standard cheese, the mean and SD were calculated from nine productions (n = 9); for cheese productions with 10%, 20% and 30% (w/w) HH milk, the mean and SD were calculated from three productions each (n = 3).

(b), the more extensive the protein denaturation was. β-LG was denatured the most ($b_{β-LG}$ = 8.9373) and showed the strongest linear correlation ($R^2_{β-LG}$ = 0.9965). Regarding the extent of denaturation, LF (b_{LF} = 8.1487, R^2_{LF} = 0.9633), IgG (b_{IgG} = 7.3422, R^2_{IgG} = 0.9590), BSA (b_{BSA} = 6.8937, R^2_{BSA} = 0.8494), and α-LA ($b_{α-LA}$ = 5.2796, $R^2_{α-LA}$ = 0.9542) followed in descending order. Compared to the IWPDs of β-LG and α-LA, the IWPD of IgG, BSA, and LF in cheese milk without HH milk were approximately 20%. The whey protein denaturation observed for IgG, BSA, and LF in cheese milk without HH milk was due to heat treatment in the pasteurization process. IgG, BSA, and LF are more heat sensitive than β-LG and α-LA (Clawin-Rädecker, Kiesner, & Schlimme, 2000). Fig. 3c shows the whey protein denaturation (WPD) for the sum of the previously mentioned individual whey proteins. The slope was b_{sum} = 8.0050, and the coefficient of determination was R^2 = 0.9945. The slope and the coefficient of determination for the sum of the five individual whey proteins were only slightly lower than for the individual β-LG. The treatment of milk at high temperatures caused the highest denaturing effect on β-LG. This is indicated by the slope for β-LG in Fig. 3a. As β-LG represents the largest fraction of whey proteins in milk (approximately 50%), the slope for the sum of the five individual whey proteins is almost identical to that for β-LG alone.

3.4. Estimation of the total whey protein content of cheese

A more pronounced heat instability of β-LG was observed compared to α-LA and was previously described in detail (Schlimme et al., 1996). In this study, comparison of the calculated IWPD slopes of α-LA and β-LG (Fig. 3a) confirms this finding. To evaluate the suitability of the developed liquid chromatographic method, a reference value for the total whey protein (TWP) content (sum of native and denatured whey protein) in Edam cheese was calculated. The manufacturing process causes the nitrogen content of the milk to be distributed in different proportions between the whey (byproduct) and the cheese (main product). Considering this distribution, two approaches were followed to estimate the TWP content of cheese. First, the TWP content was calculated based on the mass balance approach according to Equation (2) (cf., Section 2.2.8). Second, the TWP content was calculated using the ratio of NASP to DWP as present in cheese milk, which is also valid for cheese (cf., Section 2.2.9). Finally, the amount of HH milk was plotted against the estimated TWP content and evaluated with the coefficient of determination (Fig. 4).

Very low TWP values were obtained via the mass balance approach using the ASWP data from Kjeldahl analyses, with some negative values for the standard cheese. As the preparation of the whey mix from both cheese production 1 with 30% HH milk and the corresponding standard cheese production 1 differed due to technical reasons, the calculated TWP content for these two cheese productions was not considered for the linear regression. Grubbs' test was performed to detect outliers. It was found that one data point for 30% HH milk was a significant outlier ($p < 0.01$). Consequently, this TWP value at 30% HH milk was neglected. As only one TWP value could be considered for cheese containing 30% HH milk, the standard deviation could not be determined for this data point (Fig. 4a). Using ASWP data from HPLC-UV analyses resulted in higher values for the estimated TWP content. Here, R^2 was 0.9342 and negative values did not appear. An increase from approximately 0.75% TWP (standard cheese) to nearly 2.6% TWP (cheese containing 30% (w/w) HH milk) was observed (Fig. 4b).

ASPW data resulting from the HPLC-UV analyses were used to calculate the TWP content using Equation (5). This calculation was based on the assumption that the ratio of NASP to DWP, as present in cheese milk, is also valid for cheese. The resulting TWP content was slightly lower, ranging from approximately 0.6% for standard cheese to nearly 1.8% for cheese containing 30% (w/w) HH milk (Fig. 4c). The value of R^2 was 0.9926. These results indicate that the ASWP data obtained via HPLC-UV analyses led to a better correlation regarding the TWP content than that obtained by using ASWP data from Kjeldahl analyses. The TWP content was not calculated for the approach using ASWP data obtained via Kjeldahl analyses, as the Kjeldahl method for cheese provided increasing ASWP data during cheese ripening (data not shown).

