JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Estimation of Whey Protein in Casein Coprecipitate and Milk Powder by High-Performance Liquid Chromatography Quantification of Cysteine

NICOLAI Z. BALLIN*

Department of Food Chemistry, Regional Veterinary and Food Control Authority, Danish Veterinary and Food Administration, Soendervang 4, DK-4100 Ringsted, Denmark

An analytical high-performance liquid chromatography (HPLC)−fluorescence method for indirect measuring of whey protein in casein coprecipitate and milk powder was developed. Samples were hydrolyzed with HCl, and cysteyl residues were derivatized with 3,3′-dithiodipropionic acid and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The cysteine content was used to calculate the percentage of whey protein in commercial samples with use of European Union Regulation cysteine reference values in both casein and whey protein. Method validation studies were performed for caseinates and milk powder, and results indicate that the present HPLC approach can be applied as a fast method with a standard deviation of repeatability between 3.3 and 9.5%. Applicability was studied by analysis of 40 commercial caseinate samples, and all complied to European legislation with a content of whey protein not exceeding 5%. Finally, an approach used to estimate the cysteine amount in pure casein by comparison of calculated and experimental values questions the generally accepted cysteine reference value in casein, which is most likely an overestimation.

KEYWORDS: Adulteration; caseinate; casein; cysteine; HPLC; milk powder; validation; whey

INTRODUCTION

Dairy products represent a major component of human food, and its quality is of concern to consumers, governmental control authorities, and manufacturers of dairy products. However, caseinates can be an attractive target for adulteration due to the lower price of whey protein to that of casein (CN) and the fact that whey protein is a byproduct in the production of caseinates.

To enforce relevant regulations (*1*) and support dairy manufacturers, adequate analytical methods are required and the measurement of whey protein(s) has recently been studied by different techniques including high-performance liquid chromatography (HPLC) (*2*, *3*), UV spectroscopy (*4*, *5*), and electrophoresis (*6*, *7*). These methods measure specific proteins, which is an obvious approach in determining the content of whey protein and CN in dairy products as long as no protein degradation has occurred. The production of caseinates and milk powder includes elevated temperature and pressure that can result in a change of individual proteins, which is why protein analysis is complicated. This might be part of the reason that most published methods that measure CN and whey protein in dairy products describe the analysis of products other than caseinates and milk powder.

An alternative to protein analysis of caseinates and milk powder in the determination of whey protein content is the measurement of cysteine. The amount of cysteine is then used in combination with the total protein content and cysteine reference values (*1*) in both whey protein and CN to calculate the whey protein and CN contents of samples. The total protein is determined by a Kjeldahl analysis according to IDF 92:1979 (*8*). The quantification of cysteine is an advantageous parameter to measure for the purpose of determining whey protein since the content of cysteine in CN and whey protein differs by more than a factor of 10 and the fact that cysteine content is independent of protein structure as long as it does not include cysteine modifications. Another advantage of measuring cysteine is that European Union (EU) legislation defines the "milk protein content other than CN" (in practice whey protein) to be determined by measuring the −SH and the −S−S− groups linked with proteins (*1*).

Hill and Leary (*9*) developed a method based on the difference in cysteine content in CN and whey protein as a measure of the whey protein fraction in CN coprecipitate. The method was refined (*10*, *11*) and is still frequently used in control laboratories in Europe as a colorimetric method. The colorimetric method (*10*) takes several days to perform and is generally regarded as imprecise, which is why new methods based on cysteine measurement are highly welcomed as alternatives. The present method consists of a HCl hydrolysis and two derivatizing steps followed by HPLC quantification of cysteine.

To ensure that no cysteine amino acids exist as the dimer cystine, S−S bridges are formed with the derivatizing agent 3,3′-dithiodipropionic acid (DTDPA) (*12*). The following derivative is formed with 6-aminoquinolyl-N-hydroxysuccin-

* To whom correspondence should be addressed. Tel: +45 5768 2158. Fax: +45 5768 2199. E-mail: nixb@fvst.dk.

