Protein fingerprinting and quantification of β -casein variants by ultra-performance liquid chromatography–high-resolution mass spectrometry

C. Fuerer,*¹ R. Jenni, L. Cardinaux,² F. Andetsion, S. Wagnière, J. Moulin, and M. Affolter¹

Société des Produits Nestlé SA, Nestlé Research, Route du Jorat 57, 1000 Lausanne 26, Switzerland

ABSTRACT

Infant formulations are constantly evolving as novel protein ingredients are added to make them more closely mimic the protein profile of human milk; however, precise analytical methods for characterizing and quantifying the major milk proteins in such formulations are currently lacking. This article describes an ultra-performance liquid chromatography–high-resolution mass spectrometry method for intact proteins that can efficiently detect, identify, and characterize the major milk proteins and their proteoforms (phosphorylation status, degree of glycation, genetic variants among others) in ingredients and final products, with an emphasis on detecting and quantifying specific genetic variants of β -casein in infant formulas. Method sensitivity allows detection of β -casein A1 in A2-based infant formulas with a limit of detection of 2% (grams of β -casein A1 per 100 g of total β -casein). Protein glycation affects signal intensity in a linear fashion, which permits proteins to be quantified from their mass spectrometry signals after correction according to their measured glycation index. The method was validated for the quantification of β -casein in infant formulas. Repeatability ranged from 2 to 3% and intermediate reproducibility from 5 to 9%. Calculated β -casein amounts ranged between 77 and 110% of the values based on formulations and published protein profiles for milk. Altogether, this method can be used for general fingerprinting as well as specific characterization and quantification of individual major milk proteins in dairy-based ingredients and products.

Key words: A2 β -casein, milk protein, proteoform, genetic variant, high-resolution mass spectrometry

INTRODUCTION

Genetic polymorphism and posttranslational modifications of the major milk proteins can affect the

physicochemical and biological properties of milk (reviewed by Ng-Kwai-Hang and Grosclaude, 2003; Caroli et al., 2009). Approximately 80% of cow milk proteins are made of caseins (α_{S1} -, α_{S2} -, β -, and κ -CN). Caseins are encoded by 4 genes (*CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3*) that are all located in a 250-kb spanning region of chromosome 6 and show high mutation rates. As a consequence, allele and haplotype frequencies can vary significantly between and within species and breeds, and detailed analyses have revealed a geographically associated haplotype distribution that can shed light on the evolution of the bovine casein locus (Jann et al., 2004). To date, more than a dozen genetic variants of β -CN have been described (Farrell et al., 2004); A1 and A2 are the 2 most common β -CN variants (Caroli et al., 2009; Gallinat et al., 2013). The A1 variant evolved from A2 through a mutation at position 67 (Grosclaude et al., 1972), and β -CN variants are often grouped into the A1 or A2 type based on the amino acid present at this position (His₆₇ in the A1 type and Pro₆₇ in the A2 type). The histidine residue in A1-type β -CN allows enzymatic release of the preceding 7 amino acid residues in the gastrointestinal tract to generate the β -casomorphin-7 (BCM-7) peptide (Jinsmaa and Yoshikawa, 1999). Release of this peptide is either prevented or greatly reduced from A2-type β -CN (Jinsmaa and Yoshikawa, 1999; Noni, 2008; Asledottir et al., 2018). Although BCM-7 was initially associated with several adverse effects, a review by the European Food Safety Authority (2009) concluded that “a cause-effect relationship between the oral intake of BCM7 or related peptides and etiology or course of any suggested non-communicable diseases cannot be established.” Recent studies have reported that consumption of A2 milk may be associated with attenuation of gastrointestinal symptoms of milk intolerance in self-reported lactose-intolerant individuals (Jianqin et al., 2016; He et al., 2017), but studies are lacking in healthy individuals (reviewed in Brooke-Taylor et al., 2017).

Cows can be genetically selected to be homozygous for β -CN A2 (A2A2 genotype) and thereby produce milk that only contains β -CN of the A2 type (A2 milk), and dairy products manufactured from A2 milk have

Received January 9, 2019.

Accepted September 25, 2019.

*Corresponding author: christophe.fuerer@rdls.nestle.com

been on the market for many years. Analytical methods are required to assess the integrity of ingredients and final products, but unlike genetic testing done at the individual cow level, they should directly probe the protein compartment to assess the distribution of genetic variants in final products. To our knowledge, no method is currently available for directly testing intact β -CN genetic variants in final products.

Intact protein analyses by reverse-phase (RP)-HPLC or capillary electrophoresis (CE) coupled to UV detection can effectively separate most β -CN variants in raw milk samples (e.g., de Jong et al., 1993; Visser et al., 1995; Bonfatti et al., 2008; Poulsen et al., 2016). In manufactured products such as skim milk powder, processing conditions may induce reactions between proteins and reducing sugars (Maillard reaction) that yield multiple proteoforms containing one or more sugar adducts (Fenaille et al., 2006). As a consequence, protein peaks split and broaden, creating overlaps that prevent the analysis of single proteoforms (Vallejo-Cordoba, 1997; Feng et al., 2017). Coupling RP-HPLC with MS could in principle overcome this limitation provided the different proteoforms can be sufficiently resolved by the mass spectrometer. A study by Givens et al. (2013) applied HPLC-MS to the analysis of pasteurized milks but did not study UHT or powder milks that can often contain more lactosylated proteoforms (Fenaille et al., 2006).

A1:A2 β -CN ratios were analyzed in raw milk and powder infant formulas by multiple reaction monitoring after digestion of casein precipitates by thermolysin (Duarte-Vazquez et al., 2017). Although A2 raw milk contained exclusively A2-type β -CN, β -CN A1 levels were surprisingly elevated in an infant formula prototype manufactured using A2 milk. The authors attributed this observation to the presence of other milk-derived ingredients used in the formulation. This observation was also reported in another study (Trujillo-Cárdenas et al., 2018). Indeed, proteolysis of β -CN by plasmin leads to the generation of γ -CN and proteose peptones (PP8 fast, PP8 slow, and PP-5). In contrast to γ -CN, proteose peptones do not coagulate with caseins during acidification or rennet treatment and remain in the whey fraction (reviewed in Karamoko et al., 2013). PP8 slow and PP-5 correspond to sequences 29 to 105/7 and 1 to 105/7, respectively, and both species include position 67. Thus, whey products derived from milk containing β -CN A1 contain proteose peptones of the A1 type that will be detected by peptide-directed analyses after digestion with proteolytic enzymes such as trypsin, thermolysin, and GluC. Consequently, peptide-based approaches are particularly problematic for the true detection of β -CN variants in final products.