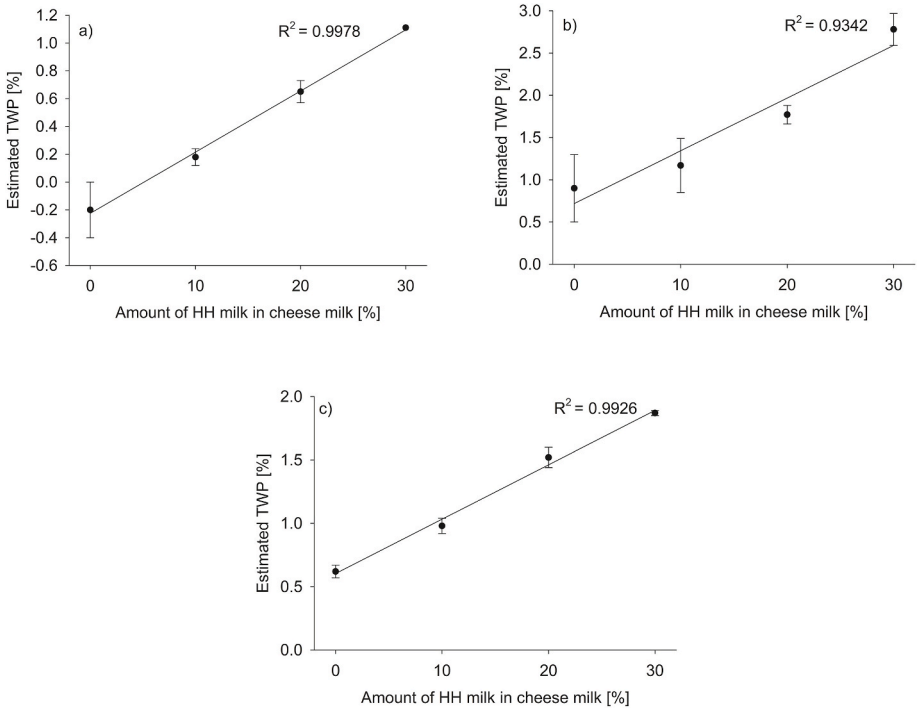

Fig. 4. Mean, SD, and coefficient of determination (R^2) for the estimated TWP content in cheese; a), b) estimation based on the mass balance approach, a) included ASWP data obtained via Kjeldahl analyses; n = 8 for standard cheese (0% (w/w) HH milk), n = 3 for cheese containing 10% and 20% (w/w) HH milk, and n = 1 for cheese containing 30% (w/w) HH milk; b) included ASWP data obtained via HPLC UV analyses; c) estimation based on a constant ratio of NASP to DWP, with included ASWP data obtained via HPLC UV analyses; n = 9 for standard cheese (0% (w/w) HH milk), n = 3 for cheese containing HH milk.

Considering the ASIWP (based on the chromatographic results), the total individual whey protein (TIWP) content was determined based on the mass balance approach (Fig. 5a–c) or based on a constant ratio of NASP to DWP (Fig. 5d–f). For all whey proteins determined in the cheese matrix (α-LA, β-LG, and IgG), better coefficients of determination were obtained when estimating based on a constant NASP to DWP ratio (R^2 for TIWP α-LA was 0.9290, R^2 for TIWP β-LG was 0.9979, and R^2 for TIWP IgG was 0.9660; Fig. 5d–f). Regarding the estimation based on the mass balance approach, R^2 for TIWP α-LA was 0.9042, R^2 for TIWP β-LG was 0.9357, and R^2 for TIWP IgG was 0.9528 (Fig. 5a–c).