10.1021/jf060199k CCC: $33.50
Published on Web 05/17/2006

imidyl carbamate (*13*) and allows the product to be visualized with the use of fluorescence detection after reversed phase HPLC separation (*14*). The method was validated with the use of three samples of caseinates and one milk powder. Applicability was tested by analysis of 40 commercial caseinates.

As an addendum to the presented method, the experimentally determined cysteine reference values 0.26 (w/w) and 3.06% (w/w) for CN and whey protein, respectively, described by de Koning and van Rooijen (*10*), were evaluated theoretically by investigation of the primary structures of major CNs and whey proteins (*15*). It is not explained by de Koning and van Rooijen (*10*) how they avoided the coprecipitation of whey protein in the CN samples used to experimentally determine the cysteine reference value of CN, which is why it is particularly relevant to examine the reliability of this reference value. Rounded off cysteine reference values are described in the Commission Regulation 1990 (*1*) as 0.25 and 3.0% for CN and whey protein, respectively, which is why these values will be used in the calculation of sample results.

MATERIALS AND METHODS

Chemicals and Standards. DTDPA (99%), *trans*-4-hydroxy-L-proline (98%), sodium acetate trihydrate (min 99%), ethylenediaminetetraacetic acid (EDTA, molecular biology reagent), triethylamine (min 99%), and L-cysteine (97%) were obtained from Aldrich (Germany). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (United Kingdom), phosphoric acid (Baker analyzed, 85%) was from Baker (Holland), and the AccQ-Fluor Reagent Kit was from Waters (Milford, MA) containing 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent and borate buffer. Lyophilized bovine β-CN (min 90%) and lyophilized bovine β-lactoglobulin (LG) (A and B) (approximately 90%) were from Sigma (Germany). The protein content of β-CN and β-LG was determined by Kjeldahl analysis to give 93.74 and 91.35%, respectively.

HPLC Instrumentation and Chromatographic Conditions. The Waters HPLC system consisted of an Alliance 2695 separation module and a 474 scanning fluorescence detector. The system was equipped with a Merck LiChroCART LiChrospher C_{18} end-capped, 5 μm, 4 mm $\times$ 250 mm column (Germany). The chromatographic system (*14*) consisted of eluents A and B. Eluent A was made from concentrated eluent ($A_{concentrated}$). $A_{concentrated}$ consisted of 1.4 M sodium acetate, 70 μM EDTA, and 0.17 M triethylamine. The adjustment to pH 5.02 $\pm$ 0.05 was performed with phosphoric acid. Eluent A was made by mixing 100 mL of $A_{concentrated}$ with 1 L of deionized water. Eluent B was 60% (v/v) acetonitrile in deionized water. Eluents were filtered through a 0.45 μm nylon filter. The flow rate was 1 mL/min, the column temperature was 35 °C, and the injection volume was 20 μL. The column eluate was measured fluorometrically (λ_{ex}, 245 nm; λ_{em}, 395 nm; gain, 100; and attenuation, 32). The chromatographic gradient (*14*) consisted of linear segments: The initial eluent was 100% A followed by 92% A at 17 min, 83% A at 21 min, 73% A at 32 min, 50% A at 34−35 min, 0% A at 37 min, and 100% A at 38 min. Injections were carried out every 45 min.

Acid Treatment and DTDPA Derivatizing. A 30 $\pm$ 1 mg amount of CN sample, 100 $\pm$ 1 mg of milk powder, 1 mL of standards (0.2, 0.3, 0.6, 1.0, 1.2, 1.6, 2.0, and 4.0 mM cysteine in 20 mM HCl), and 1 mL of 20 mM HCl as a "blank" were added to separate 50 mL bottles with screw caps. To each bottle was added 8 mL of 8.25 M HCl, 1 mL of 2% DTDPA (w/v) in 0.2 M NaOH, and 1 mL of 2 mM hydroxyproline in deionized water as an internal standard (IS). In addition, 1 mL of 20 mM HCl was added to bottles that contained sample. The bottles were sealed under nitrogen and incubated at 145 °C for 75 min (*12*). After the bottles reached room temperature, samples and standards were neutralized with 11 mL of 6 M NaOH.

