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Quantification of Milk Fat Globule Membrane Proteins Using Selected Reaction Monitoring Mass Spectrometry

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Although some of the physiological roles of milk fat globule membrane (MFGM) proteins are still unclear, there is increasing evidence that the consumption of bovine MFGM proteins has significant nutritional health benefits for humans; therefore, it may be important to be able to estimate the MFGM proteins in complex ingredients. In this study, the absolute quantification (AQUA) technique, which is typically used for the quantification of proteins in proteomic studies, was applied for the quantification of bovine MFGM proteins in butter milk protein concentrate. Six MFGM proteins (fatty acid binding protein, butyrophilin, PAS 6/7, adipophilin, xanthine oxidase, and mucin 1) were simultaneously quantified using high-resolution selected reaction monitoring mass spectrometry. Samples were rehydrated in 6.7 M urea buffer prior to dilution to 2.2 M before tryspin digestion. Direct rehydration in 2.2 M urea buffer or 2.2 M urea/20% acetonitilrile buffer reduced peptide yield digestion. Isotopically labeled peptides were used as internal standards. The coefficient of variation ranged from 5 to 15%, with a recovery of 84−105%. The limit of detection was in the range of 20−40 pg.

KEYWORDS: Milk fat globule membrane; mass spectrometry; butter milk protein

INTRODUCTION

The bovine milk fat globule membrane (MFGM) is a layer that surrounds each lipid droplet in milk. It is organized into a trilayer structure (an inner lipid monolayer and an outer lipid bilayer), consisting of a complex mixture of proteins, glycoproteins, enzymes, neutral lipids, and polar lipids such as phospholipids and glycosphingolipids (*1*, *2*).

The MFGM components have been reviewed extensively over the past few years (*2*−*8*). They have received a lot of interest for both their emulsification properties and their different health benefits (*2*,*4*−*6*). The reported bioactivity of the MFGM proteins includes anticancer effects on some cancer cell line models (*9*, *10*), antibacterial effects (*11*, *12*), and antiviral effects (*13*, *14*), whereas MFGM phospholipids and gangliosides are implicated as having various health benefits such as anticholesterolemic effects, attenuation of neuronal aging, and antibacterial properties (*3*, *15*). Some researchers have also linked some of the membrane proteins as contributors to multiple sclerosis (*6*) and coronary heart disease (*16*, *17*).

Similar bioactivity has also been demonstrated for some human MFGM proteins such as immunoglobulin, mucins, and lactadherin (PAS 6/7) (*11*−*14*, *18*, *19*). These proteins have been reported to play an important role in various cell processes and defense mechanisms against bacteria and viruses in the newborn.

Over the past decade, most of the studies on bovine MFGM and human MFGM have been focused on the identification of the proteins using proteomic techniques (*8*, *20*−*29*). Of the numerous bovine MFGM proteins reported to date, there are eight major proteins [mucin 1 (MUC 1), xanthine oxidase (XO), CD36 (PAS 4), mucin 15 (PAS 3), butyrophilin (BTN), PAS 6/7, adipophilin (ADPH), and fatty acid binding protein (FABP)].

The increasing interest in the bioactivity of the MFGM proteins means that the ability to quantify these proteins is essential if they are to be used as nutraceuticals. PAS 3 is the only MFGM protein that has been directly quantified to date, employing the Western blot technique and using in-house-purified bovine PAS 3 standards (*30*). Whereas antibodies to some of the bovine MFGM proteins are available commercially, standards for the bovine MFGM proteins are not, making their direct quantification difficult. This is further complicated by the fact that most MFGM proteins are also heavily glycosylated and, hence, are present in many isoforms (*8*).

In this study, the absolute quantification (AQUA) technique, which is typically used for proteomic-scale studies (*31*), was adapted for the simultaneous quantification of the six most abundant bovine MFGM proteins (FABP, BTN, PAS 6/7, ADPH, XO, and MUC 1) present in complex butter milk protein concentrate. This technique quantified the targeted proteins indirectly by measuring specific cleavage peptide sequences that were generated using specific enzymes. The accuracy of the technique relied heavily on the complete release of the target cleavage peptide sequence from the intact protein. These released peptides were simultaneously measured using liquid chromatography−high-resolution selected reaction monitoring−mass spectrometry (LC-HSRM-MS).

