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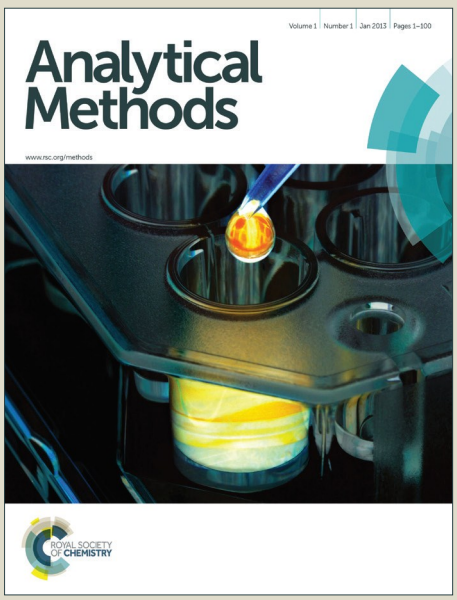

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Certification of Reference Materials for Analysis of Isoflavones Genistin and Genistein in Soy Products

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Abstract

Soy isoflavones are a class of secondary metabolites in the growth process of soybeans or other legumes. Many studies support their roles in the prevention and treatment of cancer, arterial sclerosis, osteoporosis and menopausal syndrome. Genistin and genistein, as two highest amout active ingredients in soy isoflavones, were developed into two new certified reference materials (CRMs), respectively in this work. According to the guidelines of development of CRMs mainly including ISO Guides 34:2009 and 35:2005, studies on sample preparation, homogeneity studies, stability studies, characterization, and uncertainties estimation were carried out. In the characterization, two methods based on different theories, namely, differential scanning calorimetry (DSC) and coulometric titrimetry (CT) were employed. Genistin and genistein CRMs certified values and corresponding expanded uncertainties, obtained from the combined standard uncertainty multiplied by the coverage factor (k = 2), for a confidence level of 95 %, were 99.7 % ± 0.3 % and 99.3 % ± 0.5 %. The mass balance (MB) method was employed to cross-checked the results. Genistin and genistein CRMs have been approved and assigned as a grade primary reference material by the national administrative committee. These CRMs can be applied to analysis of soy isoflavones in relative products, such as (fermenting) soybean foods or medicines.

Introduction

Soy isoflavones are a class of secondary metabolites in the growth process of soybeans or other legumes, which are classified as phytoestrogens (PE) for their estrogen-like effects [1~2]. They are responsible for many of health benefits of soy consumption including cholesterol, heart disease, breast cancers, prostate cancers, bone health, menopausal symptom, weight loss, renal function, cognitive function and so on [3~5]. Because of the increasing popularity of soy foods and the availability of isoflavone supplements, there is an important public health need to accurately quantify the isoflavone content of these soy products. Certified reference materials (CRMs) for analysis of soy isoflavones in relative products were necessary in this situation. There exist 12 kinds of isoflavones in soybean and they are divided into daidzin group, genistin group and glycitin group, respectively, in which the content of genistin group (genistin and genistein) is the highest [6]. Therefore, genistin and genistein were developed into two new certified reference materials (CRMs) in this work.

A certified reference material (CRM) is a material or substance that its one or more property values are sufficiently homogeneous, stable, and well established to be used for the

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calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials [7~9]. CRMs are essential tools to guarantee the metrological traceability of measurement results to the International System of Units (SI), which means the accuracy and comparability of results over time and space [10]. However, no CRMs of isoflavones were offered in the markets until today. Therefore, the development of genistin and genistein CRMs was carried out in this work, following the principles of ISO Guides 34:2009 and 35:2005 [11~12]. The certification progress of these CRMs was designed as described in Fig 1.