AccQ-Fluor Derivatizing of Amino Acids. Ten microliters of hydrolyzed sample and standard was pipetted into sample vials (45 mm $\times$ 15 mm) and mixed with 70 μL of AccQ-Fluor buffer. Derivatization was initiated with 20 μL of AccQ-Fluor reagent, and hydrolyzed sample and standard were diluted to 1 mL in eluent A. Filtration prior to HPLC analysis was done through a 0.45 μm nylon filter.

Estimation of Whey Protein in CN Coprecipitate and Milk Powder. The ratio between the peak area of derivatized cysteine and the derivatized IS vs cysteine concentration was used to describe the standard curve, and linear regression was used to calculate the total amount of cysteine in samples. The content of whey protein in CN coprecipitate and milk powder was calculated by the formula:

$$\% \text{ whey protein} = (X - 0.25)/(3.0-0.25) \times 100$$

where X = percentage of cysteine in CN coprecipitate or milk powder on the basis of 100% protein. The total amount of protein in samples was determined by a Kjeldahl analysis (IDF 92:1979) (*8*).

Statistics and Interpretation of Results. All validation data were statistically treated with the Microsoft Excel 2000 software. The precision validation results were evaluated according to the criteria defined in the International Organization for Standardization (ISO) 5725 (*16*).

Method Validation. *Limit of Detection/Quantification (LOD/LOQ)*. Commercial bovine β-CN was analyzed analogously to caseinates. The LOD was calculated as three times the standard deviation, and the LOQ was calculated as ten times the standard deviation. To determine the standard deviation, 14 analyses were performed under conditions of repeatability (r).

Precision. Two different technicians carried out precision experiments on seven different days with use of two different HPLC apparatuses and columns. Four validation samples, VS1, VS2, VS3, and VSmp, were tested in two duplicate analyses each day. VS1 was a Na caseinate produced in Denmark in February 2001. VS2 was a Na caseinate produced in Denmark in October 2004. VS3 was a "milk protein agglomerate type calcium caseinate", lot no. 10164010, from DMW International (Holland) produced in August 2004. VSmp was a whole milk powder (28−29% fat) commercially produced in Denmark in July 2005. Validation samples were treated like normal samples. The standard deviations of repeatability (RSD_r) and reproducibility (RSD_R) were generated in accordance with ISO 5725 (*16*) except that the RSD_R was replaced by the standard deviation of intralaboratory reproducibility (RSD_{IR}) because no other laboratories were involved in the HPLC validation process.

Recovery of Cysteine. A 300 mg amount of β-LG was dissolved in 100 mL of 0.2 M NaCl. One milliliter (3 mg/mL) of the β-LG solution was added to bottles with screw caps. Further analysis was done as described for standards; see above. Three duplicate analyses were done on four individual days.

Comparison of Results Obtained at Different Laboratories with Use of Different Methods. To compare our results of whey protein in CN coprecipitates, validation samples VS1 and VS3 were analyzed in duplicate at four different European laboratories using different methods. Laboratories A, B, and C determined the whey protein content in April/May 2005 based on a colorimetric method measuring cysteine as proposed by de Koning et al. (*11*). In October 2004, laboratory D determined the content of whey protein by an electrophoretic analysis (*17*) of lactoferrin (LF), serum albumin (SA), immunoglobulin (Ig) G, β-LG, α-lactalbumin (LA), intact CNs (α_{s2}-, α_{s1}-, β-, and κ-CN), and CN fragments [proteose peptone (PP) 5 and γ-CNs].

Samples. Forty caseinate samples (mixtures of Na, K, and Ca caseinates) from individual batches produced in Denmark from June 2004 to October 2005 were analyzed on a routine basis for law enforcement purposes.