MATERIALS AND METHODS

Materials. Butter milk protein concentrates (BPC50 and BPC60) containing 50 and 60% protein, respectively, were prepared by concentrating

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Table 1. Selected MFGM Peptide Sequences, Their Respective Peptide Transitions, and the CEs Used for HSRM Quantification[a]

peptide ID	peptide sequence	M^{2+}	selected transitions		isotopic peptide ID	M^{2+}	selected transitions		CE
β-Lg	TPEVDDEALEK	623.30	918.44	572.77	TPEV*DDEALEK	625.81	923.47	819.37	23
FABP	LGVEFDETTADDR	734.33	577.26	1198.49	LGV*EFDETTADDR	736.85	922.37	678.31	28
BTN	TPLPLAGPPR	509.81	610.37	707.40	TPLPL*AGPPR	513.32	497.28	714.44	21
PAS 6/7 (638)	IFIGNVNNSGLK	638.36	902.47	1015.55	IFIGNV*NNSGLK	640.87	907.50	1020.58	28
PAS 6/7 (597)	QFQFIQVAGR	597.32	790.46	918.52	QFQFIQV*AGR	599.84	648.42	795.48	28
ADPH (616)	SVVSGSINTVLR	616.35	859.50	1045.60	SVV*SGSINTVLR	618.87	859.50	946.53	24
ADPH (759)	SELLVDQYLPLTK	759.92	458.30	1076.60	SELLV*DQYLPLTK	762.44	977.53	1081.63	29
XO (565)	VSLSTTGFYR	565.80	542.27	831.40	VSL*STTGFYR	569.31	831.40	542.27	23
XO (696)	STLVSVAVALAAYK	696.91	735.44	992.58	STL*VSVAVALAAYK	700.42	806.48	992.58	27
MUC 1	SIWGLILQIYK	667.40	777.49	947.59	SIWGLIL*QIYK	670.91	570.85	954.62	28

[a] The isotopically labeled internal standard peptides were synthesized by incorporating either ^{120}leucine or ^{104}valine into the peptide sequence*.

Table 2. Typical R^2 Coefficient, Linear Range for the MFGM Peptide Calibration Curve, and the Limit of Detection

	MFGM protein (mg/g)							
	FABP	BTN	PAS 6/7 (638)	PAS 6/7 (597	ADPH (616)	ADPH (759)	XO (565)	MUC
R^2	0.997	0.993	0.998	0.992	0.998	0.992	0.994	0.986
linear range (ng loaded on column)	40	40	40	40	40	40	40	40
LOD (pg)	40	20	20	20	20	20	20	40

β-serum, a proprietary product enriched in milkfat globule membrane (*32*), using ultrafiltration and supercritical CO_2 extraction as described in patent WO/2006/041316 (*33*). Peptide standards including isotopically labeled peptides were obtained from Quality Control Biochemicals (Hopkinton, MA). Isotopically labeled peptides were synthesized using ^{13}C- and ^{15}N-labeled amino acids. Trypsin (TPCK treated, bovine pancreas, 12,500 units/mg solid, T1426) was purchased from Sigma-Aldrich (St. Louis, MO). All solvents used were of high-performance liquid chromatography (HPLC) grade. The urea used was of electrophoresis grade. β-Lactoglobulin A (β-Lg A) was obtained from Sigma-Aldrich.

Selection of Target MFGM Peptides for Quantification. The MFGM peptides were selected based on the uniqueness of the tryptic peptide sequence (obtained by in silico trypsin digestion using peptideMass tool and protein sequences obtained from http://us.expasy.org), the ability to detect these peptides using MS, and the absence of readily oxidizable cysteine and methionine residues and post-translational modification sites such as serine and threonine amino acid residues. The peptide sequences were also limited to no more than 15 amino acid residues. The selected peptides are shown in **Table 1**.

Standard Preparation. Individual peptide stock solutions were prepared at a concentration of 1 mg/mL in 20% acetonitrile (ACN) in water and were stored in deactivated glass vials at −80 °C. A combined intermediate standard solution consisting of 10 μg/mL of each peptide was prepared from the stock solutions (biweekly), one for the standard peptides and one for the isotopically labeled internal standard peptides. Serial dilutions of the intermediate standard solution were prepared to give a five-point calibration curve (0.63, 0.13, 0.25, 0.5, and 1 μg/mL or 1.25, 2.5, 5, 10, and 20 ng on column, respectively) for each of the MFGM peptides containing 0.25 μg/mL of their respective isotopically labeled internal standard peptide. The R^2 values for each of the MFGM peptide calibration curves were typically greater than 0.98 (**Table 2**). The linear range of the calibration standard could be extended to 40 ng (**Table 2**). A linear range beyond 40 ng was not investigated in this study.

Internal Standard. In this study, the synthetic isotopically labeled internal standard was added to the sample after digestion. This method was chosen for its simplicity over the use of peptides in which the tryptic cleavage sites are incorporated into the internal standard to create an internal standard that has cleavage characteristics more similar to those of the protein being quantified. Both techniques have been evaluated in detail for membrane proteins (*34*) and human serum albumin (*35*), with the conclusion being that the latter technique does not improve the accuracy and precision of the value obtained as compared with the isotopically labeled internal standard method.

