International Dairy Journal 97 (2019) 167–175

Contents lists available at ScienceDirect

International Dairy Journal

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

Protein profile of dairy products: Simultaneous quantification of twenty bovine milk proteins

Cornelia Bär*, [1], Déborah Mathis [1], [2], Patrick Neuhaus, Desirée Dürr, Walter Bisig, Lotti Egger, Reto Portmann

Agroscope, Schwarzenburgstrasse 161, 3003 Bern, Switzerland

ARTICLE INFO

Article history:
Received 7 August 2018
Received in revised form
19 December 2018
Accepted 4 January 2019
Available online 18 January 2019

ABSTRACT

While proteomic techniques allow the identification and relative quantification of thousands of proteins in a single run, methods for absolute quantification remain laborious. In this study, a newly developed multiple reaction monitoring (MRM) method using liquid chromatography mass spectrometry (LC-MS) that enables the simultaneous quantification of twenty key milk proteins is presented. The selected proteins comprise all individual caseins, the major whey proteins and most well-known milk fat globule membrane (MFGM) proteins. For validation, the twenty milk proteins in raw milk, raw cream, raw milk Emmental cheese and whey, were quantified as well as in eighteen commercial heat-treated dairy products. The method presented is ideally suited for various applications, for example, the comparison of the protein patterns in raw milk of cows at different stages of lactation or of different breeds.

1. Introduction

Proteins form a major class of milk components, comprising over 400 different types (Lu, 2013). They are present over a broad concentration range and can be grouped into three main classes (Casado, Affolter, & Kussmann, 2009): (i) the casein micelle proteins (CasMPs, 80–85%), organised as supramolecular, dynamic structures called casein micelles, that entrap colloidal calcium phosphate (McMahon & Oommen, 2013); (ii) the whey proteins (WPs, 13–18%), dissolved in the water phase; and (iii) proteins which are associated within the milk fat globule membrane (MFGMPs, 1–2%), a phospholipid bilayer that embeds proteins that protects the fat globules from coalescence and lipolysis (Bauman, Mather, Wall, & Lock, 2006; Dewettinck et al., 2008).

Milk proteins are of high value from a technological point of view as well as for their beneficial physiological effects (Supplementary material, Table S1). Therefore, the analysis of the different individual protein profiles in milk and dairy products is of high interest, not only for the dairy industry but also for nutritional research as well as applied biotechnology. Typical methods for the quantification of individual proteins involve ELISA or Western blot techniques. However, these methods require the availability of specific antibodies and a significant amount of time and effort, as the possibility of multiplex assays is restricted.

Other techniques utilise high-performance liquid chromatography (HPLC; Schwendel et al., 2017) or two-dimensional gel electrophoresis with the subsequent application of densitometry, dyes, fluorophores or radioactivity (Turner, MacDonald, Back, & Thomson, 2006) to attain the necessary sensitivity and resolution for protein quantification. However, due to a lack of individual certified milk protein standards, these quantifications are rarely absolute and proteins, which are insoluble or present in low concentrations are not detected.

Recently, a few laboratories developed multiple reaction monitoring (MRM) methods using liquid chromatography mass spectrometry (LC-MS) for the quantification of major milk proteins such as β-lactoglobulin and caseins (Lutter, Parisod, & Weymuth, 2011) or certain individual MFGMPs (Affolter, Grass, Vanrobaeys, Casado, & Kussmann, 2010; Fong & Norris, 2009). By targeted fragmentation of sample derived peptides as well as selected signature peptides and subsequent monitoring of their specific ions simultaneously, the MRM procedure enables the quantification of sample peptides based on their corresponding signal intensities. Therefore, MRM methods are very sensitive and increase selectivity to a level required when complex mixtures such as food matrices are analysed (Lange, Picotti, Domon, & Aebersold, 2008).

---

* Corresponding author. Tel.: +41 58 462 59 58.
E-mail address: cornelia.baer@agroscope.admin.ch (C. Bär).
[1] These authors contributed equally to this work.
[2] Current address: Kinderspital Zürich, Steinwiesstrasse 75, 8032 Zürich, Switzerland.

https://doi.org/10.1016/j.idairyj.2019.01.001

Up to now, MRM methods were developed covering only a few major milk proteins (Le, Deeth, & Larsen, 2017). To the best of our knowledge, no method is currently available for the absolute and simultaneous quantification of minor as well as the most abundant bovine milk proteins. Therefore, we developed a MRM method for the individual quantification of twenty key milk proteins at once by applying the absolute quantification (AQUA) strategy (Kirkpatrick, Gerber, & Gygi, 2005). This new method allows the simultaneous quantification of the CasMPs (α_{S1}-, α_{S2}-, β- and κ-casein) and the casein-associated lipoprotein lipase, six key WPs (β-lactoglobulin, α-lactalbumin, proteose peptone 3, serum albumin, lactoperoxidase and lactoferrin), and the most abundant MFGMPs (butyrophilin, xanthine dehydrogenase/oxidase, adipophilin, lactadherin, platelet glycoprotein 4 (CD36), polymeric immunoglobulin receptor, fatty acid binding protein, fatty acid synthase and glycoprotein 2). Designations and abbreviations are listed in Table 1, and most known functions of those milk proteins are listed in Supplementary material, Table S1.

The MRM method developed was validated by quantifying the twenty proteins in dairy products produced from raw milk and in commercial heat-treated dairy products. The dairy products raw milk (RM), raw cream (RC), Swiss Emmental (EM)—a raw milk cheese, and sweet whey (W) are particularly suitable for the evaluation of the method, since they represent all three different milk fractions, thus having different protein profiles. The investigated commercial dairy products comprise pasteurised and ultra-pasteurised milk (MI), ultra-high temperature (UHT) and ultra-pasteurised cream (CR), yoghurt (pasteurised, YOG), buttermilk (pasteurised, BM), quark (pasteurised, Q) and cottage cheese (pasteurised, COTC), each from three different manufacturers. They are listed, including the specifications of the indicated preservation processes in Supplementary material, Table S4.

2. Materials and methods

2.1. Materials

RM was obtained from the cheese dairy Uettligen (Bern, Switzerland). The RC was separated by centrifugation from the RM at 10 °C and 2000×*g* for 15 min. The W was collected after the production of Tilsit cheese (from milk that was heated at 44.5 °C) at Agroscope (Bern, Switzerland). The commercial dairy products were bought from the supermarkets Migros and Coop (Switzerland, Supplementary material, Table S4). LC-MS grade water was purchased from VWR International (Dietikon, Switzerland); LC-MS hypergrade acetonitrile (ACN), formic acid (FA), ammonium bicarbonate (ABC) and the reference proteins used for spiking [α-casein (α_{S1}- + α_{S2}-casein), β-lactoglobulin, α-lactalbumin, serum albumin, lactoferrin] from Merck (Zug, Switzerland); and trypsin Gold (MS-Grade) from Promega (Dübendorf, Switzerland).

2.2. Sample preparation for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The total protein content of each dairy product was calculated from the total nitrogen (TN) content (raw milk dairy products) or from the TN minus the non-protein nitrogen (NPN) (heat-treated dairy products) determined by Kjeldahl according to the ISO/IDF standard method ISO 8968-3:2007/IDF 20-3:2007 (ISO, 2007) and multiplied by a conversion factor of 6.38 (Supplementary material, Table S4). A precise quantity of dairy product containing 2 mg of total proteins was placed in a 1.5 mL Eppendorf tube and precipitated with 1 mL of ice-cold acetone. The resulting pellets were dissolved in 200 μL Tris−HCl (100 mmol L^{-1}, pH 7.5, 1% sodium dodecyl sulphate (SDS)); 100 μL of the sample (clear phase) was mixed with 20 μL of the sample buffer 6× (Tris−HCl 350 mmol L^{-1}, pH 6.8, SDS 10%, glycerol 50%, Dithiothreitol (DTT) 100 mmol L^{-1}) with bromophenol blue and heated at 95 °C for 5 min. Then, 3.6 μL (30 μg of protein) of each sample and 5 μL of the molecular weight marker (Benchmark™ Prestained Protein Ladder; Thermo Fisher Scientific, Reinach, Switzerland) were separated by SDS-PAGE (15% polyacrylamide) and stained with colloidal Coomassie Blue, as previously described (Egger et al., 2016).

