Evaluation of Uncertainty in the Determination of A2 β-Casein Content in Milk Powder Using UPLC-MS/MS

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Abstract [**Objectives**] To evaluate the uncertainty in the determination of A2 β-casein content in milk powder using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). [**Methods**] In accordance with JJF 1059.1-2012 and CNAS-GL006-2019, a mathematical model for uncertainty evaluation was developed. The sources of each component during the measurement process, including the purity and weighing of standard substances, preparation of standard solutions, calibration curve fitting, instruments, sample volume adjustment, weighing volume, and measurement repeatability, were analyzed. These uncertainty components were further combined and expanded. [**Results**] The concentration of A2 β-casein in milk powder was determined to be (3.01 ±0.31) g/100 g (k =2). The primary sources of uncertainty influencing these results were the concentration of the injection solution (u_{rel} =4.127%) and the sample volume adjustment process (u_{rel} = 2.873%), whereas the effects of sample weighing and measurement repeatability were comparatively small. [**Conclusions**] This method can be employed to evaluate the uncertainty in the determination of A2 β-casein in milk powder using UPLC-MS/MS, thereby offering a reference for routine analysis and quality control.

Key words UPLC-MS/MS, A2 β-casein, Uncertainty, Milk powder

0 Introduction

β-Casein accounts for approximately 35% of the total casein content in milk. To date, more than ten variants of β-casein have been identified, with types A1 and A2 being the most prevalent[1-2]. The sole difference between these two variants is found at the 67th amino acid, where A2 β-casein contains proline (Pro), whereas A1 β-casein contains histidine (His). This amino acid substitution results in notable differences in their digestive properties. A1 β-casein is more prone to releasing β-casomorphin-7 during digestion. This short peptide is hypothesized to be associated with gastrointestinal discomfort, inflammatory responses, and certain chronic diseases[3-4]. In contrast, A2 β-casein does not generate β-casomorphin-7 during digestion and, therefore, does not generate similar adverse intestinal reactions as A1 β-casein, making it more suitable for human absorption. Currently, the market for products containing A2 β-casein is expanding continuously, which has increased industry focus on issues related to authenticity verification and quality control.

At present, the detection of A2 β-casein primarily includes two approaches: genotyping and protein quantification. The former involves source screening through the identification of the CSN2 genotype in cattle[5], whereas the latter entails the qualitative and quantitative analysis of the target protein in dairy products. Regarding protein detection, there is presently no national standard serving as technical guidance. Within the industry, detection methods predominantly include liquid chromatography-tandem mass spectrometry[6-7], capillary electrophoresis[8], and liquid chromatography[9]. Among these, ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) is characterized by high specificity and sensitivity, establishing it as the prevailing detection technique.

To ensure the accuracy of detection results obtained via UPLC-MS/MS and to standardize the expression of measurement results, it is essential to evaluate the measurement uncertainty associated with A2 β-casein. This study aims to develop a rapid and straightforward evaluation method based on the synthetic uncertainty framework, thereby enhancing the proximity of measured values to their true values. In accordance with the fundamental methods and procedures in *Evaluation and Representation of Measurement Uncertainty* (JJF 1059.1-2012)[10] and *Guidelines for Quantifying Uncertainty in Chemical Analysis* (CNAS-GL006-2019)[11], the uncertainty associated with the established detection method for A2 β-casein was systematically evaluated. Various factors influencing the results were analyzed to enable their control during the detection process, thereby enhancing the overall quality of detection.

1 Materials and methods

1.1 Materials and reagents Commercially available milk powder, A2 β-casein standard substance (purity ≥96.8%), acetonitrile, formic acid (LC-MS grade; Shanghai Anpel Laboratory Technology Co., Ltd.), and ammonium bicarbonate (analytical grade; Shanghai Titan Technology Co., Ltd.) were used in this study.

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1.2 Instruments and equipment The constant temperature water bath shaker was obtained from Shanghai Boxun Medical Biological Instrument Corporation. The ELIX ESSENTIAL 10 pure water system was supplied by Merck, Germany. The Waters Xevo TQ-S ultra-high performance liquid chromatography-mass spectrometer was provided by Waters, USA.