Digestion Protocol. Unless otherwise specified, samples were rehydrated in 6.7 M urea/0.2 M ammonium bicarbonate buffer (pH 8.1) to give a concentration of approximately 40% protein, followed by reduction with β-mercaptoethanol (5 μL/mL) for 30 min at room temperature. Other rehydration buffer systems were also tested in this study (**Table 3**). Before trypsin addition, the samples were further diluted with 0.2 M ammonium bicarbonate/pH 8.1 to give a final urea concentration of 2.2 M. Timed hydrolysis of the sample was conducted over 5 h at 37 °C using a trypsin:substrate ratio of 1:20. The samples were acidified with concentrated formic acid to give a final concentration of 0.5% formic acid to stop the hydrolysis, before centrifugation (27000*g*) for 15 min at 5 °C to allow the lipid to separate. An aliquot (100 μL) of the sample was diluted with 700 μL of 5% ACN in water and 800 μL of isotopically labeled internal standard (0.25 μg/mL) prior to analysis. A hydrolysis time of 60 min was used for all of the MFGM proteins except BTN (30 min).

Table 3. Sample Solubilization/Denaturing Buffers

rehydration buffer
1. 2.2 M urea/20% ACN/ammonium bicarbonate carbonate (0.2 M, pH 8.1) in water
2. 2.2 M urea/ammonium bicarbonate (0.2 M, pH 8.1)
3. 6.7 M urea/ammonium bicarbonate (0.2 M, pH 8.1)
4. 6.7 M urea, followed by dilution with ammonium bicarbonate (0.2 M, pH 8.1) to give a final urea concentration of 2.2 M
5. 6.7 M urea, reduced with β-mercaptoethanol (ambient temperature), followed by dilution with ammonium bicarbonate (0.2 M, pH 8.1) to give a final urea concentration of 2.2 M

Reversed-Phase LC-HSRM-MS. Peptide separation was conducted on an Alliance 2690 LC system (Waters Corp., Milford, MA), equipped with a C18 column (Aqua 5 μ 125 Å, 150 mm × 1.00 mm, Phenomenex, Torrance, CA), maintained at 60 °C. The aqueous mobile phase (A) contained 0.2% formic acid, and the organic mobile phase (B) contained 90% ACN in water with 0.2% formic acid. Standards and samples (20 μL) were loaded onto the column with 5% mobile phase B for the first 5 min. Peptides were eluted from the column with a gradient of 5−35% B over 40 min, increasing to 90% over 5 min at a flow rate of 150 μL/min. The column was re-equilibrated at the initial conditions after 2 min for a period of 10 min.

The LC system was coupled to a Thermo Scientific TSQ Quantum Ultra EMR mass spectrometer (Thermo Fisher Scientific, San Jose, CA), using a heated electrospray ionization (ESI) interface. The first 12 min and the last 10 min of the LC eluant were diverted to waste. Ions were generated and focused using a positive ion spray voltage of 3000 V, a vaporizer temperature of 150 °C, a sheath gas of 18 arbitrary units, an auxiliary gas of 10 arbitrary units, and a capillary temperature of 275 °C. The HSRM mode conditions were as follows: scan width, 0.002 *m*/*z*; scan time, 0.05 s; and collision gas pressure, 1.5 mTorr. The collision energies