2.3. In-gel tryptic digestion

Pieces of polyacrylamide gel containing protein bands of interest were excised from gels (15% polyacrylamide), washed and digested with trypsin as described in Kopf-Bolanz, Schwander, Gijs, Vergères, Portmann, and Egger (2012).

2.4. Isotopically labelled peptides as internal standards

The twenty isotopically labelled AQUA peptides were manufactured by Thermo Fisher GmbH (Ulm, Germany) according to the provided sequences (Table 1). The last AA of the tryptic peptides was labelled with ^{13}C and ^{15}N, thereby producing a mass shift between the AQUA and the native peptides of +10 for arginine, +8 for lysine and +6 for valine. The labelled AQUA peptides were produced as lyophilised trifluoroacetic salts, which were dissolved in sample solution (5% ACN, 0.1% FA in water), thereby resulting in a concentration of approximately 50 pmol μL^{-1}. Following the accurate determination of the soluble concentrations by AA analyses (phenylthiocarbamyl (PTC)-derivatisation, as described by Kopf-Bolanz et al., 2012), a specific quantity of each AQUA peptide dilution was mixed to produce a peptide-mix-solution containing the labelled peptides of CASA1, CASA2, CASB, CASK, LACB, LALBA, FABP, PAS 6/7 and PIGR at a concentration of 0.2 pmol μL^{-1} and the labelled peptides of LPL, BSA, LPO, LF, BTN, XDH, ADPH, CD36, PP3, FAS and GP2 at a concentration of 0.02 pmol μL^{-1}. These concentration ratios have proven to be favourable, since the signals of the native peptides in milk and the AQUA peptides differed by no more than a factor of 10, if 5 μL of the AQUA peptide-mix-solution was injected simultaneously with each sample. A twenty-fold concentrated solution of the AQUA peptide-mix was stored at −80 °C for up to a maximum of 8 weeks.

2.5. Sample preparation for protein quantification

The total protein content of each sample was determined as described above (Supplementary material, Table S4). The EM was grated, and subsequently, precisely 2 mg of total protein (RM, RC, W, EM, YOG, Q, and COTC), or 60 μL of product (MI, CR and BM) respectively, were added into a 1.5 mL Eppendorf tube. For EM and RC, 50 μL of digestion buffer (10% ACN, 25 mM ABC in micro filtered H_2O) were added and placed in a sonication bath for 30 min, helping the matrix to dissolve and liberate the proteins. The proteins were precipitated by an addition of 1 mL of ice-cold acetone and the tube was let on ice for 1 h. The suspensions were centrifuged at 4 °C and 18,000×*g* for 20 min, the acetone was carefully discarded, and the resulting pellets were air-dried for at least 30 min. The pellets were resolubilised in 1 mL of digestion buffer by vortex and sonication in a warm water bath (40 °C). Fifty μL of protein solution (2 μg μL^{-1}) were mixed with 40 μL of digestion buffer followed by addition of 10 μL of trypsin solution (0.2 μg μL^{-1} in 0.1% Tris, pH 9, specific activity >15,000 u mg^{-1}). After overnight incubation at 37 °C, 10 μL of the digested protein solution were diluted in 990 μL of sample solution (5% ACN, 0.1% FA in water). For the quantification of the high concentrated proteins in milk (LACB, LALBA, PP3, CASA2, CASK, CASA1, CASB), 10 μL of this (100× diluted) digest solution was injected into the LC-MS, corresponding to 0.1 μg of total proteins. For the quantification of the lower concentrated proteins (FAS, TRFL, XDH, BTN, PIGR, LIPL, ADPH, FABP3, GP2, CD36, PAS6/7, BSA and PERL), 10 μL of the undiluted digest solution was injected (corresponding to 10 μg of total protein). With each sample-injection, 5 μL of the internal standard peptide-mix-solution (see above) were simultaneously injected. To minimise the experimental error, exactly the same sample was prepared and analysed multiple times (technical replicates) and each replicate was measured three times.

2.6. Analysis by liquid chromatography tandem mass spectrometry

Peptides were separated on a Rheos 2200 HPLC (Flux Instruments, Reinach, Switzerland) equipped with a XTerra MS C18 column (3.5 μm, 1.0 mm × 100 mm) and a guard column (XTerra MS C18 VanGuard Cartridge, 3.5 μm, 2.1 mm × 10 mm; both columns: Waters, Baden-Dättwil, Switzerland) with a flow rate of 80 μL min^{-1} for 30 min. A gradient from 5% to 60% solution B (ACN, 0.1% FA) in solution A (H_2O, 0.1% FA) was applied in the first 15 min, increased to 95% in the next 5 min, and returned to the initial conditions within the 21st min for a 9-min re-equilibration. The column temperature was maintained at 25 °C.

The Rheos 2200 HPLC was coupled directly to a LTQ linear ion trap mass spectrometer (QQQ-MS, Thermo Scientific, Reinach, Switzerland) using an electron spray ionisation (ESI) interface. The HPLC eluent of the first 3.5 min and the last 17 min were diverted to waste. ESI conditions were as follows: source voltage 4000 V, capillary voltage 5 V, tube lens 150 V, capillary temperature 275 °C, sheath gas flow 20 arbitrary units and auxiliary gas flow 10 arbitrary units. The ion trap mass spectrometer was operated in a positive ion mode. The MRM included liquid chromatography-tandem mass spectrometry (LC-MS/MS) runs with 2–4 segments (1–8 min) and 2–6 scan events. The following MRM conditions were included: full scan range 260–1500 *m/z*, isolation width 2 *m/z*, normalised collision energies 35.0, collision gas helium, activation time 30 ms and activation Q 0.250.

The retention time (r.t.) of the signature peptides, peptides *m/z* and transitions are shown in Table 1. The resulting peaks were integrated using the quantitative software LCquan (version 2.8) from Thermo Scientific. The performance of the LC-MS/MS-system was reviewed before and in between each batch of measurements by injection of a BSA solution (10 μL, final concentration 10 fmol μL^{-1}, tryptic digested peptides of BSA), separated with the identical gradient and flow rate on the same XTerra MS C18 column/guard column-system as peptide separation of the samples occurred. The ESI settings were the same as for the peptides samples. The MS setup was a full-scan range from 300 to 1100 *m/z* in one segment with a start delay of 1.9 min. The MS settings included isolation width 1 *m/z*, normalised collision energy 35.0, collision gas helium, activation Q 0.250 and activation time 30 ms. The measurements were conducted in the positive ion mode and the resulting spectra were evaluated by an identification search with Mascot v 2.2.04 (Matrix Science Inc., Boston, MA, USA) using the UniProt Database (search parameter settings: MS/MS ion search; trypsinisation; variable modifications: deamination, pyroglutamic acid, oxidation; average mass values; unrestricted protein mass; mass and fragment mass tolerance: ± 0.8 Da; maximum missed cleavage: 1; instrument type: ESI-TRAP). The performance was assessed by means of a BSA sequence-coverage of at least 15%.