Compared to Kjeldahl analysis, chromatographic separation offered the advantage that the individual whey proteins α-LA, β-LG, and IgG could be determined. Furthermore, separation of proteins from a large proportion of peptides formed during cheese ripening was possible. Therefore, only the T(I)WP content calculated based on AS(I)WP data was considered for calculation of the enrichment factors (EFs) (Table 2). A two-sample *t*-test was used to evaluate whether there were significant differences between the EFs calculated based on the two calculation models for the TWP, TIWP β-LG, TIWP α-LA, and TIWP IgG. No significant difference was found ($p < 0.05$). Both the mass balance approach and the approach based on a constant ratio of NASP to DWP resulted in an increase in the TWP content by a factor of three for cheese containing 30% (w/w) HH milk in relation to the standard cheese. When comparing the single whey proteins, β-LG increased the most, followed by α-LA and IgG. This was valid for both approaches. Considering the SD, the EF of the mass balance approach was in the range of the EF of the approach based on a constant ratio of NASP to DWP. In addition to intense protein denaturation, the enrichment of the individual whey proteins in the cheese matrix depends on their structural properties and the possibility of forming covalent bonds or hydrophobic interactions with κ-CN (Deeth & Lewis, 2017). A two-sample *t*-test was used to evaluate whether there were significant differences for the EF for the TWP, TIWP β-LG, TIWP α-LA, and TIWP IgG between cheeses containing 10%, 20%, and 30% HH milk. When comparing the EF for the TWP, TIWP β-LG, TIWP α-LA, and TIWP IgG between cheeses containing 10%, 20%, and 30% HH milk, calculated based on the mass balance approach, significant differences ($p < 0.05$) were found for the EF for the TWP, TIWP β-LG, and TIWP α-LA. However, the EF for the TIWP IgG showed a significant difference only between cheeses containing 10% and 30% HH milk ($p < 0.05$). When comparing the EF for the TWP, TIWP β-LG, TIWP α-LA, and TIWP IgG between cheeses containing 10%, 20%, and 30% HH milk, based on the approach of a constant ratio of NASP to DWP, significant differences ($p < 0.05$) were found for the EF for the TWP and TIWP β-LG. However, the EF for the TIWP α-LA and TIWP IgG showed significant differences only between cheeses containing 10% and 20% HH milk and between cheeses containing 10% and 30% HH milk ($p < 0.05$).

3.5. Analysis of the total whey protein content of cheese via HPLC-FLD

Both chromatographic and electrophoresis methods are commonly used to analyze the milk protein composition (casein and whey proteins) of milk and milk products (DIN, 1996; Bordin, Cordeiro Rapaso, de la Calle, & Rodriguez, 2001). To determine the authenticity of milk or milk products (e.g., cheese) from different animal species (e.g., cow, goat, buffalo, and sheep), isoelectric focusing of γ-casein is well established (Commission Regulation, 2008). Compared to the casein content, the whey protein content in cheese is too low to be validly quantified with electrophoresis methods. However, a liquid chromatographic method to quantify the total whey protein (TWP) content in ripened cheese does not yet exist.

To determine the TWP content, all proteins present in the cheese from the preliminary experiment were extracted under denaturing conditions prior to chromatographic analysis (cf., Section 2.2.6). Due to the separation, the total individual whey protein (TIWP) content of α-LA and β-LG A and B could be determined. However, a complete baseline separation of all milk proteins was not possible. The CV of the repeatability ranged from 2.2% (α-CN) to 9.0% (β-LG B). The best coefficient of determination was found for β-CN and β-LG B ($R^2 = 0.983$ for both). The protein concentration ranged from 0.024 to 1.54 mg/mL for β-CN and from 0.0007 to 0.048 mg/mL for β-LG B. For β-LG A, the coefficient of determination was $R^2 = 0.926$. Here, the protein concentration ranged from 0.001 to 0.070 mg/mL. The recovery rate ranged between 112% (α-LA) and 141% (α-CN).

Proteolysis in cheese is widely studied and is mainly characterized by the breakdown of casein(s) (Grappin et al., 1985; Upadhyay et al., 2004). In contrast, proteolysis of whey proteins in cheese has not yet