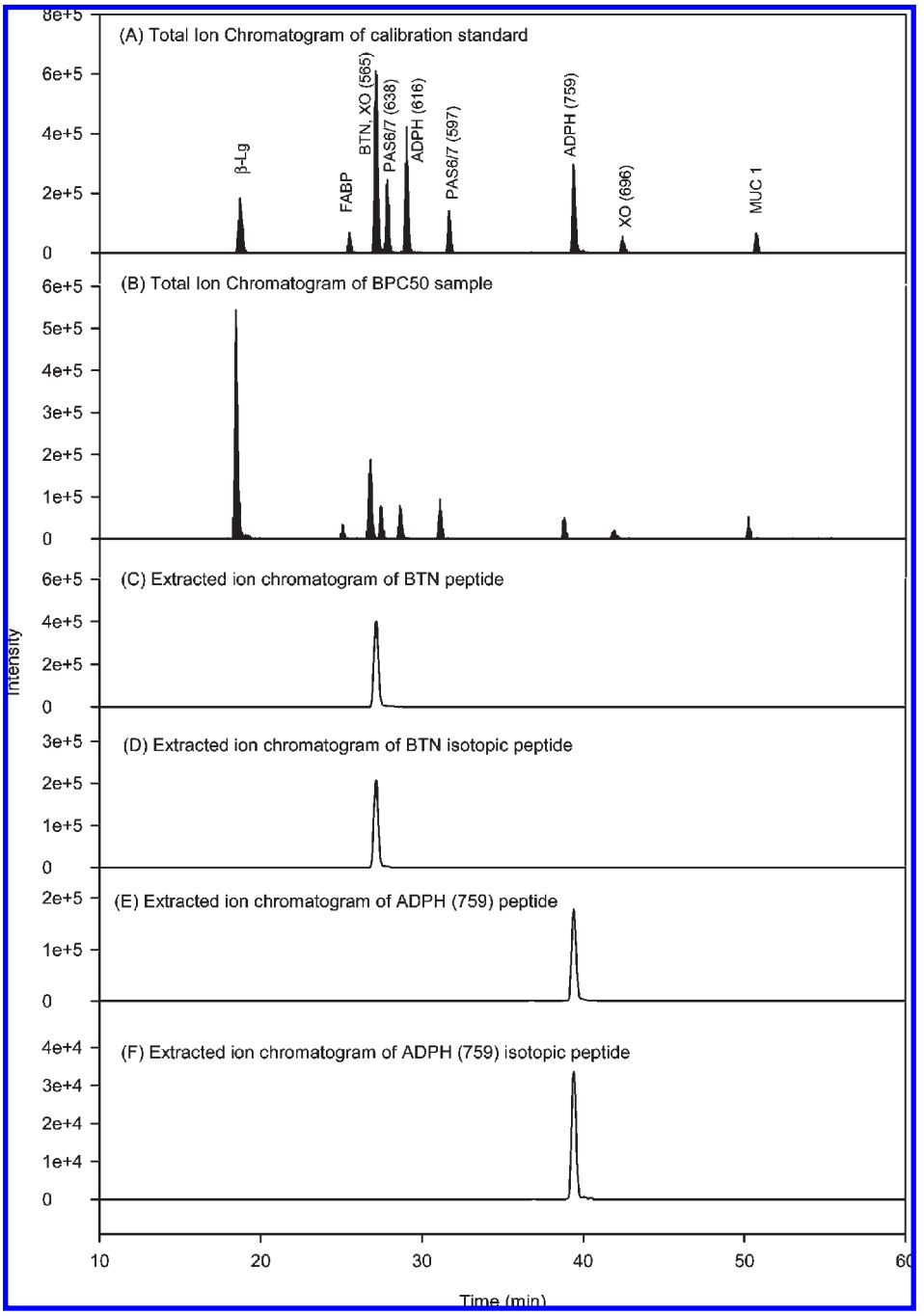

Figure 1. Typical HSRM total ion chromatogram trace of a calibration standard (5 ng, **A**) and a sample (**B**) with the extracted ion chromatograms for BTN peptide (**C**), BTN isotopically labeled peptide (**D**), ADPH (759) peptide (**E**), and ADPH (759) isotopically labeled peptide (**F**).

(CEs) were optimized by infusion of each of the peptide standards into the mass spectrometer (at 0.2 mg/mL in 50% ACN in water/0.2% formic acid in water). The optimized CEs for the selected transitions are given in **Table 1**. A typical HSRM total ion count trace and the respective extracted ion chromatograms acquired under these conditions are shown in **Figure 1**.

RESULTS AND DISCUSSION

Protein Distribution in BPC50 and BPC60 Sample. The separation of the protein component of BPC and BPC 60 using sodium dodecyl sulfate (SDS) gel electrophoresis is shown in **Figure 2**. Along with the MFGM proteins, significant amounts of casein and whey protein (β-Lg and α-Lac) can be observed in both samples.

Optimization of MFGM Protein Digestion. The complete digestion of proteins to release the target peptide sequence is a prerequisite for protein quantification using the AQUA technique. Quantifying the target peptides provides an indirect means of measuring the concentration of the proteins present in the complex BPC samples. In this study, six MFGM proteins were simultaneously quantified using this technique. The MFGM proteins were measured using nine specific tryptic peptide cleavage sequences (**Table 1**). Three of the six MFGM proteins were measured by LC-HSRM-MS using two peptide sequences for each.

Two factors may hamper the quantification of membrane proteins by trypsin digestion: Lysine and arginine cleavage sites are less frequent in membrane proteins than in cytosolic proteins (*36*), and membrane proteins are difficult to solubilize for digestion (*28*, *37*). To ensure the complete release of the target MFGM peptides from the protein, the sample