2.7. Recovery experiments

For recovery experiments, the protein content of six commercially available standard proteins (CASA1, CASA2, LACB, LALBA, BSA and LF) was determined by measuring TN and NPN with Kjeldahl (ISO, 2007). A solution containing a determined amount of the standards in digestion buffer was prepared. The concentration of each of the proteins was quantified in the solution (Supplementary material, Table S3), as well as in 60 μL milk using the developed MRM method. Increasing quantities of the prepared protein solution were added to the basis of 60 μL of milk (0–50 μL in 10 μL steps, corresponding to 0–5 aliquots in Supplementary material, Fig. S3). The proteins of the gradually spiked samples were quantified with the MRM method. For each of the six proteins, the recovery was calculated by the ratio of the added amount of protein and the quantity determined by MRM in the spiked sample after subtraction of the amount found in the milk.

3. Results and discussion

3.1. Establishment of a multiple reaction monitoring (MRM) method for the simultaneous quantification of twenty bovine milk proteins

To define the quantifiable protein set, the proteins of the four raw milk dairy products RM, RC, EM and W were separated by SDS-PAGE (Fig. 1b). Polyacrylamide gel pieces were manually excised from the most intense bands and prepared for in-gel tryptic digestion. The proteins were identified by mass spectrometry based on matching MS/MS spectra with in-silico generated spectra using the MASCOT database (Kopf-Bolanz et al., 2012). Thus, MS data for the most abundant milk proteins were obtained, which included the r.t. of the tryptic peptides, mass-to-charge ratios and their MS/MS features. The twenty milk proteins were selected for quantification, due to their technological importance, biological function or nutritional value. The selected proteins include all CasMPs (CASA1, CASA2, CASB, CASK, LPL), six major WPs (LACB, LALBA, PP3, BSA, LPO, LF) and a selection of MFGMPs (BTN, XDH, ADPH, PAS 6/7, CD36, PIGR, FABP, FAS, GP2) (Supplementary material, Table S1).

The peptide data obtained by protein identification provided the necessary information for selecting proteotypic signature peptides suitable for absolute quantification by MRM. The selection of the signature peptides was based on the uniqueness of the tryptic peptide sequence, the absence of reported post-translational modification sites (UniProt Database), and their ionisation efficiency. Furthermore, the selection was restricted to tryptic peptides up to triply charged ones with a mass-to-charge-ratio between 350 and 1000 *m/z*. In the first step, for each protein, three highly detectable peptides that met these conditions were selected and their suitability for quantification by MRM was tested. In a second step, out of the three peptides, one signature peptide was selected for each protein on the basis of peak shape, r.t. and resolution. To increase the specificity for each signature peptide, the two most intense transitions with the best resolution were chosen for monitoring and subsequent quantification with the LCquan software (Thermo Scientific). The sequence of the selected signature peptides as well as their corresponding r.t. and the specific *m/z* value for the peptides and the fragments are listed in Table 1.

3.2. Method validation

3.2.1. Specificity and selectivity

To assure specificity and selectivity of the developed MRM method, particular attention was given to the selection of the proteotypic peptides and transitions to ensure that there was no extended interaction with the matrix. The use of two transitions with at least ten data points per peak for the identification and quantification of each protein increased the specificity of the method. Moreover, the labelled peptides were spiked as internal standards into every single experiment just before co-injection to

Table 1
Data of the signature peptides for the quantification of twenty bovine milk proteins by multiple reaction monitoring (MRM).[a]

Abbreviation	Protein	Signature peptide sequence (Internal standard)	r. t. (min)	Pep. charge	Pep. *m/z*	Frag. *m/z*	Frag. *m/z*
Casein micelle proteins							
CASA1	α_{S1}-Casein	YLGYLEQLLR	10.8	2	634.6	992.1	771.9
		YLGYLEQLLR*			639.6	1002.1	781.9
CASA2	α_{S2}-Casein	FALPQYLK	9.3	2	490.7	648.8	761.9
		FALPQYLK*			494.7	656.8	769.9
CASB	β-Casein	GPFPIIV	11.2	1	742.5	625.8	441.6
		GPFPIIV*			748.5	625.8	447.6
CASK	κ-Casein	YIPIQYVLSR	10.0	2	627.1	488.6	976.1
		YIPIQYVLSR*			632.1	493.6	986.1
LPL	Lipoprotein lipase	EPDSNVIVVDWLSR	10.6	2	815.3	624.6	875.0
		EPDSNVIVVDWLSR*			820.3	624.6	885.0
Whey proteins							
LACB	β-Lactoglobulin	ALPMHIR	6.7	2	419.6	327.4	653.8
		ALPMHIR*			424.6	332.4	663.8
LALBA	α-Lactalbumin	VGINYWLAHK	8.8	2	601.4	523.1	932.1
		VGINYWLAHK*			605.4	527.1	940.1
PP3	Lactophorin (proteose peptone 3)	LPLSILK	9.0	2	392.7	335.9	573.7
		LPLSILK*			396.7	339.9	581.7
BSA	Bovine serum albumin	LGEYGFQNALIVR	9.7	2	740.8	814.0	685.8
		LGEYGFQNALIVR*			745.8	824.0	695.8
LPO	Lactoperoxidase	ASEQILLATAHTLLLR	10.4	3	584.6	611.8	498.6
		ASEQILLATAHTLLLR*			587.9	616.8	503.6
LF	Lactoferrin	YLTTLK	6.8	2	370.1	462.6	277.3
		YLTTLK*			374.1	470.6	277.3
Milk fat globule membrane proteins							
BTN	Butyrophilin (Subfamily 1 Member A1)	EIPLSPMGEDSASGDIETLHSK	8.6	3	772.4	887.9	1036.6
		EIPLSPMGEDSASGDIETLHSK*			775.1	891.9	1040.6
XDH	Xanthine dehydrogenase/oxidase	TNLSSNTAFR	6.7	2	556.4	782.8	896.0
		TNLSSNTAFR*			561.4	792.8	906.0
ADPH	Adipophilin (adipophilin differentiation related protein ADRP)	VANLPLVSSTYDLVSSAYISRK	10.4	3	795.6	994.1	1107.3
		VANLPLVSSTYDLVSSAYISRK*			798.3	998.1	1111.3
PAS 6/7	Lactadherin	NIFETPFQAR	9.5	2	611.9	996.1	719.8
		NIFETPFQAR*			616.9	1006.1	729.8
CD36	Platelet glycoprotein 4	VAIIDTYK	7.7	2	461.9	752.9	639.7
		VAIIDTYK*			465.9	760.9	647.7
PIGR	Polymeric immunoglobulin receptor	SPIFGPEEVTSVEGR	9.2	2	482.5	530.6	608.7
		SPIFGPEEVTSVEGR*			487.5	535.6	613.7
FABP	Fatty acid binding protein	SIVTLDGGK	7.2	2	445.4	689.8	590.6
		SIVTLDGGK*			449.4	697.8	598.6
FAS	Fatty acid synthase	IPALQDGR	6.4	2	435.5	378.9	659.7
		IPALQDGR*			440.5	383.9	669.7
GP2	Glycoprotein 2 (zymogen granule membrane)	DSTISVEENGVSAESR	7.2	2	840.8	549.6	1078.1
		DSTISVEENGVSAESR*			845.8	559.6	1088.1

[a] Amino acids (AAs) are abbreviated according to the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) standard; an asterisk indicates isotopically labelled by ^{13}C and ^{15}N contained in the last amino acid (AA) of each peptide. Abbreviations are: r.t., retention time; Pep., peptide; Frag., fragment.

circumvent ion suppression arising from the interaction with the sample matrix. Time segmentation was applied to optimize dwell time and S/N ratio for predefined sets of transitions, thereby resulting in improved sensitivity with minimum length of the MRM method. As the extracted ion chromatograms in Supplementary material, Fig. S2 indicate all peaks were well separated and were therefore easy to integrate. The displayed data was extracted directly from MRM experiments of commercially available buttermilk and cream samples.