1.3 Methods

1.3.1 Preparation of standard solutions. A2 β-casein standard stock solution (2 mg/mL) was prepared by accurately weighing 20 mg of A2 β-casein standard substance (accurate to 0.000 1 g) into a 10 mL volumetric flask, which was then filled to volume with ammonium bicarbonate solution (100 mmol/L). A2 β-casein isotope-specific peptide standard stock solution (500 μg/mL) was prepared by accurately weighing 5 mg of the isotope-specific peptide standard substance (accurate to 0.000 1 g) into a 10 mL volumetric flask and diluting to volume with water. A2 β-casein isotope-specific peptide intermediate working solution (50 μg/mL) was obtained by transferring 1 mL of the 500 μg/mL stock solution into a 10 mL volumetric flask and diluting to volume with water.

A2 β-casein standard curve working solutions were prepared by pipetting 10, 25, 50, 250, 500 μL, and 1 mL of A2 β-casein standard stock solution (2 mg/mL) into separate containers, each subsequently diluted to a final volume of 1 mL with ammonium bicarbonate solution (100 mmol/L). This procedure yielded A2 β-casein standard curve working solutions with concentrations of 0.02, 0.05, 0.1, 0.5, 1, and 2 mg/mL, respectively.

For the enzymatic hydrolysis of the A2 β-casein standard curve working solution, 100 μL of the aforementioned working solution was transferred into a 2 mL plastic centrifuge tube. Subsequently, 50 μL of A2 β-casein isotope-specific peptide intermediate working solution (50 μg/mL), 830 μL of ammonium bicarbonate solution (100 mmol/L), and 10 μL of alkaline trypsin solution (1 mg/mL) were added. The mixture was thoroughly mixed and subjected to enzymatic digestion at 37 ℃ (±1 ℃) for 200 min. Following incubation, the solution was allowed to cool to room temperature, after which 10 μL of pure formic acid was introduced and the reaction proceeded at room temperature for 30 min. Finally, the solution was filtered through a 0.22 μm membrane filter and collected in a bottle for subsequent injection.

1.3.2 Sample pretreatment. Approximately 1 g (accurate to 0.01 g) of the milk powder sample was weighed into a 50 mL volumetric flask, thoroughly dissolved, and then diluted to the mark with water. The solution was mixed well and, if necessary, subjected to vortex mixing to ensure complete dissolution.

100 μL of the above sample solution was transferred into a 2 mL plastic centrifuge tube. Subsequently, 50 μL of A2 β-casein isotope-specific peptide intermediate working solution (50 μg/mL), 830 μL of ammonium bicarbonate solution (100 mmol/L), and 10 μL of alkaline trypsin solution (1 mg/mL) were added and thoroughly mixed. The mixture was subjected to enzymatic digestion at 37 ℃ (±1 ℃) for 200 min, then cooled to room temperature. Thereafter, 10 μL of pure formic acid was added, and the reaction was allowed to proceed at room temperature for 30 min. Ultimately, the solution was filtered through a 0.22 μm membrane filter and collected in a bottle for injection.

1.3.3 Chromatographic conditions. The chromatographic analysis was conducted using an Acquity UPLC BEH300 C_{18} column (2.1 mm × 100 mm, 1.7 μm) maintained at a temperature of 35 ℃. The injection volume was set to 1 μL. The mobile phase consisted of solvent A1 (0.1% formic acid aqueous solution) and solvent B1 (acetonitrile), with a flow rate of 300 μL/min. The linear gradient elution program was as follows: 0 – 0.5 min, 95% A1; 0.5 – 2.5 min, 95% – 40% A1; 2.5 – 3.0 min, 40% – 0% A1; 3.0 – 5.0 min, 0% A1; and 5.0 – 7.0 min, 95% A1.