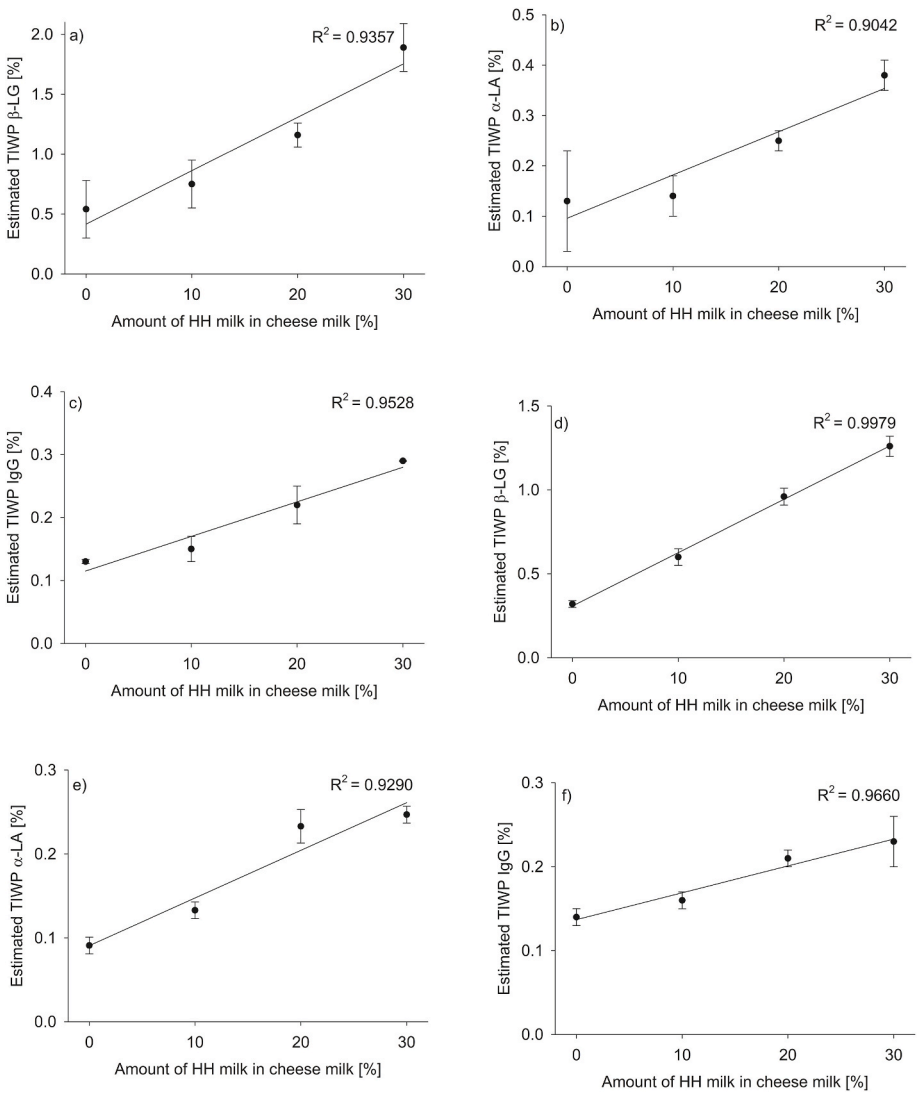

Fig. 5. Mean, SD, and coefficient of determination (R^2) for the estimated TIWP content in cheese. a)-c) Estimation based on a mass balance approach. Due to an incorrect preparation of the whey mix in production 1, the data of the corresponding production were not considered; d)-f) estimation based on a constant ratio of NASP to DWP. The ASIWP data obtained via HPLC-UV analyses were used for both calculation models. n = 9 for standard cheese (0% (w/w) HH milk), n = 3 for cheese containing HH milk.

Table 2
Enrichment factors (EFs) and standard deviation (SD) of the estimated total whey protein (TWP) and total individual whey protein (TIWP) content in cheese containing various amounts of HH milk (n = 3, each) in relation to standard cheese (n = 9).