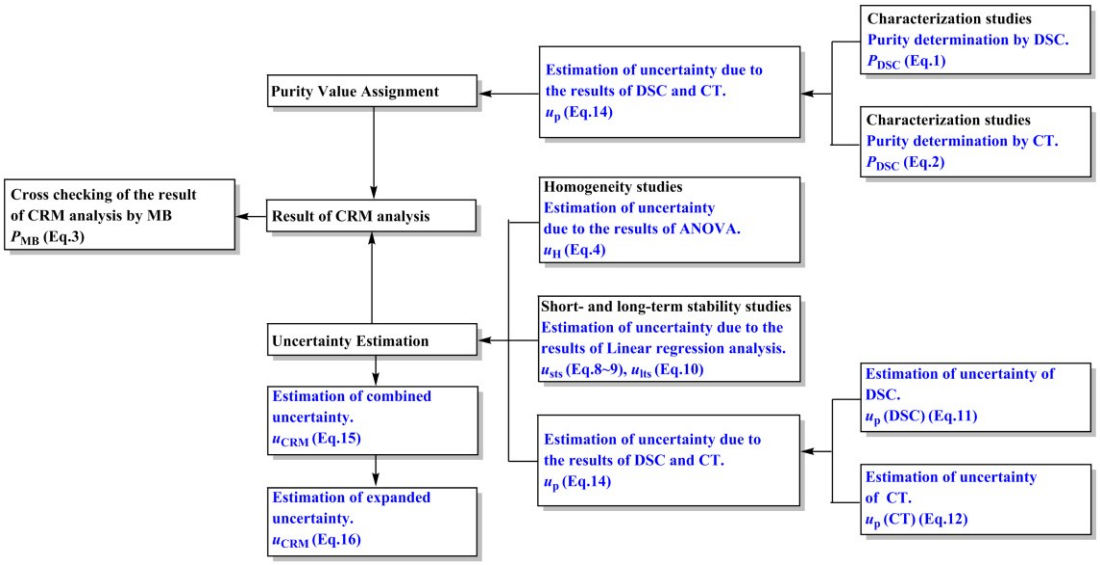

Fig. 1. Summary of certification progress of candidate CRM.

Studies on sample preparation, homogeneity studies, stability studies, characterization, and uncertainties estimation were performed. In the characterization, two certified methods based on different theories, namely, differential scanning calorimetry (DSC) and coulometric titrimetry (CT) were employed. Both methods have advantages of minimal sample requirement, short analysis time, high accuracy, good reproducibility, and do not need requirement of corresponding reference standard [13~14]. Then the uncertainty evaluations of these methods were performed carefully under the Guide to Uncertainty Measurement [15] in this work. The certification results of these CRMs were cross-checked by another independent reference method, namely, mass balance (MB) method. MB method is generally regarded as an accurate one and is recommended by World Health Organization (WHO) and European Pharmacopoeia and International Pharmacopoeia for establishment of chemical reference standards. Furthermore, it is also recommended in the comparison among national metrology institute organized by Bureau International des Poids et Mesures (BIPM) [16].

Materials and methods

Material

Genistin and genistein raw materials were obtained from the WuhanYuancheng Technology Development Co., Ltd. (Wuhan, China), whose purities are 98.0% and 98.2% determined by HPLC, respectively.

Instrumentation

The DSC curves were measured on a Mettler-Toledo DSC 1/700 calorimeter (Mettler-Toledo Inc., Switzerland). The CT analysis was conducted using a coulometer (Chinese Academy of Medical Sciences, China). HPLC measurements were made on an Agilent 1200 liquid chromatographic system with a diode-array detector (DAD) (Agilent Technologies, Inc., USA). The moisture of substances was determined by a Mettler-Toledo DL 39 Karl Fischer coulometric titrator (Mettler-Toledo Inc., Switzerland). Agilent 7890A GC system (Agilent Technologies, Inc., USA) was employed for the determination of residual solvents. A SX25-01 muffle furnace (Shanghai Shuli, Inc., China) was used for sulfated ash measurements.

Preparation of the candidate CRMs

In the work, preparation of genistin and genistein candidate CRMs used the same method as described below, by which was to obtain the candidate CRMs with the purity more than 99 %. About 60 g raw material was added to 150 ml N, N-Dimethylformamide (DMF), heated at 50 ℃, refluxed for 2 h. After complete dissolution, 750 ml mixed solution (ethanol: water: DMF = 1: 1: 0.1) was dropwise added into the solution with stirring at a uniform speed. The solution was stirred for an hour and then stood for crystallization overnight at 25 ℃. The solid obtained was flushed with 200 ml ethanol and then recrystallized for second time with the same method. Finally, the crystal was dried over 24 h under -0.1 mPa at 60 ℃, and then ground and sieved into powder within the particle size of 75 ~ 150 μm. The powder was homogenized in a multi-axle rotating mixer, dispensed and sealed into dark ampoules with 50 mg each. A total of 500 bottles was obtained for each candidate CRM.

Homogeneity studies

A homogeneity study is necessary in a batch of candidate CRM to demonstrate that the batch of bottles is sufficiently homogeneous. In the work, DSC method was used for the homogeneity study with 15 bottles selected at random. From each bottle, about 3 ~ 5 mg sample was prepared in one replicate for between-bottle homogeneity study and in triplicate for within-bottle homogeneity study.