In summary, no method currently exists for characterizing and quantifying individual milk proteins and their proteoforms in final products. Process-induced protein modifications cause peak splits, shifts, and overlaps that prevent the accurate results from established UV-based methods. Process-induced protein modifications also limit peptide-based approaches because lactosylation occurs on residues needed for trypsin proteolysis and prevents correct mass attribution. This article details a protein fingerprinting method based on ultra-performance liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) of intact proteins and its application to the analysis and quantification of β -CN genetic variants in infant formulas.

MATERIALS AND METHODS

Chemical Reagents and Samples

Guanidine hydrochloride (RDD001), trisodium citrate dihydrate (S1804), DL-dithiothreitol (DTT, 43819), and β -CN standard (C6905) were purchased from Sigma-Aldrich (St. Louis, MO), trifluoroacetic acid (TFA, 1.08178.0050) from VWR International (Radnor, PA), and LC-MS grade water (1.15333.1000) and acetonitrile (1.00029.1000) from Merck (Darmstadt, Germany).

Milk Samples

Raw milk was collected from the Teagasc Moorepark dairy farm (Fermoy, Co. Cork, Ireland) from cows that were genetically profiled as A1/A1. The milk was defatted and pasteurized using a Microthermics (UHT/HTST Electric Model 25HV Hybrid, Liquid Technologies, Wexford, Ireland) unit heated to 85°C and held for 23 s, followed by homogenization (GEA Niro Soavi S.p.A. Type: NS2006H [non-aseptic]) using 2-stage homogenization at a total homogenization pressure of 17.2 MPa (2,500 psi). The sample was spray dried to produce a skim milk powder (SMP) sample referred to as A1 SMP. Standard SMP (containing a mixture of A1- and A2-type β -CN), A2 SMP (containing only β -CN of the A2 type), and buffalo SMP were purchased commercially. Protein was quantified by the Kjeldahl method using a nitrogen conversion factor of 6.38. β -Casein genetic profiles were confirmed by the method described herein for A1 and A2 milk samples. Pasteurized breastmilk from pooled human donors (991-01-P) was purchased from Lee Biosolutions (Maryland Heights, MO). Infant formula powders suitable for infants (0–12 mo) and children (≥ 1 yr) were prototype formulas manufactured in the pilot plant or purchased commercially. Their type (source of β -CN),

protein content, casein content, and β -CN content are summarized in Table 1.

Sample Preparation

Powders were dispersed to 3.5% (wt/vol) protein in water using a volumetric flask, stirred for at least 30 min at room temperature, denatured with 4 volumes of denaturing buffer (guanidine HCl 7.5 M, trisodium citrate 6.25 mM, DTT 23 mM), incubated at room temperature for 30 min, and cleared by centrifugation at $16,000 \times g$ for 10 min.

UPLC-HRMS Analysis

Cleared samples were analyzed by UPLC-HRMS based on existing methods (Bonfatti et al., 2008; Frederiksen et al., 2011). Samples were separated on a C4 column (Acquity UPLC Protein BEH C4, 300 Å, 1.7 μ m, 2.1 mm $\times$ 150 mm; Waters Corporation, Milford, MA) using a mobile phase composed of buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile/water at 90:10) and the following gradient at 0.5 mL/min: 15, 35, 42.5, 52.5, 80, 80, 15, and 15% buffer B at 0, 4, 7, 22, 23, 24, 25, and 30 min, respectively. The MS

signal was recorded from 4 to 20 min in full MS mode on a Thermo Q-Exactive HF (ThermoFisher Scientific, Waltham, MA; heater temperature, 100°C; sheath gas, 53; auxiliary gas, 14; sweep gas, 3; spray voltage, 3.5 kV; capillary temperature, 320°C; S-lens radiofrequency level (arbitrary units), 70; mass range, 400–2,000 m/z ; resolution, 240,000; automatic gain control target, 1e6; maximum injection time, 200 ms).

Data Deconvolution with Sliding Windows

Raw data files were deconvolved using Thermo BioPharma Finder 1.0 or 3.0 software (Thermo Fisher Scientific) using the Xtract and sliding windows algorithms. The Xtract algorithm is performed on full MS spectra. It first estimates the mass of a component based on the m/z values and isotopic spacing of a peak cluster. Then, it fits an average peak distribution (Senko et al., 1995) to the observed peak cluster to determine the monoisotopic mass that best matches its profile. Finally, the algorithm combines the results of all observed charge states into a single mass value for each component. The deconvolved spectra contain the monoisotopic masses of all components identified in the source spectra.

In the sliding windows algorithm, the deconvolution is performed on time-averaged spectra that are sequentially generated along the separation time scale. The width of the time window (for averaging) and the offset between 2 time windows were optimized using infant formula samples. Deconvolved spectra are then sequentially compared along the mass axis to merge signals that belong to the same component (according to a defined tolerance interval). A minimal amount of detection events [i.e., sliding windows along the retention time (RT) axis] can also be defined to reduce noise.

The Xtract deconvolution algorithm parameters were as follows: S/N threshold, 3; relative abundance threshold, 1%; fit factor, 80%; remainder threshold, 25%; overlaps, charge states, 5 to 50; minimum intensity, 1; expected intensity error, 3; m/z , 600 to 2000; and minimum number of detected charge states, 3.

The sliding windows algorithm parameters were as follows: time, 5 to 20 min; target average spectrum width, 0.3 min; target average spectrum offset, 33%; merge tolerance, 1.5 Da; maximum RT gap, 0.5 min; minimum number of detected intervals, 3; and extracted ion chromatogram, selected.

The deconvolution process generates a list of components that includes their monoisotopic mass, total signal intensity, and apex RT. Results are exported as a .csv file and used for data visualization and proteoform attribution.