3.2.2. Precision

To review the precision of the quantification method, the coefficients of variation (CV) were determined for each protein, measuring a RM sample in six biological replicates, each injected three times (eighteen measurements). The CVs for CasMPs and WPs ranged between 5% and 15% (Supplementary material, Table S2), which are typical for such methods (Brönstrup, 2004; Yang, Hayes, Fang, Daley, Ettenberg, & Tse, 2007). Higher CVs were obtained for a few MFGMPs (ADPH, PAS 6/7, CD36, PIGR, 7–33%). This is most likely due to the low concentration of these proteins in milk. The quantification area of these minor proteins was close to the quantitation limit, thereby increasing the error. Due to the much higher concentrations of CasMPs in milk, it was not possible to increase the injection volume to improve quantification quality of those MFGMPs. As most MFGMPs are more concentrated in cream, the CV declines for these minor proteins, getting into the typical range of CV around 15% (data not shown).

3.2.3. Accuracy and recovery

Recovery experiments were conducted for six selected commercially available proteins (CASA1, CASA2, LACB, LALBA, BSA and LF) through a gradual increase in concentration of the spiked protein in a milk sample (section 2.7). The determined recovery values of the proteins in milk and the corresponding linear regression curves are plotted in Supplementary material, Fig. S3. The recovery rate ranged from 91% to 105% (Supplementary material, Table S3). The CVs of the recovery rates varied for the high abundant CasMPs between 6.6% (CASA1) and 7.9% (CASA2), for the lower abundance proteins between 9.8% and 26.7% (LF, BSA, LALBA and LACB). The higher variability could be explained by difficulties to obtain a homogenous mixture of the spiked protein with the raw milk sample.

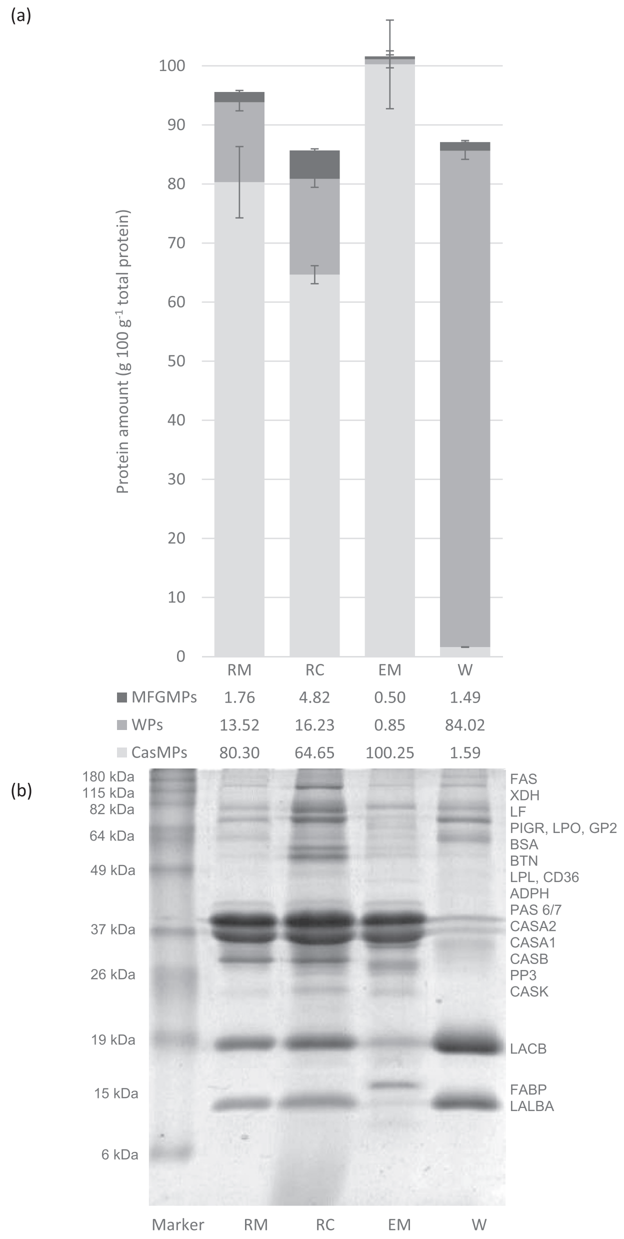

Fig. 1. Panel a: quantity (g 100 g^{-1} total protein) of casein micelle proteins (CasMPs), whey proteins (WPs) and milk fat globule membrane associated proteins (MFGMPs) in raw milk (RM), raw cream (RC), Swiss Emmental cheese (EM) and whey (W), determined by multiple reaction monitoring. Mean values and standard deviations were determined for twelve RM (in technical duplicates, each measured three times), for RC, EM and W, each sample was measured thrice in technical triplicates. Panel b: SDS-PAGE showing the protein pattern for each raw dairy product. For abbreviations for the proteins, see Table 1.

3.2.4. *Range and linearity*

The linearity of the labelled AQUA peptide quantification was determined over at least four orders of magnitude (10^{-3}–10 pmol), and the linear regression curves found had an R^2 of at least 0.96 for each single peptide (Supplementary material, Fig. S4). The upper limit of linearity was not determined due to the high cost and limited quantity of the labelled AQUA peptides. The linearity in the response of the native peptides was determined on an RM sample. Linearity is demonstrated for the most abundant milk proteins (all CasMPs, LACB, PP3; Supplementary material, Fig. S1). For minor milk proteins, it was not possible to obtain sufficient data points, since the injection of higher amounts of protein led to a saturation of the column with CasMPs.

3.2.5. *Detection and quantitation limits*

As the twenty proteins quantified in this method are present in milk in a broad concentration range, the limit of detection and quantitation were different for each individual protein. For the most abundant proteins, a detection and quantitation limit of 0.0001 μg was found, which corresponded to 0.001 μg of total injected protein. For the low concentration proteins, the detection limits ranged between 0.1 and 1 μg of total injected protein and the quantitation limits between 1 and 10 μg of total injected proteins (Supplementary material, Table S2). For the AQUA peptides, the limits of detection and quantitation were 0.0001 pmol and 0.001 pmol, respectively. Hence, to achieve a precise quantification for each protein, the same samples were injected twice with two different amounts of total proteins in the column (0.01 ug for LACB, LALBA, PP3, CASA2, CASK, CASA1, CASB and 10 ug for FAS, TRFL, XDH, BTN, LALBA, PIGR, LIPL, ADPH, FABP3, GP2, CD36, PAS6/7, BSA and PERL).

3.3. *Simultaneous quantification of twenty bovine milk proteins in raw milk and commercial heat-treated dairy products*

3.3.1. *Raw milk dairy products*

To investigate the quantification method in practice, the amounts of the twenty proteins were determined in the four raw milk dairy products already used for the method development. RM, RC, EM and W represent the different fractions of milk and, thus, are rich in milk proteins belonging to different classes. The results for the major CasMPs and WPs in RM measured with the MRM method were compared with previously obtained results from literature (Table 3), for which however the methods used were not always clearly described. Moreover, the concentration of proteins in milk depends on many factors and differs according to the course of lactation, udder health, supply of energy and crude protein, feed, season, environmental temperature and breed. Therefore, concentrations of the different milk proteins are often indicated as a range (Eigel et al., 1984; Swaisgood, 1993, Table 3).

The side-by-side comparison showed that the results for seven of the eight proteins (CASA1, CASA2, CASB, CASK, LACB, LALBA, BSA and LF) were in the expected range (Table 3). Only the amount of BSA detected in RM deviated from the published values. However, this deviation was not observed in heat-treated samples (Table 4). The reasons for the deviation found in RM could lie in the nature of the protein, as BSA has several lipid-binding sites (Spector, John, & Fletcher, 1969) as well as 35 cysteines, of which only one sulfhydryl group is free (Chevalier, Hirtz, Sommerer, & Kelly, 2009). It is therefore possible that the tight folding of the native BSA, held together by the disulphide bridges, makes it more resistant to tryptic hydrolysis. Since there is also evidence that BSA in its native form is involved in protein complexes in non-heat-treated skim milk (Chevalier et al., 2009), a lower susceptibility of the protein to precipitation and tryptic digestion may also result from a possible interaction of BSA with lipids or other native proteins present in the RM.