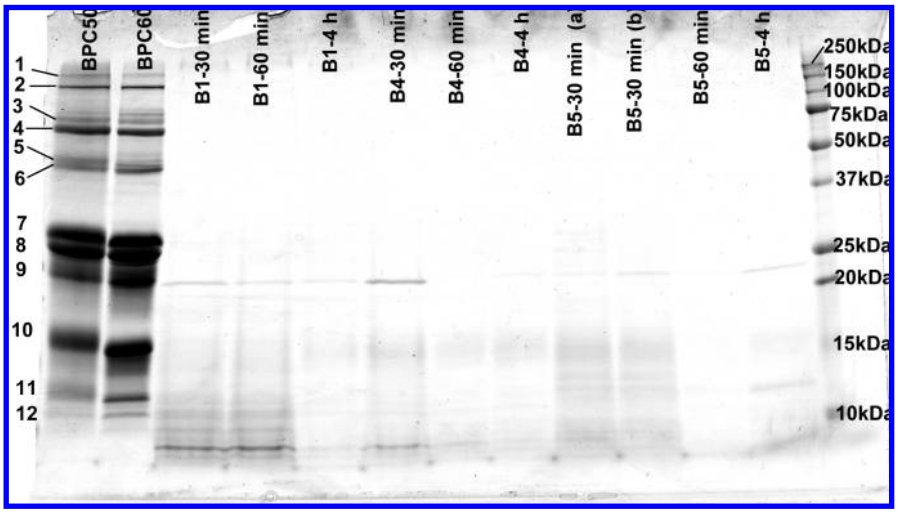

Figure 2. SDS-PAGE gel of BPC50 time hydrolysis samples. SDS-PAGE was carried out using Criterion Precast 8−16% Tris-HCl gels (BioRad Laboratories, Hercules, CA). The proteins were separated out using a current of 80 mA and were stained using Comassie Blue R250. Fifteen microliters of BPC sample (~38 μg of protein) was loaded on to the gel. The hydrolyzed samples were uploaded to an equivalent protein level of 100 μg to detect any unhydrolyzed proteins. B1, B4, and B5 samples were hydrolyzed in rehydration buffer 1, rehydration buffer 4, and rehydration buffer 5, repectively. The MFGM protein bands were identified using proteomic techniques, as described in ref (*26*). Protein band identifications were as follows: 1, 3, and 4, BTN; 2, XO; 5, PAS 6/7; 6, ADPH; 7, α-casein; 8, β-casein; 9, κ-casein; 10, β-Lg; 11, α-lactalbumin; and 12, FABP.

solubilization/hydrolysis step was optimized. A number of different solubilization/denaturing buffers were reported in the literature to be effective for the solubilization of cell membrane proteins in proteomic studies. These involved a mixture of chaotropes such as urea either with organic solvents such as ACN or methanol (*38*−*40*) or with detergents such as SDS (*34*, *41*, *42*). However, many of these buffer systems reduced the digestion efficiency of trypsin or were incompatible with MS.

In this study, a number of sample solubilization/denaturing buffers (**Table 3**) were tested to optimize the release of target MFGM peptides. Using the typical TPCK-modified trypsin hydrolysis conditions of pH 8.1 and 37 °C (*43*), the hydrolysis was monitored over 5 h using both LC-HSRM-MS and SDS−polyacrylamide gel electrophoresis (PAGE) (*44*).

It was found that the MFGM proteins were best solubilized by rehydration with 6.7 M urea/0.2 M ammonium bicarbonate buffer, followed by dilution to 2.2 M urea before digestion (rehydration buffer 5, **Table 3**). There was no significant difference between the samples that were reduced or not reduced with β-mercatoethanol prior to digestion (**Figure 3**). The temperature at which the reduction was carried out, either ambient (30 min) or 95 °C (20 min), did not make any difference to the peptide yield (data not shown). Direct rehydration with 2.2 M urea buffer (rehydration buffer 2) or 6.7 M urea buffer (rehydration buffer 3) reduced the peptide yield (data not shown). These results indicated that a significantly higher urea concentration (6.7 M) was required to first denature/solubilize the MFGM proteins.

The addition of ACN to the urea buffer system at a level of 20% (rehydration buffer 1, **Table 3**) in an attempt to improve protein solubilization significantly reduced the peptide yield for some MFGM peptides [PAS 6/7 (638), ADPH (759), and MUC 1, **Figure 3**], as compared with the yield when urea rehydration buffer 5 (**Table 3**) was used. It is likely that the presence of ACN may have inhibited the effectiveness of protein solubilization and, hence, the accessibility of trypsin to different regions of the protein. Incomplete digestion under the ACN buffer conditions was evident by the presence of a pellet after centrifugation of the sample after hydrolysis. The SDS-PAGE gel of the sample showed the presence of partially digested material in the molecular mass region spanning >25 kDa, which was not observed with the 6.7 M urea rehydration buffer 5 (**Figure 3**); consequently, the use of ACN was discontinued. In summary, the optimized digestion condition used for all further work involved rehydration of samples in of 6.7 M urea/0.2 M ammonium bicarbonate buffer, followed by dilution to 2.2 M urea before digestion.

MFGM Levels With Optimized Protein Digestion. The hydrolysis of MFGM was monitored over 5 h (duplicate analyses over 4 days). For most of the MFGM peptides, the maximum peptide yield was observed at between 30 and 60 min under the selected rehydration buffer 5 conditions (**Figure 3**). A final hydrolysis time point of 60 min was chosen, except for BTN, for which the final hydrolysis time point was 30 min.

FABP. Complete hydrolysis of FABP was observed after 30 min of hydrolysis time. For consistency with the other MFGM hydrolysis quantification time points, the 60 min hydrolysis time point was also chosen to quantify FABP in the BPC samples. FABP was measured at approximately 2 mg/g in both BPC samples, the lowest concentration of all of the MFGM proteins measured.

BTN. A significant loss of the BTN peptide (60−70%) was also observed over the 5 h hydrolysis period. However, when 20% ACN in water was added to the urea buffer system (rehydration buffer 1, **Table 3**), the loss of the BTN peptide was significantly reduced (**Figure 3**). It was possible that the ACN improved the solubility of the hydrophobic BTN peptide in the buffer system, preventing its loss by hydrophobic interaction with partially digested proteins that were centrifuged out of solution and or stuck to the walls of the digestion tubes.