Cheese containing various amounts of HH milk	Calculation based on the mass balance approach; included ASWP and ASIWP data obtained via HPLC-UV analyses				Calculation based on a constant ratio of NASP to DWP; included ASWP and ASIWP data obtained via HPLC-UV analyses			
	EF TWP	EF TIWP β-LG	EF TIWP α-LA	EF TIWP IgG	EF TWP	EF TIWP β-LG	EF TIWP α-LA	EF TIWP IgG
Cheese containing 10% (w/w) HH milk	$1.3 \pm 0.4^{a,c}$	$1.4 \pm 0.4^{a,c}$	$1.1 \pm 0.4^{a,c}$	1.1 ± 0.2^{c}	$1.6 \pm 0.2^{a,c}$	$1.9 \pm 0.3^{a,c}$	$1.5 \pm 0.2^{a,c}$	$1.2 \pm 0.2^{a,c}$
Cheese containing 20% (w/w) HH milk	$1.9 \pm 0.7^{a,b}$	$2.2 \pm 0.6^{a,b}$	$2.0 \pm 0.7^{a,b}$	1.7 ± 0.4	$2.5 \pm 0.3^{a,b}$	$3.0 \pm 0.4^{a,b}$	2.6 ± 0.3^{a}	1.5 ± 0.1^{a}
Cheese containing 30% (w/w) HH milk	$3.0 \pm 0.4^{b,c}$	$3.5 \pm 0.5^{b,c}$	$3.0 \pm 0.4^{b,c}$	2.2 ± 0.3^{c}	$3.0 \pm 0.2^{b,c}$	$4.0 \pm 0.4^{b,c}$	2.7 ± 0.3^{c}	1.7 ± 0.3^{c}

[a] Significant difference ($p < 0.05$) for EF between cheese containing 10% and 20% (w/w) HH milk.
[b] Significant difference ($p < 0.05$) for EF between cheese containing 20% and 30% (w/w) HH milk.
[c] Significant difference ($p < 0.05$) for EF between cheese containing 10% and 30% (w/w) HH milk.

been studied comprehensively. De Koning, De Boer, Both, and Nooy (1981) found that native whey proteins are resistant toward proteolytic enzymes of rennet or the starter culture. The results shown in Section 3.3 confirm this finding. However, the proteolytic stability of DWP during cheese ripening has not been comprehensively studied. The TIWP content of α-LA and β-LG during cheese ripening is shown in Fig. 6. There were variations during the ripening process, but a decrease in the TIWP α-LA and TIWP β-LG could not be detected. These variations could be caused by the individual cheese loaves sampled each time. Based on these results, DWP seem to be resistant to proteolytic degradation during ripening.

Fig. 6 shows the TIWP content of α-LA and β-LG during the ripening of the cheese from the preliminary experiment. The TIWP α-LA content in standard cheese varied from 0.18 ± 0.01 g/100 g (six weeks of ripening) to 0.32 ± 0.01 g/100 g (after brining), in cheese with 30% (w/w) HH milk, it was 0.21 ± 0.01 g/100 g (four weeks of ripening) to 0.32 ± 0.01 g/100 g (after brining). The average TIWP α-LA content during ripening was 0.24 ± 0.04 g/100 g for standard cheese and 0.27 ± 0.04

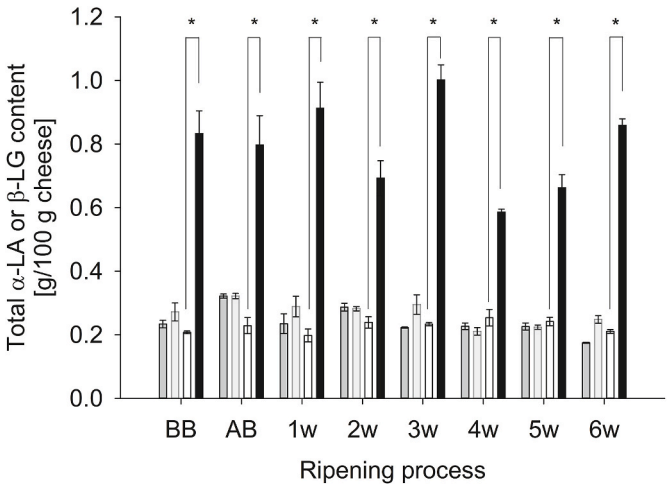

Fig. 6. Mean and SD for the total α-LA and β-LG content during cheese ripening (BB: before brining, AF: after brining, and weekly until the sixth week of ripening) for cheese in the preliminary experiment (standard cheese (n = 1) and cheese containing 30% (w/w) HH milk (n = 1)). Sample preparation was performed in duplicate and measured twice. (▒) Total α-LA content in standard cheese; (▒) total α-LA content in cheese containing 30% (w/w) HH milk; (▭) total β-LG content in standard cheese; (▬) total β-LG content in cheese containing 30% (w/w) HH milk; *the TIWP β-LG content in cheese containing 30% (w/w) HH milk was significantly higher than the TIWP β-LG content in standard cheese ($p < 0.05$).