To the best of our knowledge, no literature data in RM are available for LPL, PP3 and LPO as well as all investigated MFGMPs (BTN, XDH, ADPH, PAS 6/7, CD36, PIGR, FABP, FAS, GP2). The milk proteins in the four products analysed, RM, RC, EM and W, were attributed to the three classes CasMPs, WPs and MFGMPs and given in g 100 g^{-1} of total protein (Fig. 1a). The individual concentrations are listed in Table 2. As displayed in Fig. 1, the protein profiles of the four products correspond well with the expected class of proteins on the basis of their manufacturing technology. RM

Table 2
Quantity of twenty individual milk proteins in raw milk, raw cream, Swiss Emmental cheese and whey determined by multiple reaction monitoring (MRM).[a]

Protein	Product							
	Raw milk		Raw cream		Emmental		Whey	
	Amount	*σ*	Amount	*σ*	Amount	*σ*	Amount	*σ*
Casein micelle proteins								
CASA1	31.1	*1.8*	23.3	*1.1*	36.4	*3.1*	1.00	*0.04*
CASA2	11.1	*1.2*	9.5	*0.4*	11.6	*0.9*	0.12	*0.01*
CASB	31.2	*3.7*	25.8	*0.9*	42.5	*2.3*	0.38	*0.02*
CASK	6.9	*1.0*	6.0	*0.2*	9.6	*0.7*	0.07	*0.004*
LPL	0.052	*0.006*	0.047	*0.005*	0.066	*0.005*	0.025	*0.005*
Whey proteins								
LACB	10.2	*1.3*	10.6	*1.1*	0.39	*0.04*	58.9	*1.6*
LALBA	2.0	*0.3*	3.3	*0.6*	0.16	*0.05*	18.7	*1.0*
PP3	1.0	*0.1*	2.0	*0.2*	0.064	*0.009*	4.2	*0.2*
BSA	0.02	*0.01*	0.016	*0.005*	0.003	*0.001*	1.9	*0.5*
LPO	0.040	*0.022*	0.046	*0.008*	0.032	*0.04*	0.171	*0.024*
LF	0.25	*0.052*	0.25	*0.02*	0.20	*0.013*	0.19	*0.03*
Milk fat globule membrane proteins								
BTN	1.15	*0.19*	3.35	*1.69*	0.21	*0.11*	0.44	*0.22*
XDH	0.146	*0.03*	0.578	*0.04*	0.073	*0.008*	0.374	*0.08*
ADPH	0.00025	*0.0001*	0.0069	*0.003*	0.0036	*0.0001*	0.00008	*0.00005*
PAS 6/7	0.02	*0.01*	0.93	*0.13*	0.27	*0.04*	0.25	*0.04*
CD36	0.022	*0.006*	0.177	*0.08*	0.023	*0.008*	0.055	*0.05*
PIGR	0.31	*0.12*	0.36	*0.17*	0.05	*0.06*	0.02	*0.01*
FABP	0.03	*0.02*	0.17	*0.07*	0.05	*0.01*	0.43	*0.2*
FAS	0.027	*0.007*	0.083	*0.005*	0.023	*0.004*	0.051	*0.006*
GP2	0.023	*0.003*	0.035	*0.005*	0.005	*0.001*	0.071	*0.007*

[a] Mean values (g 100 g^{-1} total protein) and standard deviations were determined on twelve raw milk samples (3.5% total protein), each measured three times, one raw cream sample (2.2% total protein), one Swiss Emmental cheese sample (30.3% total protein), and one whey sample (0.5% total protein) in technical triplicates, measured three times. Protein abbreviations are listed in Table 1.

comprised 80.3% CasMPs, 13.5% WPs, and 1.8% MFGMPs, which is in good accordance with the existing literature (Fox, 2011). RC had a higher proportion of MFGMPs (4.8%), due to the high content of native fat globules and contained 64.7% CasMPs, 16.2% WPs. EM contained mainly CasMPs (100.3%) and only minor amounts of the other classes of proteins, namely 0.9% WPs and 0.5% MFGMPs. And as expected, whey contained mostly WPs (84%) and only 1.6% CasMPs and 1.5% MFGMPs (Fig. 1a). The protein distribution was qualitatively confirmed by SDS-PAGE (Fig. 1b).

3.3.2. *Commercial heat-treated dairy products*

In addition to the four raw milk products, the protein profiles of eighteen commercially available heat-treated dairy products with different fat contents were examined, including MI 3.5–3.9% fat, CR

Table 3
Comparison of the concentration of twenty milk proteins in raw milk determined by multiple reaction monitoring (MRM) with published literature data.[a]

Protein	Concentration in g 100 g^{-1} protein		Concentration in g L^{-1}				
	MRM	Literature data	MRM	Literature data			
		Walstra, Wouters, and Geurts (2006)		Tremblay, Laporte, Léonil, Dupont, and Paquin (2003)	Swaisgood (1995)	Eigel et al., 1984; Swaisgood, 1993	Kuczynska et al. (2012)
Casein micelle proteins							
CASA1	31.1	32	11.36	10.0	11.9	12–15	–
CASA2	11.1	8.4	4.02	2.6	3.1	3–4	–
CASB	31.2	26	11.33	9.3	9.8	9–11	–
CASK	6.9	9.3	2.5	3.3	3.5	2–4	–
LPL	0.052	–	0.019	–	–	–	–
Whey proteins							
LACB	10.2	9.8	3.7	3.2	3.2	2–4	2.68–4.12
LALBA	2.0	3.7	0.74	1.2	1.2	0.6–1.7	1.73–2.06
PP3	1.0	–	0.37	0.3	–	–	–
BSA	0.02	1.2	0.01	0.4	0.4	0.4	0.12–0.2
LPO	0.040	–	0.015	–	–	–	–
LF	0.25	–	0.09	0.1	–	–	0.19–0.33
Milk fat globule membrane proteins							
BTN	1.15	–	0.42	–	–	–	–
XDH	0.146	–	0.054	–	–	–	–
ADPH	0.00025	–	0.0001	–	–	–	–
PAS 6/7	0.02	–	0.0078	–	–	–	–
CD36	0.022	–	0.0079	–	–	–	–
PIGR	0.31	–	0.11	–	–	–	–
FABP	0.03	–	0.01	–	–	–	–
FAS	0.027	–	0.01	–	–	–	–
GP2	0.023	–	0.0086	–	–	–	–

[a] Means determined by multiple reaction monitoring (MRM) were on twelve raw milk samples, each measured three times. Data from Walstra et al. (2006) are approximate composition and those from Swaisgood (1995) are averaged values. Protein abbreviations are listed in Table 1.