Because of the observed loss of BTN over the 5 h hydrolysis period, a 30 min hydrolysis time (using rehydration buffer 5) was used to estimate the protein level in the samples. The BTN levels measured under these conditions for the BPC50 and BPC60 samples were 42 ± 5 and 66 ± 8 mg/g, respectively, which were approximately 10−15% higher than the results obtained with the 60 min hydrolysis time (**Table 4**). The BTN results obtained with the ACN buffer (rehydration buffer 1, **Table 3**) were not significantly different from the 30 min hydrolysis results.

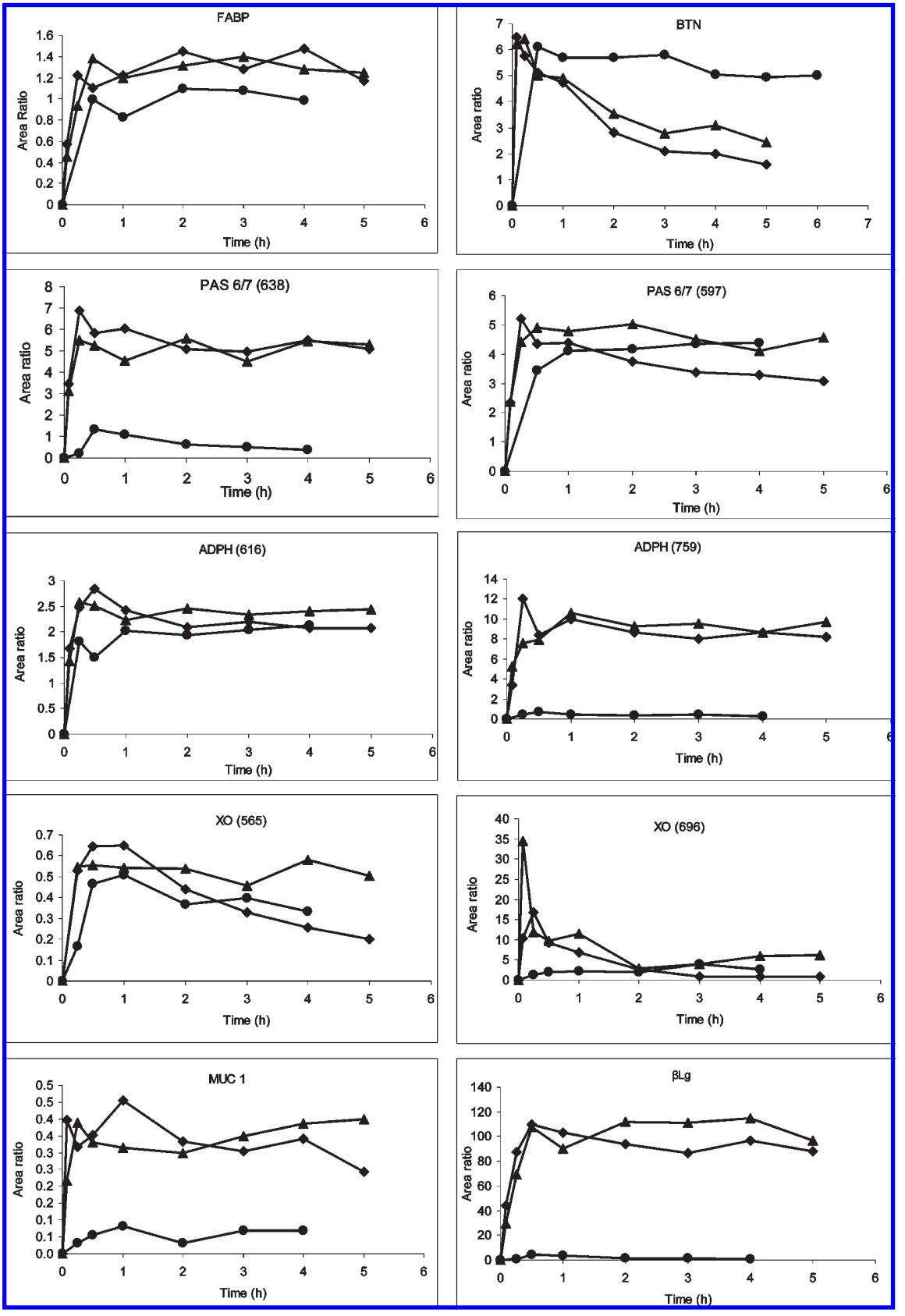

Figure 3. Timed hydrolysis (h) of BPC50 using urea buffer system with (▲) and without (♦) β-mercaptoethanol and with 20% ACN in water (●). Only one data set shown.