Calculated vs Experimental Found Values for Cysteine in CN and Whey Protein. A theoretical examination of the cysteine content in CN and whey protein was performed based on generally accepted levels of the vast majority of milk proteins (*15*) and compared to cysteine reference values 0.26 (in CN) and 3.06% (in whey protein) (*10*). Proteins included in the theoretical examination of CN and whey proteins were α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, Igs (IgG1, IgG2, IgA, and IgM), α-LA, LF, β-LG, PP, and SA, respectively. Protein databases (http://www.ncbi.nih.gov/entrez/query.fcgi) were used to find the

Table 1. Results from the Precision Study of Validation Samples VS1, VS2, VS3, and VSmp

validation sample	VS1	VS2	VS3	VSmp
number of assays	7	7	7	7
mean whey protein value (%)	6.7	2.4	2.7	14
standard deviation	0.59	0.36	0.34	0.93
RSD_r (%)	7.8	8.0	9.5	3.3
RSD_{IR} (%)	8.9	15	13	7.7

primary structures of proteins, and the molecular mass of 103.15 Da was used to calculate the cysteine content (w/w) in individual proteins.

RESULTS AND DISCUSSION

Method Validation. *Linearity*. The range between 0.2 and 4.0 mM was used to examine linearity. Standard curve linearity was satisfactory with $r^2 > 0.9996$ in seven experiments with a sum of squares of residuals between 0.042 and 0.23.

LOD/LOQ. The average content of cysteine in the purchased β-CN sample was determined to be 0.108% with a standard deviation of 0.007% resulting in a LOD and LOQ of 0.022 and 0.072% cysteine, respectively. According to the cysteine reference values (*10*), a pure caseinate contains 0.25% cysteine whereas a caseinate coprecipitate contains 0.25−3.0% cystein. This means that a LOQ below 0.25% cysteine is satisfactory.

Precision. The three validation CN coprecipitate samples (VS1, VS2, and VS3) show an acceptable precision with RSD_r values between 7.8 and 9.5% and RSD_{IR} values between 8.9 and 15%. VSmp had an RSD_r of 3.3% and an RSD_{IR} of 7.7%, which means that the method is also suitable for the analysis of whole milk powder. Results are shown in **Table 1**.

Recovery of Cysteine. A single protein with known sequence was used to investigate recovery since no certified reference CN or milk powder with a known content of cystein exists. Recovery is expressed as the percentage of total cysteine (2.8%, w/w) found in β-LG (accession no. 1BSY). To ensure a result within the standard curve range, 1 mL of β-LG solution (3 mg/mL) was used. The recovery was found to be 94% with a standard deviation of 6%.

To mimic the real concentration of amino acids in caseinates and milk powder, an experiment with 30 mg (instead of 3 mg) of β-LG added directly to destruction flasks was performed. A 10 times dilution after hydrolysis and derivatization was necessary to stay within the standard curve, and results showed a recovery within the range reported above.

Comparison of Results Obtained at Different Laboratories with Use of Different Methods. The presented HPLC cysteine method found mean values of 6.7 and 2.7% of whey protein in VS1 and VS3 (**Table 1**), respectively, which is close to results obtained by laboratories A−D using different techniques (**Table 2**). The mean whey protein values from laboratories A−D were 7.5 and 2.5% for VS1 and VS3, respectively (excluding the result 0.8% reported by laboratory A). Laboratory A measured the lowest percentage for both CN coprecipitates, and the result 0.8% for VS3 is according to Grubbs' test (*18*) ($P < 0.01$; $n = 4$), an outlier that was consequently rejected. Despite the small number of laboratories that participated in this investigation, the comparison of whey protein results in CN coprecipitates does indicate that the HPLC method based on cysteine quantification is satisfactory.

Table 2. Results from the HPLC Method and Four Different Laboratories Analyzing Validation Samples VS1 and VS3[a]

	% whey protein	
lab/method	VS1	VS3
A	4.7	0.8[b]
B	6.8	2.4
C	9.4	2.5
D	9.0	2.5
HPLC	6.7	2.7

[a] Laboratory A–C used a colorimetric method. Laboratory D used an electrophoretic method. [b] According to Grubbs' test (*18*) ($P < 0.01$; $n = 4$), the 0.8% is an outlier and consequently rejected.