Table 4
Average amount of twenty individual milk proteins in groups of heat-treated dairy products determined by multiple reaction monitoring (MRM).[a]

Protein	Product											
	MI		CR		YOG		BM		Q		COTC	
	Amount	*σ*	Amount	*σ*	Amount	*σ*	Amount	*σ*	Amount	*σ*	Amount	*σ*
Casein micelle proteins												
CASA1	20.90	*2.54*	13.98	*0.28*	19.14	*1.79*	20.58	*1.65*	23.76	*2.89*	24.61	*1.5*
CASA2	10.37	*0.63*	7.88	*0.98*	9.93	*1.35*	8.17	*0.58*	12.08	*0.81*	15.75	*0.51*
CASB	25.31	*1.43*	16.85	*0.57*	25.80	*2.94*	18.32	*1.06*	30.64	*2.55*	35.93	*2.76*
CASK	6.43	*0.26*	4.43	*0.60*	4.76	*0.35*	4.29	*0.105*	5.89	*0.48*	7.68	*0.18*
LPL	0.05	*0.005*	0.04	*0.005*	0.05	*0.003*	0.04	*0.003*	0.06	*0.007*	0.06	*0.0004*
Whey proteins												
LACB	9.23	*1.06*	7.74	*0.97*	9.38	*1.44*	10.96	*0.53*	12.69	*1.70*	2.03	*0.23*
LALBA	1.26	*0.35*	0.43	*0.22*	1.53	*0.33*	2.05	*0.25*	1.85	*0.20*	0.43	*0.06*
PP3	0.84	*0.05*	1.97	*0.35*	0.81	*0.11*	2.29	*0.06*	0.25	*0.07*	0.46	*0.008*
BSA	0.21	*0.10*	0.30	*0.028*	0.37	*0.04*	0.27	*0.005*	0.47	*0.02*	0.18	*0.02*
LPO	0.05	*0.003*	0.03	*0.003*	0.08	*0.01*	0.08	*0.008*	0.09	*0.007*	0.05	*0.004*
LF	0.33	*0.03*	0.26	*0.07*	0.38	*0.080*	0.41	*0.008*	0.30	*0.037*	0.32	*0.012*
Milk fat globule membrane proteins												
BTN	0.35	*0.05*	1.95	*0.43*	0.33	*0.02*	2.26	*0.09*	0.22	*0.018*	0.36	*0.038*
XDH	0.22	*0.05*	1.00	*0.20*	0.23	*0.028*	1.31	*0.08*	0.22	*0.018*	0.23	*0.015*
ADPH	0.0009	*0.0001*	0.0023	*0.001*	0.0006	*0.0001*	0.0014	*0.0001*	0.0007	*0.0001*	0.0003	*0.00004*
PAS 6/7	0.30	*0.05*	1.48	*0.24*	0.36	*0.04*	3.55	*0.71*	0.11	*0.016*	0.28	*0.02*
CD36	0.07	*0.005*	0.25	*0.063*	0.07	*0.02*	0.24	*0.026*	0.08	*0.012*	0.06	*0.005*
PIGR	0.22	*0.024*	0.34	*0.10*	0.59	*0.05*	0.35	*0.101*	0.13	*0.025*	0.41	*0.23*
FABP	0.09	*0.005*	0.17	*0.04*	0.09	*0.02*	0.33	*0.05*	0.25	*0.034*	0.12	*0.036*
FAS	0.02	*0.003*	0.12	*0.04*	0.01	*0.003*	0.13	*0.005*	0.02	*0.005*	0.02	*0.006*
GP2	0.02	*0.01*	0.06	*0.02*	0.03	*0.001*	0.06	*0.005*	0.04	*0.002*	0.02	*0.003*

[a] Abbreviations are: MI, milk (3.5–3.9% fat); CR, cream (35% fat); YOG, yoghurt (3.5–4% fat): BM, buttermilk (0.5% fat); Q, quark (0.1% fat); COTC, cottage cheese (4–4.5% fat). Values are in g 100 g^{-1} total protein; mean values and standard deviations for every group of dairy products were determined for three products from different manufacturers, each in technical triplicates, measured thrice. Protein abbreviations are listed in Table 1.

35% fat, YOG 3.5–4% fat, BM 0.5% fat, Q 0.1% fat, and COTC 4–4.5% fat, each product obtained from three different manufacturers. The average total amount of CasMPs, WPs and MFGMPs was calculated by the sum of the individual proteins belonging to these protein classes and plotted for each type of product (Fig. 2a). The deviations and the fact that total protein amounts that were below the expected 100% can be explained by the different origin of the products, and are therefore no measure for the quality of the experiments. A representative example for the individual protein pattern of each dairy product is shown in the SDS-PAGE in Fig. 2b.

3.3.3. Comparison of the protein concentrations found in raw and commercial heat-treated dairy products

A one-to-one comparison between the determined protein concentrations in the raw milk products and those found for the heat-treated dairy products might be tempting, but is not fully appropriate, since the investigated products are purchased at different times from different suppliers (Supplementary material, Table S4). Hence, the milk used for these products originates from different animals, possibly of different breeds, which were most likely at different stages of the lactation cycle and receiving different feeds with varying energy levels and crude protein content. Moreover, the milk was processed by different manufacturers and has thus undergone a variety of different processing steps besides heat-treatment. Nevertheless, taking these restrictions into account, it is interesting to consider a brief and cautious comparison of the measured values.

Remarkably, in comparison with the RM and the raw milk dairy products, the content of the twenty proteins found in the heat-treated dairy products were lower, with some exceptions. As indicated before, the most obvious explanation are differences in the concentration of these proteins in the milk of origin and that processing and storage might affect protein conformation and stability and possibly lead to protein degradation (Deeth & Lewis, 2017; Garcia-Risco, Ramos, & López-Fandiño, 2002). Another explanation is the potential emergence of glycated lysyl residues due to the presence of reducing carbohydrates during heat-treatment and storage as a first step of the Maillard reaction (Metha & Deeth, 2015). As a result, peptide bonds might be blocked for cleavage by trypsin, therefore preventing a precise quantification by MRM, leading to a substantial decrease in concentration of individual proteins in dairy products that have undergone a more severe thermal treatment. However, the determined quantities for the individual proteins in pasteurised milk compared with high-pasteurised milk as well as in high-pasteurised cream, compared with UHT cream, do not support this hypothesis (Supplementary material, Fig. S5). Furthermore, the observed reduction in quantity was not higher for the proteins when the selected signature peptides ended with a c-terminal lysyl residue compared with the ones with a c-terminal arginyl residue (Table 1). Nevertheless, the heat-treated dairy products of one kind shared a similar protein pattern, while the concentration of specific proteins varied in the products between the different manufacturers (Supplementary material, Fig. S5). Since RM is used as a starting material in the manufacturing process for all of these dairy products, the amount of individual proteins in the RM could be decisive for the quality of the product.

Casein micelle proteins. In the four raw milk products analysed, RM, RC, EM and W, amounts of the individual CasMPs were in accordance with previous results (Tables 3 and 4). In RC, CasMPs had a lower concentration but the same ratio compared with RM. In EM however, the ratio of CasMPs was different from RM. CASA1, CASB and CASK were higher than CASA2 and LPL. LPL, which is loosely attached to the casein micelles, is most probably partially released into whey during cheese manufacturing (Table 2). In the case of CASA2, possible hydrolysis of the indicator peptide by bacteria during cheese ripening might explain the finding. As expected, in W, only traces of CasMPs were found. Surprisingly, with the exception of the COTC samples, the total amount of CasMPs in heat-treated dairy products was lower than that in the RM, possibly due to a lower concentration of CasMPs in the milk of origin and the

