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Evaluation of Uncertainty for Determination of Stevioside Content in Fermented Milk by HPLC

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Abstract [**Objectives**] The paper was to establish an evaluation method for the uncertainty of stevioside (including stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside F, Dulcoside A, rubusoside and steviolbioside) content determination in fermented milk based on HPLC. [**Methods**] The mathematical model of stevioside content and the propagation rate of uncertainty were established, and the sources of uncertainty were analyzed. [**Results**] The uncertainty mainly came from four main aspects, including standard uncertainty $u(C)$ introduced by solution concentration C, standard uncertainty $u(V)$ introduced by sample volume V, standard uncertainty $u(m)$ introduced by sample mass m weighing and standard uncertainty $u(f_{rep})$ introduced by measurement repeatability of stevioside content after sample dissolution and constant volume. The uncertainty estimation table and fishbone chart of stevioside content X determination were established. The relative synthetic standard uncertainty of stevioside content was obtained, and the standard uncertainty was extended to form the measurement result of stevioside content and its uncertainty report. [**Conclusions**] The evaluation results can be directly applied to the daily practical detection work.

Key words HPLC, Stevioside, Uncertainty, Relative synthetic standard uncertainty, Extended standard uncertainty

1 Introduction

Stevioside is a series of tetracyclic diterpenoids found in the leaves of *Stevia rebaudiana*, a perennial herb belonging to Compositae, and its fine products are white powder. It is a kind of natural sweetener with low calories and high sweetness, widely used in low calorie drinks, yogurt, tablet candy and medicine, being an important food additive. In recent years, stevioside has been used in drinks, biscuits, candies and dairy products, *etc.* Therefore, it is necessary to conduct rapid, sensitive and effective determination of stevioside content for monitoring product quality and controlling product risks, which is of great significance to food safety and national health.

The application of uncertainty has received more and more attention in all walks of life in China. The uncertainty for determination of stevioside content in fermented milk by HPLC was discussed in this paper. The evaluation results can be directly applied to the daily practical detection work.

2 Materials and methods

2.1 Materials and reagents Acetonitrile (chromatographically pure, Knowles); sodium dihydrogen phosphate, phosphoric acid (chromatography pure, Tianjin Damao Chemical Reagent Factory); zinc acetate, potassium ferrocyanide, glacial acetic acid (guarantee reagent, Tianjin Damao Chemical Reagent Factory); stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside F, Dulcoside A, rubusoside and steviolbioside (standard, Anpel).

2.2 Instruments and equipments High performance liquid chromatography (Agilent); AB265-S analytical balance (Mettler-Toledo, Switzerland); ultrasonic cleaner (Changzhou Kaihang Instrument Co., Ltd.); MS3 vortex mixer (IKA). Measuring instruments and equipments: electronic balance (1/10 000), 50 mL volumetric flask, 10 mL volumetric flask.

2.3 Methods

2.3.1 Preparation of filtrate. About 10 g of fermented milk (accurate to 0.1 mg) were loaded into a beaker, added with 15 mL of water and mixed evenly. After treated by ultrasonic sound in 45 ℃ water bath for 15 min, the solution was mixed with 15 mL of acetonitrile, and then continuously treated by ultrasonic sound in 45 ℃ water bath for 10 min to ensure that all of rebaudioside A in the fermented milk were extracted. Approximately 2 mL of zinc acetate solution and 2 mL of potassium ferrocyanide solution were added, mixed well, and transferred to a 50 mL volumetric flask, and finally set to the constant volume with purified water. After standing, the solution was filtered through 0.45 μm organic phase microporous filter membrane, and the filtrate was to be tested.

2.3.2 Preparation of standard solution (volumetric method). (i) Standard stock solution (1.0 mg/mL): 0.05 g of standard (accurate to 0.1 mg) were placed in a 50 mL volumetric flask, completely dissolved in 30% acetonitrile aqueous solution, and set to the constant volume. (ii) Rebaudioside A standard intermediate solution: 1mL of each stevioside standard stock solution were absorbed, and set to the constant volume in 10 mL volumetric flasks separately. (iii) Rebaudioside A standard working solution: stevioside standard intermediate solution (0.2, 0.5, 1.0,

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2.0, 5.0 mL) were absorbed into 10 mL volume flasks and set to the constant volume with 30% acetonitrile aqueous solution. The series concentrations were 2.0, 5.0, 10.0, 20.0, and 50.0 μg/mL.