The HPLC cysteine method found a mean value of 14% whey protein in VSmp (**Table 1**), and a comparison with literature results generated from raw milk was done. The whey protein content in raw milk is reported in the literature to be between 12.7 and 22.0% (*2*, *19*, *20*). The large variation is inevitable due to the natural season variation (*21*), and because the result obtained by the HPLC cysteine method is within 12.7 and 22.0%, I conclude that the presented method is useful in the analysis of whey protein in milk powder. The fact that our result is in the low end of literature results (12.7 and 22.0%) is in agreement with the natural low CN-to-whey ratio variation observed for Friesian cows during midsummer (*21*). The comparison was done with raw milk and not milk powder since most methods described in the literature are based on techniques that are influenced by protein conditions and therefore are not suitable for comparison with our results, which are independent of the protein condition.

Analysis of Commercial Samples. Forty commercial Na, K, and Ca caseinates were analyzed for law enforcement purposes by comparing the sample IS and cysteine peak

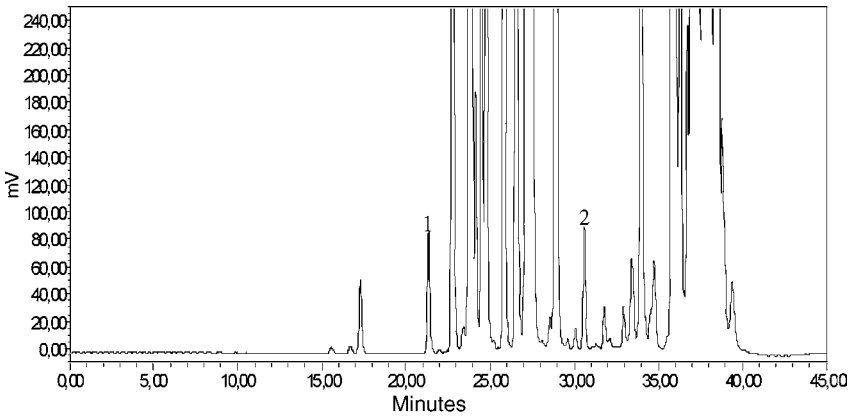

Figure 1. HPLC-fluorescence chromatogram of a typical caseinate sample (validation sample 2) after acid, DTDPA, and AccQ-FluorTM reagent treatment. IS and cysteine peaks are numbered 1 and 2, respectively.

Table 3. Composition of the Major CNs in Bovine Skimmed Milk (*23*) and Their Cysteine Content Assuming that Skimmed Milk Has the Density 1 g/mL[a]

CNs[b]	protein composition in bovine skimmed milk (g/L) min	max	cysteine (%)	accession no.
α_{s1}-CN	12	15	0.00	P02662
α_{s2}-CN$_A$	3	4	0.85	KABOS2
β-CN$_{A2}$	9	11	0.00	P02666
κ-CN$_A$	2	4	1.09	P02668
total protein (g/L)	26	34		
total cysteine (w/w) (%)	0.18	0.23		
mean cysteine content in CN (w/w) (%)	0.21			

[a] The percentage of cysteine is calculated by analysis of the primary structure of individual proteins. [b] The subscript describes the genetic variant. The different major genetic variants do not differ in cysteine content (*23*).

Table 4. Composition of Major Whey Proteins in Bovine Milk (*15*, *23*) and Their Cysteine Content Assuming that Skimmed Milk Has the Density 1 g/mL[a]

whey proteins[b]	protein composition in bovine skimmed milk (g/L) min	max	cysteine (%)	accession no.
β-LG$_A$	2	4	2.87	1BSY
SA$_A$	0.4	0.4	5.44	P02769
α-LA$_B$	0.6	1.7	5.81	NP_776803
IgG1	0.3	0.6	2.76	CAA44700; CAA44699
IgG2	0.05	0.05	2.76[c]	
IgA	0.01	0.01	2.76[c]	
IgM	0.09	0.09	2.76[c]	
LF	0.02	0.1	4.81	CAA38572
total PP	0.6[d]	1.8[d]	0.12[e]	
total protein (g/L)	4.07	8.75		
total cysteine (w/w) (%)	3.13	2.98		
mean cysteine content in whey (w/w) (%)	3.06			