PAS 6/7. As for ADPH, attempts were made to measure PAS 6/7 using two peptides, PAS 6/7 (597) and PAS 6/7 (638). Although optimum release of both peptides was observed after approximately 30 min (**Figure 3**), the PAS 6/7 peptides were not released on an equimolar basis, as anticipated. The PAS 6/7 (597) peptide gave protein results that were 2−3 times higher than those determined with the PAS 6/7 (638) peptide, even after a 5 h hydrolysis period (**Table 4**). Both peptides are located within close

Table 4. MFGM Proteins Measured in the BPC50 and BPC60 Samples and Assay Variation[a]

		MFGM protein (mg/g)							
		FABP	BTN	PAS 6/7 (638)	PAS 6/7 (597)[b]	ADPH (616)[b]	ADPH (759)	XO (565)	MUC 1[c]
BPC50	average	2	38	29	47	11	13	16	18
	SD[d]	0.2	4	3	5	1	2	2	1
	CV (%)	10	10	11	10	9	13	12	5
BPC60	average	2	56	20	56	12	10	33	11
	SD[d]	0.2	8	3	8	2	1	5	1
	CV (%)	12	14	15	14	15	13	14	12

[a] Samples were analyzed in duplicate over four different days. [b] Peptide chosen to quantify protein. [c] $n = 3$. [d] Standard deviation.

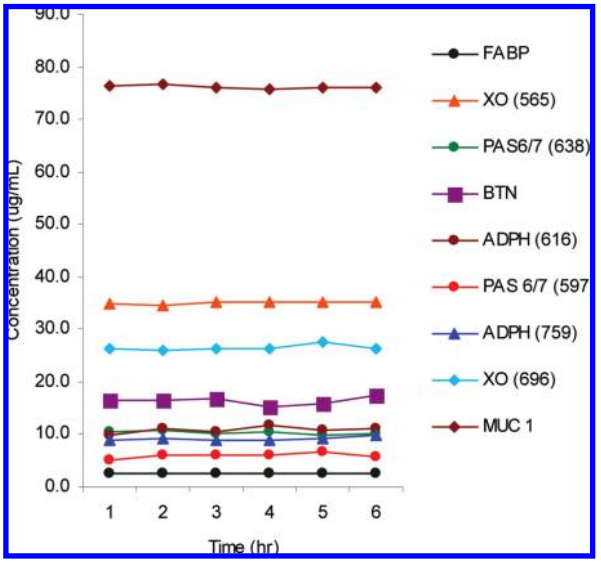

Figure 4. Changes to the MFGM peptide standards when subjected to the same extraction and hydrolysis conditions as the sample (digestion in 2.2 M urea/0.2 M ammonium bicarbonate buffer, pH 8.1). Trypsin was added to the standard at a similar mole ratio to that for the sample.

proximity to each other (VAYSTDGR[221]**QFQFIQVAG**[232]**R** SGDK [221]**IFIGNVNNSGL**[232]**K**INLFDTP) in the C1 domain of the protein, which contains two disulfide linkages (*8*). It is possible that the differences in release rate of the two PAS 6/7 peptides could have been caused by steric hindrance/insufficient reduction of the disulfide bonds or the disulfide bond reforming, as the reduced cysteine residues were not blocked. The results from the PAS 6/7 (597) peptide after 60 min of hydrolysis time were used to quantify PAS 6/7 as this peptide consistently gave higher results than the PAS 6/7 (638) peptide. PAS 6/7 made up a significant proportion of the MFGM proteins present in both BPC samples (**Table 4**).

ADPH. ADPH was measured using two peptide sequences, ADPH (616) and ADPH (759). These peptides were released at equimolar ratios (**Table 4**) as expected, with complete hydrolysis observed after approximately 60 min (**Figure 3**). Either peptide could be used to quantify this protein. The ADPH (616) peptide was used for routine analysis in this study. The levels of ADPH appeared to be roughly similar in both BPC samples (11−12 mg/g) despite the BPC60 sample having 10% more total protein than the BPC50 sample (**Table 4**).

XO. A very rapid loss of the XO (696) peptide (70%) was observed within 15 min of hydrolysis. There was no significant loss of the XO (565) peptide under the same buffer conditions. This peptide loss phenomenon was not observed when the calibration standard peptides were subjected to the same digestion conditions (**Figure 4**), suggesting that the loss of the XO (696) peptide was not due to digestion by contaminating enzyme (chymotrypsin) from the trypsin batches. This was not investigated further. XO was quantified using the XO (565) peptide. The amount of XO measured in the BPC60 sample (33 mg/g) was twice that measured in the BPC50 sample (16 mg/g) (**Table 4**).

MUC 1. Complete release of MUC 1 occurred at a hydrolysis time between 30 and 60 min (**Figure 3**). MUC 1 had been difficult to measure in this study because it was lost from the intermediate standard (10 mg/g) after 7−8 days (stored at −30 °C) and had to be reprepared fresh from the stock solution. It was the most hydrophobic of the peptides used in this study, and it was likely that it stuck to the vials or precipitated out of solution. In this study, approximately 40% more MUC 1 was measured in the BPC50 sample (18 mg/g) than in the BPC60 sample (11 mg/g).