Table 1. Infant formula (IF) powders included in this study

ID	Type	Protein (%)	Casein (%) ¹	β -CN ² (%)
IF1	A2	10.2	35	1.19
IF2	A2	15.3	60	3.06
IF3	A2	13.9	60	2.78
IF4	A2	17.0	60	3.40
IF5	A2	10.4	30	1.04
IF6	A2	15.0	50	2.50
IF7	A2	15.0	60	3.00
IF8	A1/A2	10.6	40	1.41
IF9	A1/A2	14.9	40 ³	1.99
IF10	A1/A2	15.3	70 ³	3.57
IF11	A1/A2	22.2	78	5.77
IF12	A1/A2	11.0	40	1.47
IF13	A1/A2	10.2	35	1.25
IF14	A1/A2	15.3	60	3.02
IF15	A1/A2	15.0	60	3.07
IF16	A1/A2	17.0	60	3.48
IF17	A2	9.9	30	1.01
IF18	A2	10.4	30	1.03
IF19	A2	12.8	50	1.94
IF20	A2	10.1	35	1.32
IF21	A2	11.7	60	2.41
IF22	A2	15.2	60	3.12

¹As indicated on the label, unless otherwise noted.

²Calculated by multiplying the protein content by the total casein content and then by the β -CN content (as stated in Swaisgood, 1995).

³Based on product composition and the order of ingredients on the label.

Data Visualization and Proteoform Attribution

The .csv files were visualized as bubble charts using a software developed in our research group (Protein Analyzer). All components (proteoforms) were plotted on a scatter plot according to their RT (x -axis), monoisotopic mass (y -axis), and signal intensity (bubble area). Signals less than 2 Da and 1 min apart were merged. For proteoform attribution, monoisotopic masses were first matched to a database containing the major milk proteins [α_{S1} -CN, α_{S2} -CN, β -CN, κ -CN, γ -CN, α -LA, β -LG, and casein glycomacropeptide (CGMP)]; see Supplemental Table S1, <https://doi.org/10.3168/jds.2019-16273>] using a tolerance of 1.8 Da. This step took basal modifications such as the 5 phosphate groups of β -CN into consideration. Once a proteoform was identified, various standard modifications (in particular phosphorylation, oxidation, lactosylation, and glycosylation) were tested to assess whether a signal corresponding to the newly calculated monoisotopic mass was found after deconvolution. If such a signal was found, and if the proposed modification had the expected effect on the elution (i.e., RT are decreased by lactosylation and oxidation but increased by phosphorylation), the signal was attributed to the new proteoform, and the process was repeated until the majority of signals were identified. Automatically annotated signals were reviewed, and misattributed signals were corrected manually (such verification is based on RT and proximity with the related proteoforms). Total signal intensities were extracted for the relevant proteins or proteoforms (i.e., total β -CN signal and total signal for each lactosylation state for each genetic variant). For clarity, only β -CN genetic variants A1, A2, and B are detailed in the figures. Because β -CN I has the same mass as dehydrated A2 and co-elutes with β -CN A2, it was merged with β -CN A2. Both variants have a proline at position 67 and belong to the A2 type.

Solid-State Protein Glycation

The solid-state glycation experiment was based on Fenaille et al. (2003). Briefly, 45 g of milk powder was incubated in an enclosure saturated with dipotassium carbonate for 8 to 10 d to reach 5.4% humidity (initial value was 3.8%). Nine 2.1-g aliquots of the humidified powder were incubated in 25-mL glass tubes at 60°C in an oven for 45 min to 24 h. Glycation was stopped by transferring the tubes to ice for 5 min. Then, 20 g of water was added to obtain a 3.5% (wt/vol) protein solution, which was mixed at room temperature for 1 h 30 min on a roller mixer. Solutions were stored at 4°C overnight until the end of the experiment and warmed to room temperature for 2 h before analysis. Sample

preparation was carried out as described above, except that the incubation step was 10 min at 60°C on a thermomixer (650 rpm). Mass spectrometry chromatograms were recorded on a Thermo Orbitrap Elite (heater temperature, 60°C; sheath gas, 20; auxiliary gas, 5; sweep gas, 0; spray voltage, 3.8 kV; capillary temperature, 320°C; S-lens radiofrequency level, 60; mass range, 400 to 2,000 m/z ; resolution, 240,000; AGC target, 1e6).

β -Casein Quantification

An SMP quality control (SMP_{QC}) sample was injected multiple times throughout each analytical series to normalize the signals across experiments and assess instrument performance (series were reanalyzed if SMP_{QC} normalized total ion current levels dropped below 5E8; this threshold was established based on SMP_{QC} data collected over several months on the same instrument).

An external calibration curve was established using a β -CN standard adjusted for protein purity (86%, see results section). β -Casein signal intensities were normalized with the average β -CN signal of the SMP_{QC} sample (corrected for glycation, see results section for details). This normalization allows a direct comparison between independent experiments without the need to establish a standard curve for each series. The calibration curve was created using 4 independent series and forced to 0 ($y = 0.0553x$, where x is expressed in micrograms of β -CN injected and y is dimensionless).

For each unknown sample, β -CN intensity values were first corrected for glycation and normalized with the average β -CN signal of the co-injected SMP_{QC} samples (corrected for glycation). Then, the amount of injected β -CN was calculated through the external calibration curve. This calculation can be done for total β -CN, a given variant, or a set of variants (β -CN A2 results also include the related I variant).

Method performance was evaluated in 7 infant formula samples manufactured using A2 SMP (IF1–7). Analyses were performed by 3 operators on 9 different days in triplicate.

Limit of Detection

Spiking experiments were performed by 3 operators for 7 infant formula samples (IF1–7), using single injections on 5 different days (10 for unspiked samples). Dispersed A1 skim milk powder was spiked into dispersed A2 infant formulas to a final concentration of 0, 1, 2, or 3% β -CN A1 (relative to total β -CN). Samples (including blank spike) were analyzed and deconvolved as described above.