The above working solutions were prepared immediately before use.

2.3.3 Calculation of rebaudioside A content. The content X of rebaudioside A in fermented milk was calculated by working curve.

2.3.4 Mathematical models and propagation rate of uncertainty. The formula for the determination of rebaudioside A content X by HPLC was:

$$X = (C \times V \times n)/m \quad (1)$$

where X stands for the content of rebaudioside A in the sample, mg/kg; C stands for the concentration of rebaudioside A in the test solution, μg/mL; V stands for the constant volume, mL; n stands for the dilution ratio; m stands for the weighing mass of the sample, g. The calculation result retained 3 significant digits. By combining similar influencing factors, repetitive factors of inputs C, V, m and n were combined and classified as repetitive factors of output X. Therefore, it was not necessary to evaluate the uncertainty component by repeated introduction of inputs C, V and n separately, but the uncertainty component by repeated introduction of measurement results (rebaudioside A content X) was directly assessed. The test did not involve dilution, so n was not assessed. For this reason, equation (1) was changed as follows:

$$X = (CV/m) \times f_{rep} \quad (2)$$

where f_{rep} is the correction factor of influencing factors of measurement repeatability, and is assigned with 1. From formula (2), it can be seen that the inputs C, V, m and f_{rep} were interrelated, and the standard uncertainty was synthesized by square and root method. Moreover, the formula for the content of rebaudioside A was only the product and quotient of the inputs C, V and m, so the synthetic standard uncertainty was calculated in the following simplified way.

$$u_c(X) = \sqrt{[c_1 u_{rel}(C)]^2 + [c_2 u_{rel}(V)]^2 + [c_3 u_{rel}(m)]^2 + [c_4 u_{rel}(f_{rep})]^2} = \sqrt{u_{rel}(C)^2 + u_{rel}(V)^2 + u_{rel}(m)^2 + u_{rel}(f_{rep})^2} \quad (3)$$

where sensitivity coefficients are: $c_1 = 1$, $c_2 = 1$, $c_3 = -1$, $c_4 = 1$.

3 Results and analysis

3.1 Sources of measurement uncertainty Table 1 and Fig. 1 show the sources of uncertainty and related information. The uncertainty of output X came from four aspects.

Table 1 Prediction of uncertainty for determination of rebaudioside A content X

No.	Source of uncertainty	Type	Probability distribution	Coverage factor	Standard uncertainty	
					Symbol	Numerical value
1	**Standard uncertainty of solution concentration C measurement**					
	Relative standard uncertainty component introduced by fitting calibration curve	A	–	–	$u_{1rel}(C)$	3.46%
	Relative standard uncertainty component introduced by standard solution weighing	B	Uniform	$\sqrt{3}$	$U_{2rel}(Cm)$	2.05%
	Standard uncertainty introduced by standard solution preparation	B	Triangular	$\sqrt{6}$	$U_{3rel}(Cv)$	1.30%
	Relative standard uncertainty introduced by purity of standard solution	B	Uniform	2	$U_{4rel}(C)$	0.26%
	Relative standard uncertainty of solution concentration C measurement	Synthesis	–	–	$u_{rel}(C)$	4.23%
2	**Standard uncertainty of constant volume of sample V**					
	Standard uncertainty component introduced by calibration of volumetric flask	B	Triangular	$\sqrt{6}$	$u_a(V)$	0.02 mL
	Relative standard uncertainty introduced by constant volume of sample V	Synthesis	–	–	$u_{rel}(V)$	0.04%
3	**Standard uncertainty of sample mass m weighing**					
	Standard uncertainty component introduced by balance calibration	B	Uniform	$\sqrt{3}$	$u_1(m)$	0.29 mg
	Standard uncertainty component introduced by balance resolution	B	Uniform	$\sqrt{3}$	$u_2(m)$	0.029 mg
	Relative standard uncertainty of mass m weighing	Synthesis	–	–	$u_{rel}(m)$	0.41%
4	Standard uncertainty introduced by measurement repeatability of rebaudioside A content					
	Relative standard uncertainty introduced by measurement repeatability	A	–	–	$u_{rel}(frep)$	0.61%
5	Synthetic standard uncertainty of the measurement result of rebaudioside A content X in fermented milk $u_c(X) = \sqrt{u_{rel}^2(C) + u_{rel}^2(V) + u_{rel}^2(m)^2 + u_{rel}^2(f_{rep})} = 4.29\%$					
6	The content of rebaudioside A is given by the average value of 6 samples $X_0 = 15.4$ mg/kg. The relative extended uncertainty $U_{rel} = 8.58\%$ (or extended uncertainty $U = 1.32$ mg/kg) of the measured results of rebaudioside A content is given by the synthetic standard uncertainty multiplied by the coverage factor $k(x) = 2$, providing the coverage probability of $p \approx 95\%$.					