1.3.4 Mass spectrometry conditions. Ionization mode was electrospray positive ion mode (ESI +). Detection was performed using multiple reaction monitoring (MRM). The capillary voltage was set at 3.5 kV, desolvation temperature at 500 ℃, and desolvation gas flow rate at 1 000 L/h. The source temperature was maintained at 150 ℃, and the collision chamber gas flow rate was 0.13 mL/min. Other mass spectrometry conditions are presented in Table 1.

Table 1 Mass spectrometry conditions for A2 β-casein and its isotope internal standard

Compound	Retention time//min	Ion pair//*m/z*	Cone voltage//V	Collision voltage//V
A2 β-casein specific peptide	2.80	1 330.40 > 1 428.78 *	51	32
		1 330.40 > 1 945.40		25
A2 β-casein isotope-specific peptide internal standard	2.80	1 337.10 > 1 436.70 *	51	26
		1 337.10 > 1 955.52		22

NOTE * denotes quantitative ions.

1.3.5 Qualitative and quantitative methods. Qualitative analysis was performed by examining retention time and the relative abundance of characteristic ions. Quantification was achieved through the use of the internal standard method.

2 Results and analysis

2.1 Mathematical models The content of A2 β-casein in the sample was calculated using the following Equation (1), with the blank value of the process subtracted from the calculation result. The determination result was expressed as the arithmetic mean of parallel measurements and reported to two decimal places.

$$X = (C \times V \times n \times 100)/(m \times 1\,000)$$

where X denotes the concentration of the analyte in the sample, expressed in g/100 g; C represents the concentration of the analyte in the sample injection solution, measured in μg/mL; V indicates the final volume of the sample solution, expressed in mL; n corresponds to the dilution factor; and m denotes the mass of the sample, expressed in g.

2.2 Analysis of the sources of uncertainty When analyzing milk powder samples using the above method, the primary sources of measurement uncertainty can be categorized as follows: (i) the uncertainty associated with the concentration of the injection solution (C), which encompasses uncertainties arising from the purity of the standard substance, the weighing of the standard substance, the preparation of the standard solution, the linear fitting of the calibration curve (including the addition of internal standards), and the instrument itself; (ii) the uncertainty related to the sample volume adjustment (V); (iii) the uncertainty stemming from the weighing of the sample mass (m); and (iv), the uncertainty attributable to measurement repeatability.

2.3 Evaluation of uncertainty components

2.3.1 Relative standard uncertainty $u_{rel}(C)$ introduced by the concentration of the injection soultion C of A2 β-casein. (i) Evaluation of the relative standard uncertainty component $u_{rel}(m_{standard})$ arising from the purity of the standard substance and the weighing process. According to the certificate, the purity of A2 β-casein was 96.8%. Assuming a rectangular distribution, the relative uncertainty introduced by the purity of the standard substance was calculated as $u_{rel}(m_1) = (1 - 0.968)/\sqrt{3} = 0.018\ 5$.

The electronic balance, possessing a precision of 1/100 000, was verified and deemed qualified. According to the balance verification certificate, the expanded uncertainty was 0.02 mg ($k = 2$). Consequently, the standard uncertainty associated with the weighing process was calculated as $u(m_2) = 0.02\ \text{mg}/2 = 0.01\ \text{mg}$. The mass ($m$) of the standard substance weighed was 20.66 mg, determined using the subtraction method. Therefore, the relative uncertainty introduced by the weighing of the standard substance was $u_{rel}(m_2) = \dfrac{\sqrt{0.01^2 + 0.01^2}}{20.66} = 0.000\ 7$

Ultimately, the relative standard uncertainty, $u_{rel}(m_{standard})$, arising from the purity and weighing of the standard substance was:

$$u_{rel}(m_{standard}) = \sqrt{u_{rel}(m_1)^2 + u_{rel}(m_2)^2} = \sqrt{0.018\ 5^2 + 0.000\ 7^2} \times 100\% = 1.851\%$$

(ii) Evaluation of the relative standard uncertainty component $u_{rel}(v_{standard})$ arising from the standard substance preparation process. In accordance with the *Pipette Verification Regulation* (JJG 646-2006)[12], and by considering either the expanded uncertainty value or the maximum allowable error, the relative standard uncertainties associated with each pipette and volumetric flask were sequentially calculated, as presented in Table 2.