RESULTS

UPLC-HRMS Method, Data Deconvolution, and Visualization

To analyze intact proteins, infant formulas and SMP samples were dispersed in water to 3.5% (wt/vol) protein, denatured in 6 *M* guanidine, reduced with DTT, and cleared by centrifugation. Intact proteins were separated by UPLC on a C4 column using a water/acetonitrile gradient with 0.1% TFA and analyzed by high-resolution MS (Figure 1A). The UV and total ion current profiles of skim milk are shown in Figure 1B as an example, with β -CN eluting between 10.5 and 12.5 min. The β -CN signal was distributed among several charge states (here from MH13+ to MH30+, Figure 1C), and each charge state contained all β -CN proteoforms (genetic variants, lactose adducts, etc.; Figure 1D). The β -CN signals were isotopically resolved and centered around the most abundant peak (1,143.05 *m/z* for the MH21+ peak of β -CN A2, Figure 1E, crossed circle), which corresponded to the average mass of the protein at 23,983 Da. The monoisotopic signal (circle and arrow) represented less than 0.001% of all isotopes and was therefore not directly detectable. Chromatograms were sequentially deconvolved using the Xtract and sliding windows algorithms to obtain the monoisotopic masses of the proteoforms (Figure 1F, detailed in Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-16273>). Finally, proteoforms were visualized on bubble charts, with their RT on the *x*-axis, monoisotopic size on the *y*-axis, and signal intensity as bubble area (Figure 1G).

Proteoform identification was performed by first matching the measured monoisotopic masses against a small database of the major secreted milk proteins and their constitutive modifications (for instance, the 5 phosphate groups of β -CN; see Supplemental Table S1, <https://doi.org/10.3168/jds.2019-16273>). Once a proteoform was identified, various standard modifications (in particular, phosphorylation, oxidation, lactosylation, and glycosylation) were tested to assess whether a signal corresponding to the newly calculated monoisotopic mass was present. If so, the signal was attributed to the new proteoform, and the process was repeated until the majority of signals were identified. Proteoform attributions were further refined manually if necessary.

Signals of all proteoforms for a given protein can be summed to calculate the total protein signal. Alternatively, the signal can be split according to the genetic variant or to a particular modification such as the number of lactose adducts. The percentage of unglycated

protein signal is given by the summed signals of all the nonglycated proteoforms over the total signal of the protein.

Global Fingerprinting

Figure 2A shows a typical bubble chart for cow raw milk with the major milk proteins (α_{S1} -, α_{S2} -, β -, κ -, and γ -CN; α -LA; and β -LG) highlighted. Zooming into the β -CN region (Figure 2B) allows detailed analysis of the various β -CN proteoforms, including genetic variants and protein modifications (process-induced lactosylated adducts were detectable in all SMP samples). Mass accuracy is shown in Table 2 for the raw milk sample. A 1-Da difference may be due to a misattributed monoisotopic signal propagated throughout the deconvolution because a merging tolerance of 1.5 Da was applied between the sliding windows to avoid splitting signals into 2 masses that were 1 Da apart. The method could be used to profile a wide variety of ingredients and final products (Supplemental Figure S2; <https://doi.org/10.3168/jds.2019-16273>). Interestingly, this method readily detected CGMP, the C-terminus part of κ -CN released by rennet enzyme during the cheesemaking process. This polypeptide is notoriously challenging to detect by gel staining or UV profiling.

Detection of β -Casein A1 in β -Casein A2 Infant Formulas

Seven infant formula samples manufactured using A2 SMP (IF1–7) were spiked with A1 SMP to 2% β -CN A1 (2 g of β -CN A1 in 100 g of total β -CN). At this concentration, the β -CN A1 signal was detected in all cases (Figure 3). The limit of detection was validated using spikes at 1, 2, and 3% β -CN A1 on 5 d using single injections. One formula (IF4) scored positive in some unspiked samples, suggesting that it contained residual β -CN A1 close to the limit of detection. Excluding IF4, β -CN A1 was detected in all cases when infant formulas were spiked at 2% or more β -CN A1. Lower β -CN A1 levels were only detected in 10% of the cases (Table 3; details in Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16273>).

Altogether, these results show that the method has a limit of detection of 2% β -CN A1 with a false-negative rate and a false-positive rate below 5%. The method does not reliably detect 1% β -CN A1.

Glycation and Protein Quantification

Protein quantification not only requires a suitable standard to establish a calibration curve but also simi-

Figure 1. Description of the ultra-performance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) method steps: (A) sample preparation; (B) MS (total ion current) and UV chromatograms; (C) spectrum of the β -CN region (10.5–12.0 min, shown as a red interval in B); (D) detail of the MH21+ peak series (red interval in C); the genetic variants A1, A2, and B and the number of lactose adducts (L, 2L) are specified; (E) detail of the β -CN A2 MH21+ peak (red interval in D) showing the average mass (crossed circle), with the monoisotopic mass (circle and arrow) situated at 1,142.3 m/z ; (F) deconvoluted spectrum showing monoisotopic masses and relative summed intensities; and (G) bubble chart representation of the β -CN proteoforms (bubble areas represent summed signal intensities). DTT = DL-dithiothreitol; ACN = acetonitrile; TFA = trifluoroacetic acid.

Figure 2. (A) Bubble chart of a raw milk sample for global fingerprinting. Major milk proteins are well separated according to their retention time (RT) and mass. (B) Bubble chart of the β -CN region showing different proteoforms for β -CN; A1, A2, and B are genetic variants; superscript notations correspond to the number of lactose (L) adducts (L, 2L). SMP = skim milk powder.

lar signal responses from different proteoforms. Such similarity is necessary to apply the calibration curve to unknown samples given that the standard and the samples are likely to have different proteoform distributions. Alternatively, the method should provide a way to correct for the proteoform distribution.

Table 2. Mass accuracy in cow raw milk sample

Item	Expected (Da)	Detected (Da)	Δ Mass (Da)	Relative Δ Mass ¹ (ppm)
β -CN A1 (5P)	24,008.16	24,007.00	-1.16	-48.51
β -CN A2 (5P)	23,968.15	23,967.10	-1.06	-44.09
α _{S1} -CN (8P)	23,600.21	23,599.64	-0.57	-24.18
α _{S2} -CN (11P)	25,212.98	25,212.12	-0.86	-34.17
α -LA	14,176.81	14,176.87	0.06	4.44
β -LG A	18,355.46	18,355.54	0.08	4.09
β -LG B	18,269.42	18,269.49	0.07	3.78
κ -CN A	19,025.53	19,025.62	0.09	4.85
κ -CN B	18,993.58	18,993.73	0.15	7.86
γ ₂ -CN	11,816.25	11,816.25	0.00	-0.42
γ ₃ -CN	11,551.10	11,551.13	0.03	2.60

¹Delta mass relative to total protein mass; calculated as Δ Mass/Expected $\times 10^6$.