3.1.1 Standard uncertainty $u(C)$ introduced by solution concentration C after sample dissolution and constant volume. Standard uncertainty $u(C)$ introduced by solution concentration C after sample dissolution and constant volume included four sources: standard uncertainty introduced by least square fitting calibration curve $u_{1rel}(C)$; relative standard uncertainty introduced by standard solution weighing $U_{2rel}(Cm)$; standard uncertainty introduced by standard stock solution and working solution volume in standard solution preparation process $U_{2rel}(Cv)$; standard uncertainty introduced by standard solution concentration $u_{3rel}(C)$.

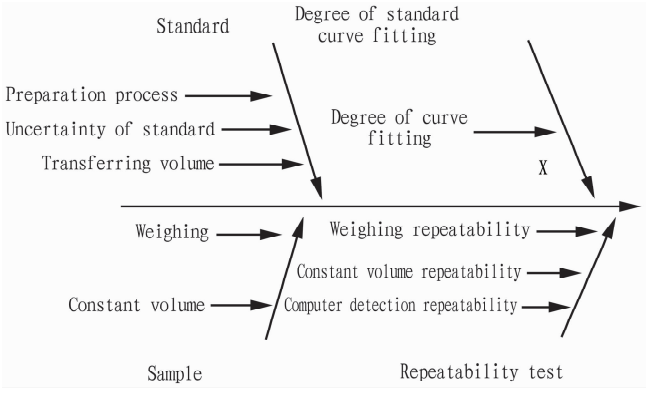

Fig. 1 Fishbone chart of influencing factors of uncertainty

3.1.2 Standard uncertainty $u(V)$ introduced by sample volume V. Standard uncertainty $u(V)$ introduced by sample volume V included three sources: calibration, repeatability and temperature effect. Repeatability was classified into the repeatability of rebaudioside A content $u(f_{rep})$. Since the laboratory was equipped with effective moisture and temperature control systems such as air supply and exhaust and ice water, the standard uncertainty introduced by temperature could be ignored, and only the standard uncertainty $u(V)$ introduced by calibration of pipette and constant volume flask was evaluated, namely the uncertainty component introduced by constant volume of the sample.

3.1.3 Standard uncertainty $u(m)$ introduced by sample mass m weighing. Because the mass m could not be measured directly, it was actually obtained by using the decrement method through two weighing, that is, by one reset weighing. The uncertainty of these measurements was derived from three sources: calibration, repeatability, and resolution, and repeatability was classified into the repeatability of rebaudioside A content $u(f_{rep})$. Therefore, only the standard uncertainty introduced by calibration of analytical balance $u_1(m)$ and that introduced by resolution of analytical balance $u_2(m)$ were evaluated. Because the same balance was used to weigh within a narrow range, the sensitivity of balance mass difference could be negligible.

3.1.4 Standard uncertainty introduced by measurement repeatability of rebaudioside A content $u(f_{rep})$.

3.2 Evaluation of uncertainty

3.2.1 Evaluation of standard uncertainty $u(C)$ for determination of solution concentration C of rebaudioside A sample after constant volume. (i) Evaluation of relative standard uncertainty component $u_{1rel}(C)$ of sample measurement introduced by fitting calibration curve. The experimental data of rebaudioside A are shown in Table 2.