[a] The percentage of cysteine (w/w) is calculated by analysis of the primary structure of individual proteins. [b] The subscript following the protein describes the genetic variant. The different major genetic variants do not differ in cysteine content (*23*). [c] Because of a large homology in the primary structure between domains in different Igs, the percentage 2.76 calculated from IgG1 is used. [d] The amount of PP is described by Swaisgood (*15*). [e] On the basis of the composition of total PP in pasteurized milk stored at 37 °C for 7 days (*24*).

retention time ratio with that of a standard; the cysteine peak was positively verified. Hydroxyproline is a suitable IS since it is not naturally found in milk and it does not interfere with other substances in the chromatogram (**Figure 1**).

The mean whey protein content was found to be 2.6% with a range from 0.7 to 5.0%. European legislation states that "casein and caseinates shall have a milk protein content other than casein not exceeding 5% of total protein content" (*22*), so results show that the level of whey protein in the analyzed caseinates produced in Denmark from June 2004 to October 2005 is acceptable.

Calculated vs Experimental Found Values for Cysteine in CN and Whey Protein. The accuracy of the reference values representing cysteine content in CN and whey protein is important for obtaining accurate results. The cysteine reference values reported by de Koning and van Rooijen (1971) (*10*) are 0.26 and 3.06% for CN and whey protein, respectively. These cysteine reference values were obtained by analysis of CN and whey protein prepared by standard methods in the laboratory of de Koning and van Rooijen. The fact that the purity of the CN and whey protein subject to analysis is not well-described (*10*) questions the validity of the determined cysteine reference values. This fact prompted me to investigate whether the experimentally based cysteine reference values could be accounted for theoretically. On the basis of compositional milk protein values in bovine skimmed milk (*23*), the calculated mean cysteine values for CN and whey protein are 0.21 and 3.06% (w/w), respectively (**Tables 3** and **4**). Consistency between the theoretical and the experimental cysteine values for whey protein support the value of 3.06%. The theoretical value for cysteine in CN is between 0.18 and 0.23% with an average of 0.21%, which is lower than the experimental value (0.26%). This result questions 0.26% as the proper cysteine reference value and indicates that the CN analyzed by de Koning and van Rooijen (*10*) might have contained a fraction of protein normally regarded as whey protein. Replacing the cysteine reference value 0.26% with 0.21% will result in a nonignorable increase in the whey protein amount found when samples of CN coprecipitate and milk powder are analyzed.

ABBREVIATIONS USED

CN, casein; Ig, immunoglobulin; IS, internal standard; LA, lactalbumin; LF, lactoferrin; LG, lactoglobulin; PP, proteose peptone; SA, serum albumin.

ACKNOWLEDGMENT

I thank Solveig Nielsen and Malene Jensen for technical support and Kirsten Halkjær Lund and Erik Wolthers for critical comments.

LITERATURE CITED

(1) Commission Regulation (EEC) No. 2921/90 of 10 October 1990 on aid for the production of casein and caseinates from skimmed milk. L279. *Off. J. Eur. Commun.* **1990**, 22−27.

(2) Bordin, G.; Cordeiro, R. F.; de la, C. B.; Rodriguez, A. R. Identification and quantification of major bovine milk proteins by liquid chromatography. *J. Chromatogr. A* **2001**, *928*, 63−76.

(3) Torre, M.; Cohen, M. E.; Corzo, N.; Rodriguez, M. A.; Diez-Masa, J. C. Perfusion liquid chromatography of whey proteins. *J. Chromatogr. A* **1996**, *729*, 99−111.

(4) Miralles, B.; Bartolome, B.; Ramos, M.; Amigo, L. Determination of whey protein to total protein ratio in UHT milk using fourth derivative spectroscopy. *Int. Dairy J.* **2000**, *10*, 191−197.

(5) Meisel, H. Application of fourth derivative spectroscopy to quantitation of whey and casein in total milk protein. *Milchwissenschaft* **1995**, *50*, 247−251.