Lactose adducts are inevitably found in powder dairy samples. These adducts are often a consequence of thermal processes such as spray drying that are essential for prolonging the shelf life of a perishable product for use in food formulations. Spray drying conditions may favor early Maillard reactions. Lactose adducts occur mostly on lysine residues and could potentially affect protein ionization and detection. To explore whether protein lactosylation influences MS signal intensity, glycation was induced in an SMP sample as outlined in the materials and methods section. Samples were collected at different time points for analysis (Figure 4A), and the protein glycation profile of each major protein was established. The increase in glycated β -CN (Figure 4B) was accompanied by a decrease in the total β -CN signal (Figure 4C). Plotting the total β -CN signal against the percentage of unglycated β -CN signal revealed a linear relationship (Figure 4D) that was converted into a fold signal reduction (Figure 4E) of the form $y = 1/(ax + b)$, with a and b corresponding to 0.606 and 0.394, respectively. The fold signal reduction was calculated against 100% unglycated β -CN signal.

Figure 3. Bubble charts (β -CN region) of 7 A2 infant formula (IF) samples either untreated (blank) or spiked at 2% β -CN A1 (2 g of β -CN A1 per 100 g of total β -CN) with A1 skim milk powder. Signals attributed to β -CN A1 are shown in black with arrowheads. RT = retention time.

For β -CN, a glycation correction factor (**GCF**) can thus be calculated from the percentage of unglycated β -CN signal (%UG $_{\beta}$) using Equation [1]:

$$\text{GCF} = \frac{1}{0.606 \times \% \text{UG}_{\beta} + 0.394} \quad [1]$$

Multiplying the measured β -CN signal ($\beta\text{CN}_{\text{meas}}$) by the GCF yields a corrected β -CN signal ($\beta\text{CN}_{\text{corr}}$),

which corresponds to the expected signal if all β -CN was unglycated (Equation [2]):

$$\beta\text{CN}_{\text{corr}} = \beta\text{CN}_{\text{meas}} \times \text{GCF} \quad [2]$$

This signal reduction phenomenon was observed for all other milk proteins studied (Supplemental Figure S3; <https://doi.org/10.3168/jds.2019-16273>). At higher glycation rates, the relationship between the total sig-

Table 3. Method performance for the detection of low-abundance β -CN A1 in A2 infant formulas

Item	0% A1	1% A1	2% A1	3% A1
Total samples	60	30	30	30
False negatives	—	27	0	0
False positives	1	—	—	—
True positives	—	3	30	30
True negatives	59	—	—	—
False-negative rate (%)	—	90	0	0
Sensitivity (%)	—	10	100	100
False-positive rate (%)	2	—	—	—
Specificity (%)	98	—	—	—

nal detected for each protein and the degree of glycation tended to become nonlinear. This relationship was particularly obvious for β -LG, which is the most readily glycosylated protein. For β -CN, the point at 18% unglycosylated protein was not included in the linear regression (Figure 4D) although it remained closely aligned with the regression line.

β -Casein Quantification

An external calibration curve was established using commercial purified β -CN. This standard also contained α _{S1}-CN, κ -CN, and various unidentified peptides and protein fragments, most of which matched the size of potential β -CN cleavage products (Figure 5A, Supplemental Table S3; <https://doi.org/10.3168/jds.2019-16273>). The fraction of the total signal attributable to intact β -CN in the 7 most concentrated calibration samples reached $86.0 \pm 0.5\%$, and the concentration of standard β -CN was adjusted with this purity factor. No glycation was apparent in this standard.

The calibration curve was established between 0 and 18 μ g of β -CN from 4 different experiments. To account for variations in MS absolute signals and to ensure that the calibration curve can be applied to future analyses, each β -CN value was normalized to the average β -CN value of 2 injections of the SMP_{QC} sample that had been prepared at the same time (values were corrected for glycation according to Equation 2). Normalized values were plotted against the amount of β -CN loaded on the column. The calibration curve (Figure 5B) was forced through zero and showed good linearity ($R^2 = 0.999$) with a random distribution of the residuals.

For each quantitative analysis, the SMP_{QC} sample used during the establishment of the calibration curve was prepared and injected 2 times within the analytical sequence. Each unknown sample was injected once. For each injection, the amount of β -CN injected on the column (in μ g) was calculated from the calibration curve using Equation [3]:

$$\beta\text{CN}_{\text{inj}} (\mu\text{g}) = \frac{\beta\text{CN}_{\text{corr, norm}}}{0.0553}, \quad [3]$$

where $\beta\text{CN}_{\text{corr, norm}}$ is the corrected signal for β -CN (Equation [2]) divided by the average of the corrected signals for the SMP_{QC} samples injected in the same series (Equation [4]):

$$\beta\text{CN}_{\text{corr, norm}} = \frac{\beta\text{CN}_{\text{corr}}}{\beta\text{CN}_{\text{SMP}_{\text{QC}}, \text{corr}}}. \quad [4]$$

Direct quantification of β -CN through an external calibration curve injected within the same series yielded values very similar to those obtained through this normalization procedure.

Quantification of β -CN genetic variants in a dairy matrix was assessed by mixing A1 and A2 SMP while keeping the total protein amount constant (Figure 5C). The response for β -CN A2 showed good linearity ($R^2 = 0.998$), indicating that the matrix effect was negligible. The measured and expected A1/A2 ratios showed a very strong linear relationship ($\%A2_{\text{meas}} = 1.04 \times \%A2_{\text{theo}}$, $R^2 = 0.998$). This outcome means that the response factors of both variants are very close and suggests that the calibration curve is also suitable to quantify individual genetic variants. Quantification of β -CN in the 2 SMP samples yielded an average value of 24.0 g of β -CN per 100 g of protein (Table 4), in line with the 27% value described by Swaisgood (1995).

Method Performance (β -Casein Quantification)

Method performance was assessed in 7 infant formulas containing only β -CN A2. Each sample was prepared 9 different times in triplicate. The median, coefficient of variation (CV) of repeatability, and CV of intermediate reproducibility were obtained using robust statistics (Table 5); the CV of repeatability ranged from 2 to 3% and CV of intermediate reproducibility from 5 to 9%. The measured amount of β -CN was generally in line (77–110%) with theoretical values (Table 1) calculated by multiplying the protein content by the casein content and by 33%, which represents the generally accepted proportion of β -CN in the total casein (Swaisgood, 1995) (Figure 6A).