The equation of calibration curve was:

$$A_i = 2.036\ 8 \times C_i - 0.196\ 2.$$

where A_i is the peak area of rebaudioside A; C_i is the concentration of rebaudioside A, and the correlation coefficient is 0.999 8.

$$A_i = aC_i + b \tag{4}$$

Six ($m=6$) parallel measurements were made on the sample, and the average A_0 and average sample concentration C_0 were obtained as follows: $A_0 = 6.0$, $C_0 = 3.067\ 1$ μg/mL (Table 3).

The standard uncertainty component $u_1(C)$ for determination of solution concentration C introduced by least square fitting calibration curve can be calculated.

Table 2 Experimental data of rebaudioside A

No.	C_i // μg/mL	A_i	$(C_i - \overline{C_i})^2$	$(A_i - A_X)^2 \times 10^{-3}$
1	1.562 1	3.0	209.981 0	0.211 8
2	1.562 1	3.1	209.981 0	13.122 1
3	1.562 1	3.2	209.981 0	46.032 4
4	4.981 6	9.5	122.571 9	202.726 2
5	4.981 6	9.1	122.571 9	722.927 2
6	4.981 6	9.2	122.571 9	562.876 9
7	9.516 3	19.3	42.726 1	12.886 3
8	9.516 3	19.3	42.726 1	12.886 3
9	9.516 3	19.2	42.726 1	0.182 7
10	17.542 7	36.1	2.219 7	319.708 3
11	17.542 7	36.3	2.219 7	585.879 3
12	17.542 7	36.3	2.219 7	585.879 3
13	46.661 4	94.6	936.885 2	59.168 5
14	46.661 4	94.7	936.885 2	20.519 3
15	46.661 4	94.6	936.885 2	59.168 5
Σ	240.792 3	487.5	3 943.151 7	3 204.174 9
Average value	16.052 8	32.500 0	–	–

Note: A_i refers to the peak area measured on the computer, and $A_x = aC_i + b$

Table 3 Independent repeated measurement results of the content of 6 samples

Sample No.	A_i	C_i // μg/mL
1	6.0	3.050 8
2	6.1	3.099 9
3	5.9	3.001 7
4	6.1	3.099 9
5	6.0	3.050 8
6	6.1	3.099 9
Average value	6.0	3.067 1

The standard deviation of regression was:

$$S = \sqrt{\frac{1}{n-2}\sum_{i=1}^{n}(A_i - A_x)^2} = 0.410\ 7 \tag{5}$$

$$u_1(C) = s(C_0) = \frac{s}{a}\sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(A_0 - \overline{A_i})^2}{a^2\sum_{i=1}^{n}(C_i - \overline{C_i})^2}}$$

$$= \frac{0.410\ 7}{2.036\ 8}\sqrt{\frac{1}{6} + \frac{1}{15} + \frac{(6.0 - 32.5)^2}{2.036\ 8^2 \times 3\ 943.151\ 7}}$$

$$= 0.106\ \text{μg/mL} \tag{6}$$

where A_x is the peak area calculated by formula (4) when $C = C_i$; $n = 15$ is the number of data pairs (C_i, A_i); $m = 6$ is the number of parallel measurements of the sample. Therefore, relative standard uncertainty introduced by fitting calibration curve was:

$$u_{1rel}(C) = u_1(C)/C_0 = 0.106/3.067\ 1 = 3.46\% \tag{7}$$

(ii) Evaluation of relative standard uncertainty component $u_{2rel}(C)$ introduced by preparation of standard solution.

Evaluation of relative standard uncertainty component $u_{2rel}(Cm)$ introduced by standard solution weighing: Because the mass m could not be measured directly, it was actually obtained by using the decrement method through two weighing, that is, by one

reset (empty flask) weighing. The uncertainty of these measurements was derived from three sources: calibration, repeatability, and resolution, and repeatability was classified into the repeatability of rebaudioside A content $u(f_{rep})$. Therefore, only the standard uncertainty introduced by calibration of analytical balance $u_1(m)$ and that introduced by resolution of analytical balance $u_2(m)$ were evaluated. The sensitivity of balance mass difference could be negligible.