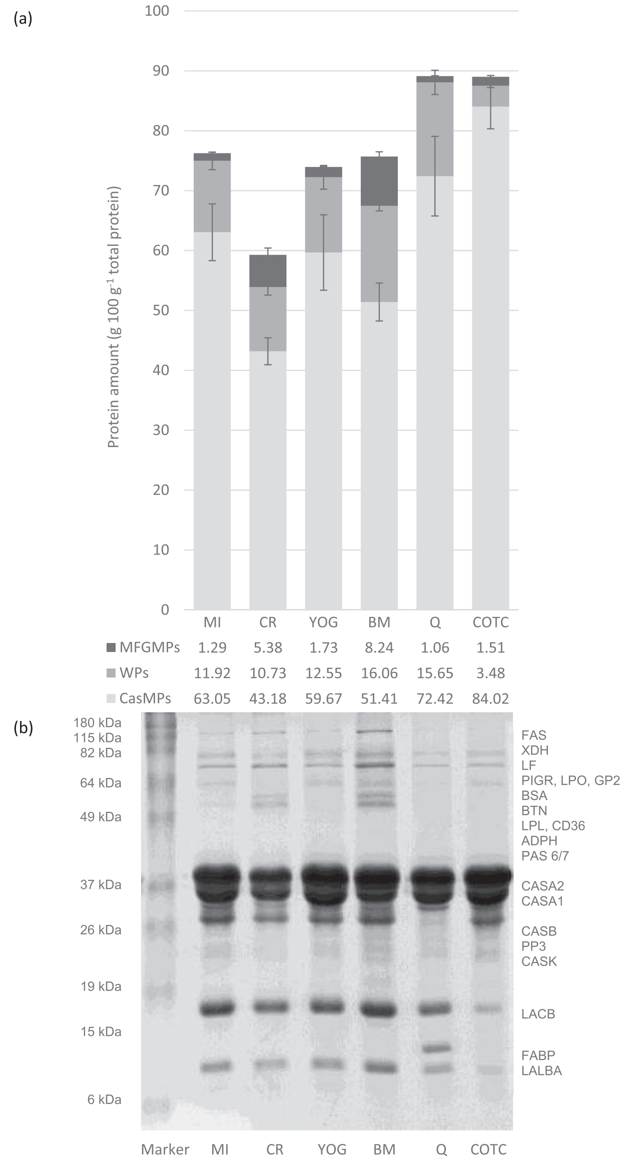

Fig. 2. Panel a: average amount (g 100 g^{-1} total protein) of casein micelle proteins (CasMPs), whey proteins (WPs) and milk fat globule membrane associated proteins (MFGMPs) in heat-treated milk (MI), cream (CR), yoghurt (YOG), buttermilk (BM), quark (Q) and cottage cheese (COTC) determined by multiple reaction monitoring. Mean values and standard deviations for every group of dairy products were determined for three products from different manufacturers, each in technical triplicates, measured thrice. Panel b: SDS-PAGE showing a representative protein pattern for each type of dairy product. For protein abbreviations, see Table 1.

degradation during heating, processing and storage (Meltretter, Schmidt, Humeny, Becker, & Pischetsrieder, 2008). In fermented dairy products such as BM, YOG and Q, a possible hydrolysis of the indicator peptides by the added starter cultures could also contribute to the lower protein concentrations. The quantities of the individual CasMPs in the heat-treated samples did not follow a clear pattern that could be explained by the technological transformations and no clear tendency could be deduced.

Whey proteins. In line with expectations, the highest concentration of all serum proteins was found in W, while most of them were absent in the EM (Table 2). PP3 were found to be slightly more concentrated in RC than in RM and MI (Table 4), thereby suggesting that it might be partially associated with the MFGM (Table 2), as previously described by Dewettinck et al. (2008). This is also supported by the results for PP3 in CR and BM and the observed low concentration in Q (Table 4). Remarkably, in the experiments reported here, LF behaved neither as a real WP nor a real MFGMP, since it was found in similar concentrations in all four milk fractions (RM, RC, EM and W) and was only slightly increased in BM and YOG. In the literature, the localisation of LF is controversially discussed: some publications associate LF with the MFGM and some with the milk serum fraction (Casado et al., 2009). LPO is present in all investigated products at similar levels with a higher concentration in W. Its activity is used as an indicator for high temperature treatment, for example, for ultra-pasteurisation (Fox & Kelly, 2006). With the exception of W, BSA levels detected in raw milk products were below the expected values reported from literature (Table 3). In contrast to that, the values found in heat-treated samples (MI, BM, CR, YOG and Q) were higher and matched the expected values. As mentioned before, the reason for this finding might lie in the tight folding of the protein or a possible interaction of the protein with lipids or other proteins present in raw milk, thereby interfering with the precipitation and hydrolysis by trypsin.

Milk fat globule membrane proteins. During butter manufacture, an important part of the MFGM is released into the serum phase. Therefore, it is not surprising that BM contains the highest concentration of MFGMPs of all investigated dairy products (Table 4, Fig. 2). As the higher fat content of cream suggests, MFGMPs were also found in high concentrations in RC and CR (35%) (Fig. 1a, Table 2 and Fig. 2a, Table 4, respectively). However, thus far, only a few methods were available for the quantification of MFGMPs and thus the quantity of the individual MFGMPs in different milk products is mostly unknown in literature.

In the products investigated here, the most prominent MFGMPs were BTN, XDH and PAS 6/7. BTN is a protein involved in the formation and stabilisation of the MFGM (Robenek et al., 2006) and had highest levels in RC and BM. However, XDH, a protein that amplifies the antibacterial effect of LPO, and PAS 6/7, which is known to have anti-infectious properties (Fox & Kelly, 2006; Mather, 2000), were most prominent in BM and CR. Moreover, BTN and XDH varied the most in the different CR samples. A possible reason for this variance might be the fact that BTN and XDH form a high molecular weight aggregate induced by heat-treatment (Ye, Singh, Taylor, & Anema, 2002). The content of PAS 6/7 differed the most between the BM samples of different manufacturers (Supplementary material, Fig. S5). Since, during the process of homogenisation or butter making, the MFGMs are destroyed and not entirely rearranged around the newly formed droplets, the dissolved proteins transferred to the serum are more vulnerable to degradation by enzymes, microorganisms and heat. This indicates that the observed high variance of MFGMPs in commercial heat-treated products might not only be attributed to different manufacturers and milk origin. Remarkably, the concentration of CD36, FABP, FAS and GP2 was comparatively high in W (Table 2). As these are non-transmembranous MFGMPs, they might have been liberated in the W during the manufacture of cheese.

4. Conclusions

In the last decade, more and more nutritional- and health-related aspects of milk proteins have been discovered (Supplementary material, Table S1), making it more relevant for research and for dairy manufacturers to define dairy products as detailed as possible to satisfy the increasing awareness of the consumer for health and nutrition. While methods for relative quantification of major milk proteins date back to 1944 (Warner, 1994), methods for quantification of the minor milk proteins are rare.

The LC-MS method presented in this study provides a simultaneous absolute quantification of twenty bovine milk proteins without elaborate or time-consuming sample preparations. The selected proteins belong to all three main milk protein classes, CasMPs, WPs, and in particular MFGMPs, for which a quantification method was lacking so far. The MRM results for RM are in good accordance with literature data. Unfortunately, the precise quantification of BSA was not yet satisfying in RM and needs to be further investigated, possibly by adding a heat-treatment during sample preparation leading to protein unfolding and reshuffling of the disulphide bridges, as the BSA concentrations in heat-treated milk samples were within the expected range (Table 4).

The method achieves a good precision in RM for proteins present at higher concentrations (CVs of 5–15%; Supplementary material, Table S2). For the minor MFGMPs (ADPH, PAS 6/7, CD36, PIGR), the results obtained showed higher CVs (up to 33%) due to the low individual protein concentration in milk, being close to the quantitation limit. The accuracy of the method was checked by recovery experiments and ranged between 91% and 105%. In the future, the method can be applied for screening purposes assessing the individual protein content of milk produced under different feeding regimes, at different lactation stages or between different breeds. Moreover, the method is suited for the quantification of specific milk proteins in the whole range of different dairy products and can as such be used for the evaluation of bioactive effects, since most known bioactive peptides are embedded in the sequence of major milk proteins. Therefore, the presented straightforward quantification method is a valuable and convenient tool for the simultaneous quantification of the minor and most abundant milk proteins, and will contribute to a better understanding of the impact of protein composition on the technological and nutritional quality of milk and dairy products.

Acknowledgements

We thank Michael Sutter (Bern University of Applied Sciences, School of Agricultural, Forest and Food Sciences HAFL, Switzerland) for collecting the raw milk samples, Urs Kaempfer's group (University of Bern, Switzerland) for AA analysis of the isotopically labelled AQUA peptides, and Beat Reidy (HAFL) for the collaboration, IP-Suisse, the Commission for Technology and Innovation and the Migros Cooperative Association we thank for the joint funding of the research in this article (Grant number CTI 15703.2 PFLS-LS).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.01.001.