(6) Miralles, B.; Rothbauer, V.; Manso, M. A.; Amigo, L.; Krause, I.; Ramos, M. Improved method for the simultaneous determination of whey proteins, caseins and para-kappa-casein in milk and dairy products by capillary electrophoresis. *J. Chromatogr. A* **2001**, *915*, 225−230.

(7) Garcia-Ruiz, C.; Torre, M.; Marina, M. L. Analysis of bovine whey proteins in soybean dairy-like products by capillary electrophoresis. *J. Chromatogr. A* **1999**, *859*, 77−86.

(8) Caseins and caseinates—Determination of protein content (reference method). *Int. IDF Standard* **1979**, *92*.

(9) Hill, R. D.; Leary, J. A Method for estimating the approximate content of whey in coprecipitate. *Aust. J. Dairy Technol.* **1968**, 160−161.

(10) de Koning, P. J.; van Rooijen, P. J. Estimation of whey proteins in casein coprecipitate or in mixtures with milk powder by the use of a modified ninhydrin reaction. *Milchwissenschaft* **1971**, *26*, 1−6.

(11) de Koning, P. J.; van Rooijen, P. J.; Draaisma, J. T. M. An improved manual method for the determination of the cystine plus cysteine content of proteins. *Milchwissenschaft* **1976**, *31*, 261−263.
(12) Tuan, Y. H.; Phillips, R. D. Optimized determination of cystine/cysteine and acid-stable amino acids from a single hydrolysate of casein- and sorghum-based diet and digesta samples. *J. Agric. Food Chem.* **1997**, *45*, 3535−3540.
(13) Cohen, S. A.; Michaud, D. P. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Anal. Biochem.* **1993**, *211*, 279−287.
(14) Liu, H. J.; Chang, B. Y.; Liu, X. X.; Yan, H. W.; Yu, F. H. Determination of amino acids in food and feed by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and reversed-phase liquid chromatographic separation. *J. AOAC Int.* **1995**, *78*, 736−744.
(15) Swaisgood, H. E. Chemistry of the caseins. In *Advanced Dairy Chemistry-Proteins*; Fox, P. F., Ed.; Blackie & Son Ltd.: London, United Kingdom, 1992.
(16) ISO 5725-2. Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. *Int. Org. Standardization* **1994**.
(17) DIN 10472. Determination of casein and whey protein contents of milk and milk products—Electrophoretic method. *Deutches Institut für Normung* **1996**, *1997-09*.
(18) Grubbs, F. E.; Beck, G. Extension of sample sizes and percentage points for significance tests of outling observations. *Technometrics* **1972**, *14*, 847−854.
(19) Lüthi-Peng, Q.-Q.; Puhan, Z. The 4th derivative UV spectroscopic method for the rapid determination of protein and casein in milk. *Milchwissenschaft* **1999**, *54*, 74−77.
(20) Miralles, B.; Bartolome, B.; Amigo, L.; Ramos, M. Comparison of three methods to determine the whey protein to total protein ratio in milk. *J. Dairy Sci.* **2000**, *83*, 2759−2765.
(21) Auldist, M. J.; Walsh, B. J.; Thomson, N. A. Seasonal and lactational influences on bovine milk composition in New Zealand. *J. Dairy Res.* **1998**, *65*, 401−411.
(22) Brendel, V.; Bucher, P.; Nourbakhsh, I.; Blaisdell, B. E.; Karlin, S. Methods and algorithms for statistical analysis of protein sequences. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2002−2006.
(23) Farrell, H. M., Jr.; Jimenez-Flores, R.; Bleck, G. T.; Brown, E. M.; Butler, J. E.; Creamer, L. K.; Hicks, C. L.; Hollar, C. M.; Ng-Kwai-Hang, K. F.; Swaisgood, H. E. Nomenclature of the proteins of cows' milk—sixth revision. *J. Dairy Sci.* **2004**, *87*, 1641−1674.
(24) Andrews, A. T.; Alichanidis, E. Proteolysis of caseins and the proteose-peptone fraction from bovine milk. *J. Dairy Res.* **1983**, *50*, 275−290.

Received for review January 23, 2006. Revised manuscript received April 12, 2006. Accepted April 20, 2006.

JF060199K