Evaluation of standard uncertainty $u_1(m)$ introduced by electronic balance calibration: The electronic balance had been verified to be qualified. It can be seen from the balance verification that the maximum allowable error was ± 0.5 mg in the weighing range of $0 \leqslant m \leqslant 5 \times 10^4 e$ ($e = 1$ mg), obeying uniform distribution, with the section half width of $a_{m1} = 0.5$ mg and the coverage factor of $k_{m1} = \sqrt{3}$. The resulting standard uncertainty was:

$$u_1(m) = a_{m1}/k_{m1} = 0.05 \text{ mg}/\sqrt{3} = 0.29 \text{ mg} \quad (8)$$

Evaluation of standard uncertainty $u_2(m)$ introduced by balance resolution: It can be seen from the balance specification that its resolution was 0.1 mg, conforming to uniform distribution, with the section half width of $a_{m2} = 0.05$ mg and the coverage factor of $k_{m2} = \sqrt{3}$. The resulting standard uncertainty was:

$$u_2(m) = a_{m2}/k_{m2} = 0.05 \text{ mg}/\sqrt{3} = 0.29 \text{ mg} \quad (9)$$

Therefore, the standard uncertainty introduced by electronic balance calibration was:

$$u_{2rel}(Cm) = \sqrt{u_1(m)^2 + u_2(m)^2} = \sqrt{0.29^2 + 0.029^2} = 0.29 \text{ mg} \quad (10)$$

The mass of rebaudioside A standard was actually given by the decrement method through two measurements, so the relative standard uncertainty component of m weighing was:

$$u_{2rel}(Cm) = (0.29 \text{ mg} \times \sqrt{2})/20 \text{ mg} = 2.05\% \quad (11)$$

Evaluation of standard uncertainty $u_{2rel}(Cv)$ introduced by calibration of pipette and volumetric flask in the preparation of standard curve:

Table 4 Uncertainty introduced by calibration of pipette in quasi-solution preparation

Range of pipette (transferring volume) // mL	Allowable error μL	Standard uncertainty μL	Relative standard uncertainty %	Symbol of relative standard uncertainty
200 μL pipette (200)	3	1.224 7	0.612 4	$u_{rel\,200}(V)$
1 000 μL pipette(500)	10	4.082 5	0.816 5	$u_{rel\,500}(V)$
1 000 μL pipette (1 000)	10	4.082 5	0.408 2	$u_{rel\,1\,000}(V)$
5 000 μL pipette (2 000)	30	12.247 4	0.612 4	$u_{rel\,2\,000}(V)$
5 000 μL pipette (5 000)	30	12.247 4	0.244 9	$u_{rel\,5\,000}(V)$

The relative standard uncertainty of sample volume V introduced by calibration of volumetric flasks when preparing stock solution and constant volume of working solution (Table 4) was as follows.

The allowable error of 50 mL grade A volumetric flask used in this experiment was 0.05 mL. The triangular distribution was adopted, $a_1(V) = 0.05$ mL, and the coverage factor was $k_1(V) = \sqrt{6}$, so the uncertainty $u_1(V)$ introduced by the volume of volumetric flask was: $u_1(V_{10}) = 0.05/\sqrt{6} = 0.020\,4$ mL.

The relative standard uncertainty was: $u_{rel}(V_{10}) = 0.020\,4/50 = 0.040\,8\%$.

The allowable error of 10 mL grade A volumetric flask used in this experiment was 0.02 mL. The triangular distribution was adopted, $a_1(V) = 0.02$ mL, and the coverage factor was $k_1(V) = \sqrt{6}$, so the uncertainty $u_1(V)$ introduced by the volume of volumetric flask was: $u_2(V_{10}) = 0.02/\sqrt{6} = 0.008\,16$ mL.

The relative standard uncertainty was: $u_{rel}(V_{10}) = 0.008\,16/10 = 0.081\,6\%$.

In the measurement process, the standard products used 50 mL volumetric flask once, 10 mL volumetric flasks 6 times, 200 μL pipette once, 1 000 μL (transferring 500 μL) pipette once, 1 000 μL (transferring 1 000 μL) pipette once, 5 000 μL (transferring 2 000 μL) pipette once, 5 000 μL (transferring 5 000 μL) pipette once. Therefore, the uncertainty was caused by the volume in the preparation process.