References

Affolter, M., Grass, L., Vanrobaeys, F., Casado, B., & Kussmann, M. (2010). Qualitative and quantitative profiling of the bovine milk fat globule membrane proteome. *Journal of Proteomics, 73*, 1079–1088.

Bauman, D. E., Mather, I. H., Wall, R. J., & Lock, A. L. (2006). Major advances associated with the biosynthesis of milk. *Journal of Dairy Science, 89*, 1235–1243.

Brönstrup, M. (2004). Absolute quantification strategies in proteomics based on mass spectrometry. *Expert Review of Proteomics, 1*, 503–512.

Casado, B., Affolter, M., & Kussmann, M. (2009). OMICS-rooted studies of milk proteins, oligosaccharides and lipids. *Journal of Proteomics, 73*, 196–208.

Chevalier, F., Hirtz, C., Sommerer, N., & Kelly, A. L. (2009). Use of reducing/nonreducing two-dimensional electrophoresis for the study of disulfide-mediated interactions between proteins in raw and heated bovine milk. *Journal of Agricultural and Food Chemistry, 57*, 5948–5955.

Deeth, H. C., & Lewis, M. J. (2017). Changes during heat treatment of milk. In H. C. Deeth, & M. J. Lewis (Eds.), *High temperature processing of milk and milk products* (1st ed., pp. 177–260). Chichester, UK: John Wiley & Sons.

Dewettinck, K., Rombaut, R., Thienpont, N., Le, T. T., Messens, K., & Van Camp, J. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal, 18*, 436–457.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., et al. (2016). The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Research International, 88*, 217–225.

Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell, H. M., JR., Harwalkar, V. R., Jenness, R., et al. (1984). Nomenclature of proteins of cows' milk: 5th revision. *Journal of Dairy Science, 67*, 1599–1631.

Fong, B. Y., & Norris, C. S. (2009). Quantification of milk fat globule membrane proteins using selected reaction monitoring mass spectrometry. *Journal of Agricultural and Food Chemistry, 57*, 6021–6028.

Fox, P. F. (2011). Bovine milk. In J. W. Fuquay, P. F. Fox, & P. L. H. McSweeney (Eds.), *Encyclopaedia of dairy sciences* (2nd ed., pp. 478–483). Amsterdam, the Netherlands: Elsevier Science.

Fox, P. F., & Kelly, A. L. (2006). Indigenous enzymes in milk: Overview and historical aspects. Part 1. *International Dairy Journal, 16*, 500–516.

Garcıa-Risco, M. R., Ramos, M., & López-Fandiño, R. (2002). Modifications in milk proteins induced by heat treatment and homogenization and their influence on susceptibility to proteolysis. *International Dairy Journal, 12*, 679–688.

ISO. (2007). *Milk – determination of nitrogen content – Part 3: Block digestion method (semi-micro rapid routine method). ISO 8968-3:2007/IDF 20-3:2007.* Geneva, Switzerland: International Standardisation Organisation.

Kirkpatrick, D. S., Gerber, S. A., & Gygi, S. P. (2005). The absolute quantification strategy: A general procedure for the quantification of proteins and post-translational modifications. *Methods, 35*, 265–273.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2012). Validation of an in vitro digestive system for studying macronutrient decomposition in humans. *Journal of Nutrition, 142*, 245–250.

Kuczynska, B., Puppel, K., Golebiewski, M., Metera, E., Sakowski, T., & Sloniewski, K. (2012). Differences in whey protein content between cow's milk collected in late pasture and early indoor feeding season from conventional and organic farms in Poland. *Journal of the Science of Food and Agriculture, 92*, 2899–2904.

Lange, V., Picotti, P., Domon, B., & Aebersold, R. (2008). Selected reaction monitoring for quantitative proteomics: A tutorial. *Molecular Systems Biology, 4*, 222.

Le, T. T., Deeth, H. C., & Larsen, L. B. (2017). Proteomics of major bovine milk proteins: Novel insights. *International Dairy Journal, 67*, 2–15.

Lu, J. (2013). *The biology of milk synthesis from a proteomics perspective* (PhD Thesis). Wageningen, the Netherlands: Wageningen University.

Lutter, P., Parisod, V., & Weymuth, H. (2011). Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. *Journal of the Association of Official Agricultural Chemists, 94*, 1043–1059.

Mather, I. H. (2000). A review and proposed nomenclature for major proteins of the milk-fat globule membrane. *Journal of Dairy Science, 83*, 203–247.

McMahon, D. J., & Oommen, B. S. (2013). Casein micelle structure, functions, and interactions. In P. L. H. McSweeney, & P. F. Fox (Eds.), *Advanced dairy chemistry – proteins: Basic aspects* (4th ed., Vol. 1A, pp. 185–209). Boston, MA, USA: Springer.

Meltretter, J., Schmidt, A., Humeny, A., Becker, C. M., & Pischetsrieder, M. (2008). Analysis of the peptide profile of milk and its changes during thermal treatment and storage. *Journal of Agricultural and Food Chemistry, 56*, 2899–2906.

Metha, B. M., & Deeth, H. C. (2015). Blocked lysine in dairy products: Formation, occurrence, analysis, and nutritional implications. *Comprehensive Reviews in Food Science and Food Safety, 15*, 206–218.

Robenek, H., Hofnagel, O., Buers, I., Lorkowski, S., Schnoor, M., Robenek, M. J., et al. (2006). Butyrophilin controls milk fat globule secretion. *Proceedings of the National Academy of Sciences, 103*, 10385–10390.

Schwendel, B. H., Wester, T. J., Morel, P. C. H., Fong, B., Tavendale, M. H., Deadman, C., et al. (2017). Pasture feeding conventional cows removes differences between organic and conventionally produced milk. *Food Chemistry, 229*, 805–813.

Spector, A. A., John, K., & Fletcher, J. E. (1969). Binding of long-chain fatty acids to bovine serum albumin. *Journal of Lipid Research, 10*, 56–67.

Swaisgood, H. E. (1993). Review and update of casein chemistry. *Journal of Dairy Science, 76*, 3054–3061.

Swaisgood, H. E. (1995). Nitrogenous components of milk. F. Protein and amino acid composition of bovine milk. In R. G. Jensen (Ed.), *Handbook of milk composition. (Food science and technology series)* (1st ed., pp. 464–467). San Diego, CA, USA: Academic Press.

Tremblay, L., Laporte, M. F., Léonil, J., Dupont, D., & Paquin, P. (2003). Quantitation of proteins in milk and milk products. In P. L. H. McSweeney, & P. F. Fox (Eds.), *Advanced dairy chemistry, Vol. 1. Proteins* (3rd ed., pp. 49–138). Boston, MA, USA: Springer.

Turner, S.-A., MacDonald, K. A., Back, P. J., & Thomson, N. A. (2006). Effect of cow genotype and feed allowance on milk composition. *Proceedings of the New Zealand Society of Animal Production, 66*, 397–402.

Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006). Milk components. Proteins. In *Dairy science and technology* (2nd ed., pp. 63–108). Boca Raton, FL, USA: CRC Press.

Warner, R. C. (1944). Separation of α- and β-casein. *Journal of the American Chemical Society, 66*, 1725–1731.

Yang, Z., Hayes, M., Fang, X., Daley, M. P., Ettenberg, S., & Tse, F. L. S. (2007). LC–MS/MS Approach for quantification of therapeutic proteins in plasma using a protein internal standard and 2D-solid-phase extraction cleanup. *Analytical Chemistry, 79*, 9294–9301.

Ye, A., Singh, H., Taylor, M. W., & Anema, S. (2002). Characterization of protein components of natural and heat treated milk fat globule membranes. *International Dairy Journal, 12*, 393–402.