Table 2 Uncertainty arising from preparation of standard solution by volumetric flasks and pipettes

No.	Volume of pipette or volumetric flask	Expanded uncertainty or maximum allowable error	Number of uses	Relative standard uncertainty
1	100 μL pipette (25 μL)	0.4 μL	1	$\frac{0.4}{2 \times 25} = 0.008\ 0$
2	100 μL pipette (50 μL)	0.4 μL	1	$\frac{0.4}{2 \times 50} = 0.004\ 0$
3	100 μL pipette (100 μL)	0.6 μL	1	$\frac{0.6}{2 \times 100} = 0.003\ 0$
4	10 μL pipette (10 μL)	0.4 μL	1	$\frac{0.4}{2 \times 10} = 0.020\ 0$
5	1 000 μL pipette (250 μL)	0.6 μL	1	$\frac{0.6}{2 \times 250} = 0.001\ 2$
6	1 000 μL pipette (500 μL)	0.8 μL	1	$\frac{0.8}{2 \times 500} = 0.000\ 8$
7	1 000 μL pipette (750 μL)	0.8 μL	1	$\frac{0.8}{2 \times 750} = 0.000\ 8$
8	1 000 μL pipette (830 μL)	1.0 μL	1	$\frac{1.0}{2 \times 830} = 0.000\ 5$
9	1 000 μL pipette (950 μL)	1.0 μL	1	$\frac{1.0}{2 \times 950} = 0.000\ 6$
10	1 000 μL pipette (975 μL)	1.0 μL	1	$\frac{1.0}{2 \times 975} = 0.000\ 5$
11	1 000 μL pipette (990 μL)	1.0 μL	1	$\frac{1.0}{2 \times 990} = 0.000\ 5$
12	10 mL volumetric flask (Type A)	±0.02 mL	1	$\frac{0.02}{\sqrt{6} \times 10} = 0.000\ 8$

During this measurement process, the relative standard uncertainties associated with the volume, contributed by both the pipette and the volumetric flask, were combined as follows:

$$u_{rel}(v_{standard}) = \sqrt{1\times0.008\ 0^2+1\times0.004\ 0^2+1\times0.003\ 0^2+1\times0.020\ 0^2+1\times0.001\ 2^2+1\times0.000\ 8^2+1\times0.000\ 5^2+1\times0.000\ 6^2+1\times0.000\ 5^2+1\times0.000\ 5^2+1\times0.000\ 5^2+1\times0.000\ 8^2}\times100\% = 2.221\%$$

(iii) Relative standard uncertainty component $u_{rel}(C_{standard})$ arising from the linear fitting of the calibration curve. The standard solution was analyzed to determine the uncertainty associated with the standard curve fitting. The standard curve comprised five mass concentration points, each measured in duplicate. The concentration and response values of the standard solution were fitted using the least squares method. The data for the standard curve are presented in Table 3. When employing this standard curve for measurement, the test sample solution was measured twice (Table 4). The optimal estimated mass concentration of A2 β-casein in the sample was determined using the regression equation, yielding a value of 0.595 8 mg/mL. The uncertainty was calculated based on the formula derived from fitting the standard curve via the least squares method.

$$u(C_0) = \frac{S(A)}{b}\sqrt{\frac{1}{p}+\frac{1}{mn}+\frac{(y-\bar{y})^2}{b^2\sum_{i=1}^{n}\sum_{i=1}^{m}(C_{ij}-C_0)^2}} = 0.015\ 1$$

$$S(A) = \sqrt{\frac{\sum_{i=1}^{n}\sum_{i=1}^{m}(y_{ij}-y_i)^2}{mn-2}} = 5.321\ 6$$

$$\bar{y} = \frac{\sum_{i=1}^{n}\sum_{i=1}^{m}y_{ij}}{mn} = 0.595\ 8$$

Therefore, the relative standard uncertainty associated with the calibration curve fitting was