Samples were analyzed in 2 separate series over the period of 1 yr. The amount of unglycosylated protein signal decreased on average by 17% over that time (Supplemental Figure S4A; <https://doi.org/10.3168/jds.2019-16273>), but this decrease had no effect on protein quantification (Supplemental Figure S4B; <https://doi.org/10.3168/jds.2019-16273>). Measured values increased by

Figure 4. (A) Bubble charts (β-CN region) show increased lactosylation during solid-state glycation experiment. (B) The fraction of unglycated β-CN signal (solid black, patterns correspond to the legend of panel A) decreases over time. (C) Total β-CN signal decreases over time and (D) has a linear relationship with the degree of glycation of the protein. (E) β-Casein signal reduction plotted as a function of the percentage of unglycated β-CN signal allows calculating a glycation correction factor. RT = retention time.

3% on average, a difference that was not significant ($P > 0.1$) except for sample IF1 ($P = 0.02$). This outcome further indicates that correcting the measured signal with a glycation correction factor allows consistent measurement over time.

Method Application

To demonstrate the applicability of the method, skim milk samples from 7 different countries were analyzed and found to have comparable β-CN content across

samples (average β-CN content: 29.9 ± 1.3 g per 100 g of protein, Figure 6B).

Finally, the method was applied on a set of commercially available infant formulas (IF8–22, manufactured either with standard SMP typically containing A1 and A2 β-CN, or with A2-specified SMP). Results were generally in line with the theoretical values listed in Table 1 (Figure 6C; for hatched values, the percentage of whey was estimated from the product composition and the order of ingredients on the label). The differences between measured and theoretical β-CN content

Figure 5. Characterization of the β -CN standard and establishment of the β -CN calibration curve. (A) Analysis of the β -CN standard revealed that about 15% of total signal could not be attributed to intact β -CN. (B) A linear calibration curve was established from 4 different experiments using normalized β -CN MS signal and adjusted β -CN amounts. (C) Cross-mixing experiments between A1 and A2 skim milk samples indicated that the relationship remained linear in a dairy matrix.

were the largest for samples with the smallest β -CN content. This finding might be due to the less abundant proteoforms being too close to noise to be measured effectively, particularly in samples with multiple genetic variants such as IF8 and IF13.

DISCUSSION

One of the most challenging aspects of protein analysis in processed food matrices (and particularly in spray-dried dairy powders) is the presence of sugar adducts that affect protein behavior. As a result, signals from single proteins are split into multiple peaks that eventually overlap, making it challenging to successfully characterize and quantify intact proteins with

UV-based methods. Sugar adducts also affect peptide-based protein quantification in final products both at the level of peptide release (glycated lysines are not recognized by trypsin) and detection, making this approach problematic in products in which early Maillard reaction occurred. As an example, absolute quantification of 20 key milk proteins by multiple reaction monitoring corresponded to ~95% of the total protein content in raw milk, but this proportion dropped to ~75% in pasteurized milk (Bär et al., 2019).

This article describes a UPLC-HRMS method for intact protein fingerprinting and demonstrates its applicability in a variety of dairy matrices such as fresh milk, SMP, whey, and infant formulas. Sample preparation is easy and quick, and it is fully compatible with

Table 4. Quantification of β -CN in A1 and A2 skim milk powder (SMP)¹

Item	Measured β -CN signal, %UG _{β} , GCF, and corrected β -CN signal			Normalized to SMP _{QC}		β -CN on column (μ g)	
	A2 SMP	A1 SMP	SMP _{QC}	A2	A1	A2	A1
Repeat 1 (total signal intensity)	179,989,098	135,352,838	171,804,237				
%UG _{β} (%)	77	82	76				
GCF	1.16	1.12	1.17				
β CN _{corr} (total signal intensity)	208,788,142	151,474,606	200,661,210	1.03	0.75	18.6	13.5
Repeat 2 (total signal intensity)	188,765,075	149,089,324	173,498,704				
%UG _{β} (%)	77	75	77				
GCF	1.16	1.18	1.16				
β CN _{corr} (total signal intensity)	219,222,015	175,200,725	201,985,059	1.08	0.86	19.5	15.6
Repeat 3 (total signal intensity)			177,428,152				
%UG _{β} (%)			77				
GCF			1.16				
β CN _{corr} (total signal intensity)			206,601,765				
Average (total signal intensity)			203,082,678	1.05	0.80	19.1	14.5
Protein loaded on column (μ g)						70.0	70.0
β -CN content (%)						27	21

¹Glycation correction factors (GCF) were calculated from the percentages of unglycated β -CN signal (%UG _{β}) using Equation [1]. The measured β -CN signal was multiplied by the GCF to obtain the corrected signal, β CN_{corr}, according to Equation [2]. Values were normalized by the average value of the SMP quality control (SMP_{QC}) sample injected in the same series (Equation [4]) and used to calculate the amount of injected β -CN according to Equation [3].

optional downstream applications such as peptide profiling if needed.

Complex MS spectra are deconvolved using the Xtract and sliding windows algorithms to calculate monoisotopic masses. An RT is associated with each signal through the extracted ion chromatogram. Results are readily visualized as bubble charts and further analyzed to identify all major milk proteins (α _{S1}-, α _{S2}-, β -, κ -, and γ -CN, as well as α -LA, β -LG, and CGMP) and their associated proteoforms. Proteoform annotation is further facilitated by the direction of the shift in RT compared with the parent protein or proteoform. The deconvolved results can be used for general fingerprinting, detection of low-abundance proteoforms, or quantification of specific proteins.