g/100 g for cheese with 30% (w/w) HH milk. Levene's test showed that the variances of the TIWP α-LA content in the standard cheese and that containing 30% HH milk were not significantly different ($p < 0.05$). The mean TIWP α-LA content in the standard cheese and that containing 30% HH milk before brining and after six weeks of ripening were compared by a two-sample *t*-test. No significant difference was found ($p < 0.05$). Additionally, Neumann's trend test did not show any trend in the TIWP α-LA content during ripening ($p < 0.05$). The TIWP β-LG content in standard cheese varied from 0.20 ± 0.02 g/100 g (one week of ripening) to 0.24 ± 0.02 g/100 g (two weeks of ripening); in cheese with 30% (w/w) HH milk, it was from 0.59 ± 0.01 g/100 g (four weeks of ripening) to 1.00 ± 0.05 g/100 g (three weeks of ripening). The average TIWP β-LG content during ripening was 0.23 ± 0.02 g/100 g for standard cheese and 0.79 ± 0.14 g/100 g for cheese with 30% (w/w) HH milk. Levene's test showed that the variances in the TIWP β-LG content in the standard cheese and that containing 30% HH milk were not significantly different ($p < 0.05$). The mean values of the TIWP β-LG content in the standard cheese and that containing 30% HH milk before brining and after six weeks of ripening were compared via a two-sample *t*-test. No significant difference was found ($p < 0.05$). Moreover, Neumann's trend test did not show any trend for the TIWP β-LG content during ripening ($p < 0.05$). The TIWP β-LG content of cheese containing 30% (w/w) HH milk was significantly higher ($p < 0.05$) than that of standard cheese. However, cheese containing 30% (w/w) HH milk did not have a significantly higher TIWP α-LA content than standard cheese. These results were confirmed with a *t*-test. The EFs for β-LG and α-LA were 3.5 and 1.1, respectively. Regarding β-LG, the EF was in the range of the estimated EF for β-LG. Regarding α-LA, the EF was much lower than the estimated EF for α-LA based on both calculation models. This indicates that the cheese matrix particularly affects the chromatographic determination of the TIWP α-LA.

4. Conclusion

To determine the whey protein content in matured cheese, traditional HPLC analyses with DAD or FLD detection were used to analyze the ASWP or TWP content (under denaturing conditions). Native and denatured whey proteins were not proteolytically degraded throughout the ripening process of Edam-type cheese. Thus, the determination of whey protein content in matured Edam-type cheese is possible and is not affected by the proteolytic degradation of whey proteins. A direct comparison of standard cheese with cheese containing 30% HH milk showed a significant enrichment of the TIWP β-LG ($p < 0.05$). However, the complex cheese matrix and proteolytic effects of caseins prevented valid whey protein quantification in mature cheese. For future research on determination of the whey protein content in cheese, a proteomic mass spectrometric approach is suggested to overcome the matrix-related influences that occur during cheese analysis. For example, proteomic strategies can be used to identify marker peptides suitable for valid quantification of whey proteins.

Funding

The project was supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme (FKZ 281A107816).

CRediT authorship contribution statement

Tobias von Oesen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Mascha Treblin:** Conceptualization, Writing – review & editing. **Alexandra Staudacher:** Formal analysis, Investigation, Writing – review & editing. **Ingrid Clawin-Rädecker:** Conceptualization, Methodology, Writing – review & editing. **Dierk Martin:** Funding acquisition, Project administration, Writing – review & editing. **Wolfgang Hoffmann:** Methodology, Funding acquisition. **Katrin Schrader:** Methodology, Writing – review & editing. **Katja Bode:** Writing – review & editing. **Ralf Zink:** Writing – review & editing. **Sascha Rohn:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Jan Fritsche:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank their colleagues from the Max Rubner-Institut (MRI) Kiel, especially Norbert Johannsen, Sandra Kleiner and Laura Kurch for manufacturing the model cheese. Furthermore, we thank Nico Bahr, Maren Böttcher, Kerstin Hansen, Sabrina Kähler, Freya Klinge, Jana von Knebel, Katja Laß, Phillip Mittelstedt, Wiebke Petersen, Longina Reimann, Andrea Schwall, Sabine Splitzer, Angelika Thoß, Cornelia Voß, Nina Winter, and Yvonne Wölk for the sample management and wet-chemical analyses. We also thank Björn Neumann for the technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114347.

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