$$u_{rel}(C_{standard}) = u(C_0)/\bar{y}\times100\% = 2.534\%$$

where $S(A)$ denotes the standard deviation of the curve; y_i refers to the peak area at each concentration point as calculated by the linear equation; y_{ij} represents the peak area measured at each concentration point; $\bar{y}$ indicates the average response value y_{ij} at each concentration point of the standard curve; b corresponds to the slope of the standard curve; p denotes the number of parallel determinations of the sample; m signifies the number of repeated measurements of the standard solution; n represents the number of mass concentrations of the standard solutions; y_0 indicates the average response value of the test sample solution; C_{ij} corresponds to each concentration point on the standard curve; and C_0 represents the average concentration of the test sample solution.

Table 3 Data for the standard curve of A2 β-casein ($n=2$)

Concentration of standard solution (C)//mg/mL	Response value of standard solution (y)	
	1	2
0.020	8.428	8.524
0.020	13.936	13.819
0.100	28.673	28.645
0.500	132.826	131.927
0.999	284.816	285.087

NOTE The equation of the curve is $y=284.052\ 85x-0.637\ 45$, where the slope (b) is 284.052 85, the intercept (a) is −0.637 45, and the correlation coefficient is 0.999 0.

Table 4 Determination results of samples ($n=2$)

Sample No.	Response value	Concentration of sample solution//mg/mL
1	168.704	0.596 16
2	168.501	0.595 45

(iv) Relative standard uncertainty component $u_{rel}(MS)$ arising from instrument. According to the detection results obtained from the tandem mass spectrometer, the relative expanded uncertainty was determined to be 3%, with a coverage factor (k) of 2. Consequently, the relative standard uncertainty was calculated as $u_{rel}(MS)=3\%/2=1.5\%$.

Ultimately, based on the above calculation results, the relative standard uncertainty $u_{rel}(C)$ associated with the injection concentration (C) of A2 β-casein was determined to be:

$$u_{rel}(C) = \sqrt{u_{rel}(m_{standard})^2+u_{rel}(v_{standard})^2+u_{rel}(C_{standard})^2+u_{rel}(MS)^2} = 4.127\%$$

2.3.2 Evaluation of the relative standard uncertainty component $u_{rel}(v)$ arising from the sample volume adjustment process. During the adjustment process of the sample volume, in accordance with the *Pipette Verification Regulation* (JJG 646-2006)[12], and considering either the expanded uncertainty value or the maximum allowable error, the relative standard uncertainties contributed by each pipette and volumetric flask were sequentially calculated, as presented in Table 5.

During this measurement process, the relative standard uncertainties associated with the volume, contributed by both pipettes and volumetric flasks, were combined as follows:

$$u_{rel}(v) = \sqrt{1\times0.004\ 0^2+1\times0.003\ 0^2+2\times0.020\ 0^2+1\times0.000\ 6^2+1\times0.000\ 4^2}\times100\% = 2.873\%$$

2.3.3 Evaluation of the relative standard uncertainty component $u_{rel}(m)$ arising from the weighing process of samples. The electronic balance, possessing a precision of 1/10 000, was verified and deemed qualified. According to the balance verification certificate, the expanded uncertainty was 0.2 mg ($k=2$). Therefore, the standard uncertainty associated with the weighing process was calculated as $u(m)=0.2\ mg/2=0.1\ mg$. The mass of the sample (m) was 1 000 mg. The subtraction method was utilized for weighing, so the relative standard uncertainty associated with the sample weighing was:

$$u_{rel}(m) = \frac{\sqrt{0.1^2+0.1^2}}{1\ 000}\times100\% = 0.014\%$$

2.3.4 Evaluation of the relative standard uncertainty component $u_{rel}(f_{rep})$ arising from measurement repeatability. Seven identical

samples were weighed and processed concurrently, followed by testing under uniform liquid chromatography conditions. The analytical results are presented in Table 6.