Detection of the β -CN A1 genetic variant in A2-based infant formulas was validated at a limit of detection of 2%, with less than 5% of samples having false-negative results. Less than 5% of unspiked samples returned false-positive results. Altogether, this approach demonstrates that intact protein MS data can be used to detect low-abundance genetic variants. When the method was applied to A2 SMP, a low-abundance signal was often detected around 24,333 Da, which corresponds to the mass of β -CN A1 bearing 1 lactose (A1^L). We believe that this signal belongs to a protein compound different from A1^L because (1) a similar signal was detected in liquid milk samples from A2A2 individual cows, and a signal with a similar mass shift was detected in liquid milk samples from A1A1 individual cows; (2) given

Table 5. β -Casein quantification method performance (robust statistics)

Item	Statistic ¹	Infant formula (IF)						
		IF1	IF2	IF3	IF4	IF5	IF6	IF7
β -CN (g/100 g of formula)	Median	0.97	2.96	2.4	3.41	1.08	2.69	3.18
	SD(r)	0.03	0.07	0.07	0.07	0.04	0.06	0.08
	CV(r) (%)	3	2	3	2	3	2	3
	SD(iR)	0.07	0.26	0.14	0.25	0.08	0.15	0.16
	CV(iR) (%)	8	9	6	7	8	5	5
% Unglycated β -CN signal	Median (%)	40	39	30	28	50	50	51
	SD(r)	0.00	0.00	0.01	0.00	0.00	0.01	0.01
	CV(r) (%)	1	1	2	1	1	2	1
	SD(iR)	0.02	0.03	0.02	0.01	0.01	0.02	0.01
	CV(iR) (%)	5	7	7	4	2	3	3

¹SD(r) is the standard deviation of repeatability; CV(r) is the coefficient of variation of repeatability; SD(iR) is the standard deviation of intermediate reproducibility; CV(iR) is the coefficient of variation of intermediate reproducibility.

that the majority of β -CN A2 was not lactosylated in those samples, a signal for unlactosylated A1 would be expected as well; and (3) peptide-based analyses failed to detect β -CN A1 sequences. Thus, in the absence of unglycated β -CN A1, this signal is likely to represent a different protein compound with a size close to that of β -CN A1^L.

This study also establishes the proof-of-concept that deconvolved mass spectrometry data can be used for absolute protein quantification, using β -CN as an example. Protein quantification is complicated by the presence of process-induced modifications that affect the signal response of the modified proteins. Protein lactosylation was shown to reduce the overall signal intensity, but this can be corrected based on the degree of glycation of the protein. The advantage of this approach is that both values (total signal and degree of glycation) come from the same experiment and do not

require supplementary measurements. Sample analyses performed after 1 yr showed similar β -CN content after correction for glycation, further validating this approach and demonstrating the robustness of the method. Glycation correction factors can be calculated for each protein using data from solid-state glycation experiments. Those correction factors will improve with increasing number of experiments and also by starting with powders that are as little glycated as possible.

β -Casein quantification was evaluated using 7 different infant formula samples. The repeatability varied between 2 and 3%, and the intermediate reproducibility between 5 and 9%. The calculated absolute values were generally in line with expected values based on product composition and generic β -CN content, but the absence of certified reference material makes it impossible to further assess the trueness of this method. The discovery of a large fraction of undeclared impurities in β -CN

Figure 6. Quantification of β -CN in infant formulas and skim milk powders (SMP). (A) β -CN was quantified in the 7 infant formula (IF) samples used to establish the limit of detection, where each sample was analyzed in triplicate 9 times. The error bars represent the coefficient of variation of intermediate reproducibility and theoretical values are based on formulations and generic milk protein composition. (B) β -Casein was quantified in 8 skim milk powder samples from different countries—Canada (CAN), Switzerland (CH), Germany (DE), Denmark (DK), France (FR), India (IN), and United States (USA and USAb). (C) A second set of infant formulas (both A2-based or based on SMP containing a mixture of genetic variants) was analyzed in triplicate in a single day. The error bars represent the 95% tolerance interval. Hatched bars represent estimations based on product composition and the order of ingredients on the label.

commercial standard (~15% of the total MS signal) also highlights the need for certified standard materials for the purpose of absolute quantification.

Finally, further method improvement should include the spiking of an exogenous protein or an isotopically labeled milk protein into each sample and calibration standard to allow better normalization across samples.

CONCLUSIONS

The UPLC-HRMS intact protein method presented herein is an effective method for the identification and quantification of proteins in dairy samples such as raw milk, SMP, whey powder, final products, and samples from other species such as buffalo or human. It allows a straightforward and unambiguous detection of the major milk proteins (below 30 kDa) and their associated proteoforms, with low-abundance genetic variants being detectable around 2%. Absolute quantitation of individual proteins in processed food matrices is possible provided that the total signal of a protein is corrected by a factor related to its glycation index.

ACKNOWLEDGMENTS

We thank Jonathan O'Regan (Wyeth Nutritionals, Ireland) for critical reading of the manuscript and sample procurement, Guillermo Julián Moreno for the Protein Analyzer Software (developed during his Master internship while at the École Polytechnique Fédérale de Lausanne, EPFL), Donagh Berry at Teagasc Moorepark (Ireland) for providing the A1 skim milk sample, Jennifer Sutton at Thermo Fisher Scientific (San Jose, CA) for BioPharma Finder software support, and Peter Erdmann and Marco Kneitschel (both from Nestlé Nutrition, Switzerland) for fruitful discussions and sample procurement.