In summary, we measured an increase in the total MFGM protein in BPC60 (170 mg/g) as compared to BPC50 (132 mg/g). This reflects the difference in total protein between the two samples. As well as a change in MFGM protein amount, relative proportions of some of the individual MFGM proteins changed between the two samples. Although these proportions generally reflect their relative abundance in the millk fat globule membrane (*8*), direct comparison with literature values was difficult because of the different sample types and sample extraction protocols, which influences MFGM protein recovery (*28*, *45*).

Validation. *Assessment of Matrix Effects*. Internal standard methodologies are normally used to circumvent the matrix problems (mainly ion suppression) that are generally associated with ESI-MS methods. To ensure that any matrix suppression issues had been eliminated, standard addition was carried using the BPC50 sample. Standards were spiked at four different levels into the sample solution. The concentration values for the MGFM proteins obtained by standard addition were not significantly different from the internal calibration standard method results, indicating the absence of any major matrix effects using the isotopically labeled internal standard protocol (**Table 5**). An example of standard addition for the ADPH (616) peptide is shown in **Figure 5**.

Assessment of AQUA Technique Using β-Lg. β-Lg was used to check the AQUA technique. This protein was present in both BPC samples, and commercially available standards and an alternative method (SDS-PAGE) were available to quantify this protein (*44*). The results from the SDS-PAGE correlated well with those from the current technique (**Table 6**), giving confidence in the LC-HSRM method.

Limit of Detection. The limits of detection for the peptides were in the range 20−40 pg loaded on column (**Table 2**). This was determined by loading a decreasing amount of the standards onto the LC-HSRM system until the signal was three times the noise level.

Table 5. Comparison of MFGM Protein Concentration Determined by Standard Addition Technique with Current Internal Standard Method for the BPC50 Sample[a]

	β-Lg	FABP	BTN	PAS 6/7 (638)	PAS 6/7 (597)	ADPH (616)	ADPH (759)	XO (565)	MUC 1
standard addition (mg/g)	66	2	39	26	54	12	13	20	16
internal standard (±SD) (mg/g)	68 ± 11	2 ± 0.2	37 ± 5	27 ± 4	47 ± 5	11 ± 1	13 ± 2	16 ± 2	18 ± 1

[a] The standard addition sample was spiked with four different levels of the peptide standard solution (**Figure 5**).

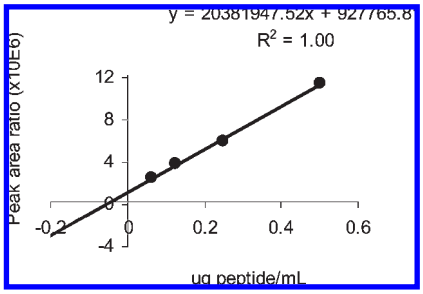

Figure 5. Typical standard addition experiment for the ADPH (616) peptide. The sample was spiked with 0.06, 0.13, 0.25, and 0.5 μg peptide/mL of standard.

Table 6. Level of β-Lg in BPC50 and BPC60 Samples, Measured Using the AQUA Technique and SDS-PAGE

	current method (mg/g)	SDS-PAGE (mg/g)
BPC50	72 ± 6 (*n* = 8)	69 ± 3 (*n* = 2)
BPC60	78 ± 7 (*n* = 6)	74 ± 5 (*n* = 3)

Table 7. Recoveries of MFGM Peptides Spiked into Infant Formula and Casein Hydrolyzate[a]

	FABP	BTN	PAS 6/7 (638)	PAS 6/7 (597)	ADPH (616)	ADPH (759)	XO (565)	MUC 1
				infant formula[b]				
average (*n* = 4)	98	102	94	105	92	105	90	94
CV (%)	7	7	5	4	2	7	7	7
				casein hydrolyzate[c]				
average (*n* = 4)	93	96	94	99	86	99	84	90
CV (%)	5	9	10	11	5	8	3	12

[a] The standards were spiked at 0.25−2% of the protein level. [b] 5.2% protein. [c] 95% protein.

Recovery. Recovery experiments were conducted in commercial infant formula and casein hydrolyzate. Standard peptides were spiked into both of these samples at 0.25−2% of the protein level in the sample. Recovery levels ranging from 84 to 105% (**Table 7**) were observed.

Assay Variation. The coefficient of variation determined from duplicate measurements across four different days ranged from 5 to 15% (**Table 4**). The variations measured in this study are typical for such methods (*46*, *47*) and arise mainly from the day-to-day variations in the sample hydrolysis step.

CONCLUSIONS

In the absence of direct methods for the quantification of MFGM proteins, the AQUA technique proved to be an effective means of measuring multiple proteins simultaneously. The selectivity and the sensitivity of the method also allow MFGM proteins to be quantified at low levels in complex sample matrices. Ongoing improvement of the digestion protocol will improve the assay reproducibility.

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