Table 5 Uncertainty arising from preparation of standard solution by volumetric flasks and pipettes

No.	Volume of pipette or volumetric flask	Expanded uncertainty or maximum allowable error	Number of uses	Relative standard uncertainty
1	100 μL pipette (50 μL)	0.4 μL	1	$\frac{0.4}{2\times50}=0.004\ 0$
2	100 μL pipette (100 μL)	0.6 μL	1	$\frac{0.6}{2\times100}=0.003\ 0$
3	10 μL pipette (10 μL)	0.4 μL	2	$\frac{0.4}{2\times10}=0.020\ 0$
4	1 000 μL pipette (830 μL)	1.0 μL	1	$\frac{1.0}{2\times830}=0.000\ 6$
5	50 mL volumetric flask (Type A)	±0.05 mL	1	$\frac{0.05}{\sqrt{6}\times10}=0.000\ 4$

Table 6 Results of measurement repeatability

Sample No.	Response value	Test result//g/100 g
1	169.266	3.029
2	167.821	3.003
3	168.523	3.016
4	167.340	2.994
5	168.704	3.019
6	168.501	3.015
7	168.094	3.008

The A2 β-casein content in seven samples was independently and repeatedly measured. The average value of these measurements was reported as $X_0=3.01$ g/100 g. The standard deviation for a single measurement was calculated using Bessel's correction as follows:

$$s(X)=\sqrt{\frac{1}{7-1}\sum_{i=1}^{7}(X_i-X_0)^2}=0.011$$

$$s(\overline{X})=\frac{S(X)}{\sqrt{n}}=\frac{0.011}{\sqrt{7}}=0.004\ 0$$

Therefore, the relative standard uncertainty $u_{rel}(f_{rep})$ associated with the measurement repeatability of A2 β-casein content was determined as follows:

$$u_{rel}(f_{rep})=\frac{S(\overline{X})}{Xo}=0.133\%$$

2.3.5 Evaluation of relative combined standard uncertainty. Utilizing the formula for the synthesis of relative combined standard uncertainties, each component of relative standard uncertainty was combined, yielding the following result:

$$U_{crel}(X)=\sqrt{u_{rel}(C)^2+u_{rel}(v)^2+u_{rel}(m)^2+u_{rel}(f_{rep})^2}=\sqrt{4.127\%^2+2.873\%^2+0.014\%^2+0.133\%^2}=5.03\%$$

2.3.6 Evaluation of expanded uncertainty. At a 95% confidence level, using a coverage factor $k(x)=2$, the relative expanded uncertainty of the A2 β-casein content measurement was calculated as $U_{rel}=U_{crel}(X)\times k(x)=5.03\%\times2=10.1\%$.

2.3.7 Content measurement results and uncertainty report. The measurement result for A2 β-casein was determined as the average of seven samples, yielding a value of $X=3.01$ g/100 g. The relative expanded uncertainty (U_{rel}) associated with this measurement was 10.1%, corresponding to an expanded uncertainty (U) of 0.31 g/100 g. This expanded uncertainty was calculated by multiplying the combined standard uncertainty by a coverage factor $k(x)=2$, which corresponds to a coverage probability of approximately 95%.

3 Conclusions

In the determination of A2 β-casein in milk powder, the uncertainties associated with various factors, including the concentration of the injection solution (C), the constant volume (V), the weighing of sample mass (m), and measurement repeatability, were systematically evaluated and analyzed. The analysis revealed that the primary sources of uncertainty influencing the test results were the injection concentration (C) and the sample volume adjustment process. The corresponding relative standard uncertainties, $u_{rel}(C)$ and $u_{rel}(v)$, were determined to be 4.127% and 2.873%, respectively. Ultimately, this study determined that the relative expanded uncertainty (U_{rel}) of the method was 10.1%, corresponding to an expanded uncertainty (U) of 0.31 g/100 g, with a coverage factor $k(x)=2$. The development of this calculation model not only offers a reference framework for evaluating the uncertainty in the determination of A2 β-casein content in milk powder but also ensures the reliability of routine analytical data, thereby significantly contributing to laboratory quality control.

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(To page 35)

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(From page 30)

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