Short- and long-term stability studies

Stability testing aims to investigate the stability of the candidate CRM after preparation under the transport condition (short-term stability) and the storage condition (long-term stability).

Short-term stability study was performed by DSC method. Three specified conditions were set as high temperature (60 ℃), high humidity (90 % ± 5 %, 25 ℃) and high illumination (4500 lx ± 500 lx, 25 ℃), respectively. Six bottles were introduced in each condition separately for 14 days. Three bottles were taken out and individually analyzed with DSC on every 7days. Three other bottles kept at 25 ℃ were used as controls. The DSC sample analysis was performed as described above for the between-bottle homogeneity study.

For the long-term stability study, 36 bottles were kept at 25 ℃ for one year. Each set of six bottles was analyzed, as described for the between-bottle homogeneity study, at 25 ℃ on 0, 1, 2, 4, 6 and 12 months, respectively.

Characterization of the candidate CRM

Table 1

Equation used in the certification studies of genistin and genistein CRMs

Equation	No.	Description
$P_{DSC}\% = (1 - x_{si}) \times 100\%$ where $x_{si} = \frac{QMF\Delta T}{mRT_0^2}$	(1)	P_{DSC} is the purity of the main component determined by DSC x_{si} is the content of solid impurities ΔH_f is the molar enthalpy of fusion of the main component in the sample F is melted fraction $\Delta T = T_0 - T_f$ is the depression of melting point Q is the heat of fusion of the sample m is the mass of the sample R is the gas constant M is the molar mass of the main component
$P_{CT}\% = \left(\frac{i \times t \times M}{n \times F} \Big/ \frac{m_1}{V_2} \times V_1 \right) \times 100\%$	(2)	P_{CT} is the purity of the main component determined by CT M is the molecular weight of the reactive substance F is Faraday's constant i is electric current (A) t is the time (s) of reaction n is the number of shifted electrons V_1 is the injection volume (μL) of the sample solution V_2 is the solution volume (μL) of the sample m_1 is the mass of the sample
$P_{MB}\% = (1 - x_{oi})(1 - x_{vi} - x_{sa}) \times 100\%$	(3)	P_{MB} is the purity of the main component determined by MB x_{oi} is the amount of main component x_{vi} is the amount of organic impurities x_{sa} is the amount of main component sulfated ash
$u_h = \sqrt{(MS_{within} - MS_{between})/n}$	(4)	u_h is the uncertainty of homogeneity MS_{within} is the mean square within groups $MS_{between}$ is the mean square between groups n is the number of replicates
$t = \frac{\lvert \overline{x_1} - \overline{x_2} \rvert}{\sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \cdot \frac{n_1 + n_2}{n_1 n_2}}}$	(5)	t is statistic of t-test x_1 is the mean of purity of the candidate CRM determined at the first time x_2 is the mean of purity of the candidate CRM determined at the second time s_1 is the standard deviation of the purity determined at the first time s_2 is the standard deviation of the purity determined at the second time n_1 is times of the measurements at the first time n_2 is times of the measurements at the second time
$S^2 = \frac{\sum_{i=1}^{n} (Y_i - b - aX_i)^2}{n-2}$	(6)	S is the standard deviation of the straight line Y_i is the purity of the candidate CRM X_i is the time a is the slope b is the intercept n is times of the measurements
$S_{(b)} = \frac{S}{\sqrt{\sum_{i=1}^{n} (X_i - \overline{X})^2}}$	(7)	$S_{(b)}$ is the slope uncertainty X is the time
$u_{sts}(temp) = u_{sts}(humi) = u_{sts}(photo) = S_{(b)} t$	(8)	$u_{sts}(temp)$ is the uncertainty of stability at the condition of high temperature $u_{sts}(humi)$ is the uncertainty of stability at the condition of high humidity $u_{sts}(photo)$ is the uncertainty of stability at the condition of high photolysis t is the time of short-term stability studies
$u_{sts} = \sqrt{u_{sts}^2(temp) + u_{sts}^2(humi) + u_{sts}^2(photo)}$	(9)	u_{sts} is the uncertainty of short-term stability
$u_{lts} = S_{(b)} t$	(10)	u_{lts} is the uncertainty of long-term stability t is the time of long-term stability studies
$\left(\frac{u(x_{si})}{x_{si}}\right)^2 = \left(\frac{u(Q)}{Q}\right)^2 + \left(\frac{u(M)}{M}\right)^2 + \left(\frac{u(F)}{F}\right)^2 + \left(\frac{u(\Delta T)}{\Delta T}\right)^2 + \left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(T_0)}{T_0}\right)^2 + \left(\frac{u(f)}{f}\right)^2$	(11)	$u(x_{si})$ is the uncertainty of solid impurities determination by DSC
$\frac{u(CT)}{P_{CT}} \approx \left(\frac{u(i)}{i}\right)^2 + \left(\frac{u(t)}{t}\right)^2 + \left(\frac{u(M)}{M}\right)^2 + \left(\frac{u(m_1)}{m_1}\right)^2 + \left(\frac{u(V1)}{V1}\right)^2 + \left(\frac{u(V_2)}{V_2}\right)^2 + \left(\frac{u(f_2)}{f_2}\right)^2$	(12)	$u_{(CT)}$ is the uncertainty of purity determination by DSC
$p_{CRM}\% = \frac{p_{DSC}\% + P_{CT}\%}{2}$	(13)	P_{CRM} is the purity of the main component as CRM
$u_p = P_{CRM} \sqrt{\left(\frac{u_p(DSC)}{P_{DSC}}\right)^2 + \left(\frac{u_p(CT)}{P_{CT}}\right)^2}$	(14)	u_p is the uncertainty of value assignments $u_p(DSC)$ is the uncertainty of purity determination by DSC $u_p(CT)$ is the uncertainty of purity determination by CT
$u_{CRM} = \sqrt{u_p^2 + u_h^2 + u_{sts}^2 + u_{lts}^2}$	(15)	u_{CRM} is the combined standard uncertainty of certified property value
$U_{CRM} = u_{CRM} k$	(16)	U_{CRM} is the expanded uncertainty of certified property value k is the coverage factor