The uncertainty introduced by the volume of pipette was:

$$u_{rel}(VQ) = \sqrt{u_{rel200}(V)^2 + u_{rel500}(V)^2 + u_{rel1000}(V)^2 + u_{rel2000}(V)^2 + u_{rel5000}(V)^2} = 1.28\% \quad (12)$$

The uncertainty introduced by the volume of volumetric flask was:

$$u_{rel}(VR) = \sqrt{6u_{rel}(V_{10})^2 + u_{rel}(V_{50})^2} = 0.20\% \quad (13)$$

The uncertainty of standards introduced by pipette and volumetric flask was:

$$U_{3rel}(C_V) = \sqrt{u_{rel}(VQ)^2 + u_{rel}(VR)^2} = 1.30\% \quad (14)$$

(iii) Evaluation of relative standard uncertainty $u_{3rel}(C)$ introduced by standard concentration.

According to the standard certificate of sodium hyaluronate, the extended uncertainty of sodium hyaluronate standard solution was 0.5%, $k = 2$, purity of standard product 98%, and the relative standard uncertainty was:

$$u_{3rel}(C) = 0.5\%/(98\% \times 2) = 0.26\% \quad (15)$$

(iv) Evaluation of synthetic standard uncertainty $u_{rel}(C)$ of solution concentration C:

$$u_{rel}(C) = \sqrt{u_{1rel}^2(C) + u_{2rel}^2(Cm) + u_{3rel}^2(CV) + u_{4rel}^2(C)} = \sqrt{3.46\%^2 + 2.05\%^2 + 1.30\%^2 + 0.26\%^2} = 4.23\% \quad (16)$$

3.2.2 Standard uncertainty evaluation of sample volume V. The standard uncertainty $u(V)$ of sample volume V included two sources: calibration and repeatability effect. Repeatability was included in f_{rep} repeatability of rebaudioside A content, and only the standard uncertainty $u(V)$ introduced by calibration was assessed.

The allowable error of 50 mL grade A volumetric flask used in this experiment was ± 0.05 mL. The triangular distribution was adopted, $a(V) = 0.05$ mL, and the coverage factor was $k(V) = \sqrt{6}$, so the uncertainty $u_a(V)$ introduced by the volume of volumetric flask was:

$$u_a(V) = 0.05/\sqrt{6} = 0.020 \text{ mL} \quad (17)$$

The relative standard uncertainty was:

$$u_{rel}(V) = 0.020/50 = 0.04\% \quad (18)$$

3.2.3 Evaluation of standard uncertainty $u(m)$ introduced by sample mass m weighing. Because the mass m could not be measured directly, it was actually obtained by using the decrement method through two weighing, that is, by one reset (empty flask) weighing. The uncertainty of these measurements was derived from three sources: calibration, repeatability, and resolution, and repeatability was classified into the repeatability of rebaudioside A content $u(f_{rep})$. Therefore, only the standard uncertainty introduced by calibration of analytical balance $u_1(m)$ and that introduced by resolution of analytical balance $u_2(m)$ were evaluated. The sensitivity of balance mass difference could be negligible.

Evaluation of standard uncertainty $u_1(m)$ introduced by electronic balance calibration: The electronic balance had been verified to be qualified. It can be seen from the balance verification that the maximum allowable error was ± 0.5 mg, obeying uniform distribution, with the section half width of $a_{m1} = 0.5$ mg and the coverage factor of $k_{m1} = \sqrt{3}$. The resulting standard uncertainty was:

$$u_1(m) = a_{m1}/\sqrt{3} = 0.5\ mg/\sqrt{3} = 0.29\ \text{mg} \quad (19)$$

Evaluation of standard uncertainty $u_2(m)$ introduced by balance resolution: It can be seen from the balance specification that its resolution was 0.1 mg, conforming to uniform distribution, with the section half width of $a_{m2} = 0.05$ mg and the coverage factor of $k_{m2} = \sqrt{3}$. The resulting standard uncertainty $u_2(m)$ was:

$$u_2(m) = a_{m2}/\sqrt{3} = 0.5\ \text{mg}/\sqrt{3} = 0.029\ \text{mg} \quad (20)$$