REFERENCES

- Asledottir, T., T. T. Le, N. A. Poulsen, T. G. Devold, L. B. Larsen, and G. E. Vegarud. 2018. Release of β -casomorphin-7 from bovine milk of different β -casein variants after ex vivo gastrointestinal digestion. *Int. Dairy J.* 81:8–11.
- Bär, C., D. Mathis, P. Neuhaus, D. Dürr, W. Bisig, L. Egger, and R. Portmann. 2019. Protein profile of dairy products: Simultaneous quantification of twenty bovine milk proteins. *Int. Dairy J.* 97:167–175.
- Bonfatti, V., L. Grigoletto, A. Cecchinato, L. Gallo, and P. Carnier. 2008. Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. *J. Chromatogr. A* 1195:101–106.
- Brooke-Taylor, S., K. Dwyer, K. Woodford, and N. Kost. 2017. Systematic review of the gastrointestinal effects of A1 compared with A2 beta-casein. *Adv. Nutr.* 8:739–748.
- Caroli, A. M., S. Chessa, and G. J. Erhardt. 2009. Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *J. Dairy Sci.* 92:5335–5352.
- de Jong, N., S. Visser, and C. Olieman. 1993. Determination of milk proteins by capillary electrophoresis. *J. Chromatogr. A* 652:207–213.
- Duarte-Vazquez, M. Á., C. Garcia-Ugalde, L. M. Villegas-Gutierrez, B. E. Garcia-Almendarez, and J. L. Rosado. 2017. Production of cow's milk free from beta-casein A1 and its application in the manufacturing of specialized foods for early infant nutrition. *Foods* 6:E50. <https://doi.org/10.3390/foods6070050>.
- European Food Safety Authority. 2009. Scientific report of EFSA prepared by a DATEX Working Group on the potential health impact of β -casomorphins and related peptides. EFSA Scientific Report 231:1–107.
- Farrell, H. M. Jr., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteins of cows' milk—Sixth revision. *J. Dairy Sci.* 87:1641–1674.
- Fenaille, F., F. Morgan, V. Parisod, J. C. Tabet, and P. A. Guy. 2003. Solid-state glycation of beta-lactoglobulin monitored by electrospray ionisation mass spectrometry and gel electrophoresis techniques. *Rapid Commun. Mass Spectrom.* 17:1483–1492.
- Fenaille, F., V. Parisod, P. Visani, S. Populaire, J.-C. Tabet, and P. A. Guy. 2006. Modifications of milk constituents during processing: A preliminary benchmarking study. *Int. Dairy J.* 16:728–739.
- Feng, P., C. Fuerer, and A. McMahon. 2017. Quantification of whey protein content in infant formulas by sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE): Single-laboratory validation, first action 2016.15. *J. AOAC Int.* 100:510–521.
- Frederiksen, P. D., K. K. Andersen, M. Hammershoj, H. D. Poulsen, J. Sorensen, M. Bakman, K. B. Qvist, and L. B. Larsen. 2011. Composition and effect of blending of noncoagulating, poorly coagulating, and well-coagulating bovine milk from individual Danish Holstein cows. *J. Dairy Sci.* 94:4787–4799.
- Gallinat, J. L., S. Qanbari, C. Drogemuller, E. C. Pimentel, G. Thaller, and J. Tetens. 2013. DNA-based identification of novel bovine casein gene variants. *J. Dairy Sci.* 96:699–709.
- Givens, L., P. Aikman, T. Gibson, and R. Brown. 2013. Proportions of A1, A2, B and C beta-casein protein variants in retail milk in the UK. *Food Chem.* 139:549–552.
- Grosclaude, F., M. F. Mahe, J. C. Mercier, and B. Ribadeau-Dumas. 1972. Characterization of genetic variants of a S1 and bovine caseins. *Eur. J. Biochem.* 26:328–337. [In French]
- He, M., J. Sun, Z. Q. Jiang, and Y. X. Yang. 2017. Effects of cow's milk beta-casein variants on symptoms of milk intolerance in Chinese adults: A multicentre, randomised controlled study. *Nutr. J.* 16:72.
- Jann, O. C., E. M. Ibeagha-Awemu, C. Ozbeyaz, P. Zaragoza, J. L. Williams, P. Ajmone-Marsan, J. A. Lenstra, K. Moazami-Goudarzi, and G. Erhardt. 2004. Geographic distribution of haplotype diversity at the bovine casein locus. *Genet. Sel. Evol.* 36:243–257.
- Jianqin, S., X. Leiming, X. Lu, G. W. Yelland, J. Ni, and A. J. Clarke. 2016. Effects of milk containing only A2 beta casein versus milk containing both A1 and A2 beta casein proteins on gastrointestinal physiology, symptoms of discomfort, and cognitive behavior of people with self-reported intolerance to traditional cows' milk. *Nutr. J.* 15:35.
- Jinsmaa, Y., and M. Yoshikawa. 1999. Enzymatic release of neocasomorphin and beta-casomorphin from bovine beta-casein. *Peptides* 20:957–962.
- Karamoko, G., P. Anihouvi, and C. Blecker. 2013. Évolution des connaissances sur les fonctionnalités de la fraction protéose-peptone du lait: Propriétés technofonctionnelles et biologiques (synthèse bibliographique). *Biotechnol. Agron. Soc. Environ.* 17:373–382.
- Ng-Kwai-Hang, K. F., and F. Grosclaude. 2003. Genetic polymorphism of milk proteins. Pages 739–816 in *Advanced Dairy Chemistry—1 Proteins: Part A/Part B*. P. F. Fox and P. L. H. McSweeney, ed. Springer US, Boston, MA.
- Noni, I. D. 2008. Release of beta-casomorphins 5 and 7 during simulated gastro-intestinal digestion of bovine beta-casein variants and milk-based infant formulas. *Food Chem.* 110:897–903.
- Poulsen, N. A., A. K. Rosenggaard, B. D. Szekeres, V. R. Gregersen, H. B. Jensen, and L. B. Larsen. 2016. Protein heterogeneity of bovine

- β -casein in Danish dairy breeds and association of rare β -casein F with milk coagulation properties. *Acta Agric. Scand. A Anim. Sci.* 66:190–198.
- Senko, M. W., S. C. Beu, and F. W. McLafferty. 1995. Determination of monoisotopic masses and ion populations for large biomolecules from resolved isotopic distributions. *J. Am. Soc. Mass Spectrom.* 6:229–233.
- Swaigood, H. E. 1995. Protein and amino acid composition of bovine milk. Pages 464–468 in *Handbook of Milk Composition*. R. G. Jensen, ed. Academic Press, San Diego, CA.
- Trujillo-Cárdenas, L., M. Camacho-Barrón, C. Cano-Sarmiento, M. A. Duarte-Vázquez, R. Viveros-Contreras, and J. L. Rosado-Loria. 2018. Development of an infant formula high in alpha-lactalbumin with only A2 beta-casein by spray drying, designed to resemble the protein composition of human milk. *Rev. Mex. Ing. Quim.* 18:151–165.
- Vallejo-Cordoba, B. 1997. Rapid separation and quantification of major caseins and whey proteins of bovine milk by capillary electrophoresis. *J. Capillary Electrophor.* 4:219–224.
- Visser, S., C. J. Slangen, F. M. Lagerwerf, W. D. Van Dongen, and J. Haverkamp. 1995. Identification of a new genetic variant of bovine beta-casein using reversed-phase high-performance liquid chromatography and mass spectrometric analysis. *J. Chromatogr. A* 711:141–150.

ORCIDS

- C. Fuerer <https://orcid.org/0000-0003-1717-6718>
L. Cardinaux <https://orcid.org/0000-0003-4272-1749>
M. Affolter <https://orcid.org/0000-0003-1568-4516>