Differential scanning calorimetry analyses

Purity assessment by DSC is based on the fact of melting or freezing point depression of a pure material caused by the presence of impurities, which can be approximatively described by the Van't Hoff equation. Because the total amount of solid impurities can be determined by DSC, the purity of the main component was calculated by the derived formula as Eq. (1).

The general performance of the instrument is evaluated quarterly using thermal analysis indium [GBW (E) 130182] with the programmed In Check method stored in STAR^e software according to the instruction manual. Heat flow, temperature, and enthalpy are calibrated by the test.

DSC purity determination was performed under a constant atmosphere of high-purity nitrogen gas at a flow rate of 50 ml $\cdot min^{-1}$. The instrument was cooled using a refrigerated cooling system. Approximately 3 ~ 5 mg of the candidate CRM was accurately weighed to 0.01 mg using a Mettler 40 μL aluminum crucible, hermetically sealed with an appropriate aluminum lid, and crimped. An empty crucible and lid of the same type were used as reference. The heating rates were set to 5 K $\cdot min^{-1}$ and 8 K $\cdot min^{-1}$ for analysis of genistin and genistein, respectively.

Coulometric titrimetry analyses

Coulometric titrimetry is an important method for electrochemical analysis. The relationship between reactive substances and consumption of electricity can be described as Faraday's law of electrolysis, and therefore the purity of sample can be calculated as Eq. (2).

Purity determination by CT method is based on the substitution reaction between a hydrogen atom in the sample structure with bromine, which is produced by potassium bromide (KBr) electrolyte; the instrument can automatically record the reaction time, which is used to calculate the purity.

Due to the introduction of glucose on 7-position of genistein, genistin could not directly react with bromine in the coulometric titration as well as genistein. Therefore, hydrolysis reaction was conducted so as to transform genistin into genistein, whose conditions are ultrasonic hydrolysis for 6 hours at 70 ℃ in 6 mol $\cdot L^{-1}$ hydrochloric acid (HCl).

The current of the coulometric titrator was 0.9839 mA, and calibration was performed before the experiment using arsenious acid solution (GBW 08666). The end point of the titration was indicated by the increase in current. The electrode material was platinum. The composition of the electrolyte solution, for the analysis of genistin, was KBr (1 mol $\cdot L^{-1}$) and methonal and glacial acetic acid in a 9 : 3 : 1 ratio, and for the analysis of genistein, was KBr (1 mol $\cdot L^{-1}$) and HCl (2 mol $\cdot L^{-1}$) in a 1:1 ratio.

Cross-checked method

For proving the accuracy of results, mass balance (MB) method, the third method based on different theory, was employed to cross-checked the purities of the candidate CRMs.

For the sample under purity analysis, the total of the measured volatile impurities, as well as water and solvent residues, organic and inorganic impurities, and main component should amount to 100 %. Therefore, the purity of the main component can be confirmed by subtracting the sum of all of the impurities from 100 % as Eq. (3).