Evaluation of relative standard uncertainty component $u(m)$ introduced by sample mass m weighing: The uncertainty components $u_{1m}(m)$ and $u_{2m}(m)$ were interrelated, and the synthetic standard uncertainty introduced by sample mass m weighing was obtained by square and root method:

$$\begin{aligned} u(m) &= \sqrt{u_{1m}^2(m) + u_{2m}^2(m)} \\ &= \sqrt{0.29^2 + 0.029^2} \\ &= 0.29\ \text{mg} \end{aligned} \quad (21)$$

The sample mass $m = 10.000\ 0$ g $= 10\ 000$ mg, which was actually obtained by subtracting two measurements using the decrement method, so the uncertainty component $u_{rel}(m)$ of sample mass m weighing was:

$$u_{rel}(m) = \frac{\sqrt{2}u(m)}{m} = \frac{\sqrt{2} \times 0.29}{10\ 000} = 0.41\% \quad (22)$$

3.2.4 Six copies of samples were weighed, processed simultaneously, and tested under the same conditions. The analysis results were as follows.

Table 5 Independent repeated determination of rebaudioside A content in 6 samples

Sample No.	A_i	C_i // μg/mL	Result // mg/kg
1	6.0	3.050 8	15.4
2	6.1	3.099 9	15.5
3	5.9	3.001 7	15.1
4	6.1	3.099 9	15.8
5	6.0	3.050 8	15.3
6	6.1	3.099 9	15.5
Average value	6.0	3.067 1	15.4

The content of rebaudioside A in 6 samples was determined independently and repeatedly, and the average value of the measured results was $X_0 = 15.4$ mg/kg (Table 5). The standard deviation of a single measurement was calculated by using Bessel's formula.

$$s(X) = \sqrt{\frac{1}{6-1}\sum_{i=1}^{6}(X_i - X_0)^2} = 0.23\ \text{mg/kg} \quad (23)$$

The relative standard uncertainty introduced by measurement repeatability of content was:

$$r_{rel}(f_{rep}) = \frac{S(X)}{\sqrt{n} \times \overline{X}} = \frac{0.23}{\sqrt{6} \times 15.4} = 0.61\% \quad (24)$$

3.3 Evaluation of synthetic uncertainty and extended uncertainty

3.3.1 Evaluation of relative synthetic standard uncertainty of outputs. The relative synthetic standard uncertainty of rebaudioside A content was calculated by formulae (16), (18), (22) and (24):

$$\begin{aligned} u_c(X) &= \sqrt{u_{rel}(C)^2 + u_{rel}(V)^2 + u_{rel}(m)^2 + u_{rel}(f_{rep})^2} \\ &= \sqrt{4.23\%^2 + 0.04\%^2 + 0.41\%^2 + 0.61\%^2} \\ &= 4.29\% \end{aligned} \quad (25)$$

3.3.2 Evaluation of extended standard uncertainty for determination of rebaudioside A content in fermented milk. Because of coverage factor $k(x) = 2$ and coverage probability $p \approx 95\ \%$, the relative extended uncertainty U_{rel} of the measurement results of rebaudioside A content in raw materials was: $U_{rel} = u_{crel}(X) \times k(x) = 4.29\% \times 2 = 8.58\%$.

3.3.3 Measurement results of rebaudioside A content and uncertainty report. The content of rebaudioside A was given by the average value of 6 samples $X_0 = 15.4$ mg/kg. The relative extended uncertainty $U_{rel} = 8.58\%$ (or extended uncertainty $U = 1.32$ mg/kg) of the measured results of rebaudioside A content was given by the synthetic standard uncertainty multiplied by the coverage factor $k(x) = 2$, providing the coverage probability of $p \approx 95\%$.

4 Conclusions

There were four main sources of uncertainty in the analysis of stevioside content (rebaudioside A as an example). The above evaluation process showed that the standard uncertainty for determination of solution concentration C was the main source. It can be clearly seen from Table 1 that the relative standard uncertainty component introduced by fitting calibration curve and that introduced by standard solution weighing were the main sources of standard uncertainty for determination of solution concentration C. Since the transferring volume of standard solution and the weighing sample of standard product were very small in the test, it was an effective means to control the uncertainty of this experiment by strictly controlling the preparation of standard solution and the weighing procedure of standard product, operating in accordance with the standard specification of weighing, and periodically calibrating balance and pipette.

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