The candidate CRMs were analyzed using an Agilent 1200 HPLC system equipped with an Agilent Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) column. The injection volume was 10 μL. The column temperature was 30 ℃. The flow rate was 1 mL min^{-1}. The mobile phase was composed of 0.5 % acetic acid aqueous solution and acetonitrile at a ratio of 80 : 20 for analysis of genistin CRM, and composed of 0.5 % acetic acid aqueous solution and methanol at a ratio of 29 : 71 for analysis of genistein CRM. The chromatographic profiles were registered at 258 nm and 260 nm for analysis of genistin and genistein CRM, respectively. Volatile impurities mainly composed of residual solvents were determined by GC. The moisture was determined by Karl Fischer coulometric titrator. The inorganic impurities were acquired through the routine method of residue on ignition.

Results and discussion

Table 2

Certification results of genistin and genistein candidate CRMs

	Parameters	Results	
		Genistin	Genistein
Homogeneity studies	F / F_{crit}	1.47 / 2.04	1.19 / 2.04
	p-value	0.18	0.33
	$MS_{between}$ / MS_{within} (n)	3.14×10^{-8}/2.13×10^{-8} (n = 3)	1.61×10^{-8}/1.36×10^{-8} (n = 3)
Short-term stability studies	t_{crit}	2.776	2.776
	t (60 ℃)	0.956 (0,7) / 0.500 (0,14)	0.632 (0,7) / 0.316 (0,14)
	t (90 % RH)	1.809 (0,7) / 0.277 (0,14)	0.632 (0,7) / 0.426 (0,14)
	t (4500 lx)	0.152 (0,7) / 0.369 (0,14)	0.250 (0,7) / 0.800 (0,14)
Long-term stability studies	$S_{(b)}$	3.89×10^{-5}	1.92×10^{-5}
	t (month)	12	12
Purity determination	*DSC* method	99.68 % (n = 10, s = 0.000233)	99.30 % (n = 10, s = 0.000357)
	CT method	99.68 % (n = 10, s = 0.000679)	99.33 % (n = 10, s = 0.000642)
Value assignment	t_{crit} / t	2.20 / 0.00	2.20 / 1.29
	P_{CRM}	99.7%	99.3%
Cross cheking	*MB* method	99.66 %	99.32 %
	HPLC method	99.95 % (n = 3, s = 0.000058)	99.86 % (n = 3, s = 0.000173)
	Water	0.12 %	0.19 %
	Sulphate ashes	0.20 %	0.12 %
	Residual solvents	0.17 %	0.23%
Uncertainty estimation	u_h	5.78×10^{-5}	2.91×10^{-5}
	u_{sts}	8.98×10^{-4}	3.27×10^{-4}
	u_{lts}	4.66×10^{-4}	2.30×10^{-4}
	u_p(DSC)	0.03%	0.04%
	u_p(CT)	0.11%	0.21%
	u_p	0.11%	0.21%
	u_{CRM}	0.15%	0.22%
	U_{CRM}	0.3%	0.5%
Results of CRM analysis	k = 2, P = 0.95	99.7 % ±0.3 %	99.3 % ±0.5 %

Homogeneity studies

A one-way analysis of variance (ANOVA, F-test) was used to evaluate the homogeneity of CRMs. The mean square between bottles ($MS_{between}$) was larger than the mean square within bottles (MS_{within}), which means the method has good repeatability. The ratios of mean square (F) were smaller than the critical value (F_{crit}), which mean the homogeneities of CRMs were

good. In this case, Eq. (4) was used to calculate u_h. Table 2 shows the results of homogeneity studies of CRMs.

Short- and long-term stability studies

Mean uniformity method (*t*-test) was used to evaluate the short-term stability. According to Eq. (5), the calculated values of *t* on different conditions were all less than the critical value (t_{crit}) from the table of bilateral quantile distribution, which have indicated that the CRMs have good short-term stabilities.

Linear regression analysis was used to evaluate the long-term stability. The slope and standard deviation of the data points obtained in the long-term stability studies were calculated using Eq. (6) ~ (7). The absolute values of slope were smaller than the product of time (12/month) and slope uncertainty, which have indicated that the CRMs have good long-term stabilities. The result showed that these CRMs were stable for up to one year under the condition in this study.

The uncertainties of short- and long-term stabilities were evaluated by Eq. (8) ~ (10). Table 2 shows the results of short- and long-term stabilities of CRMs.

Characterization Studies

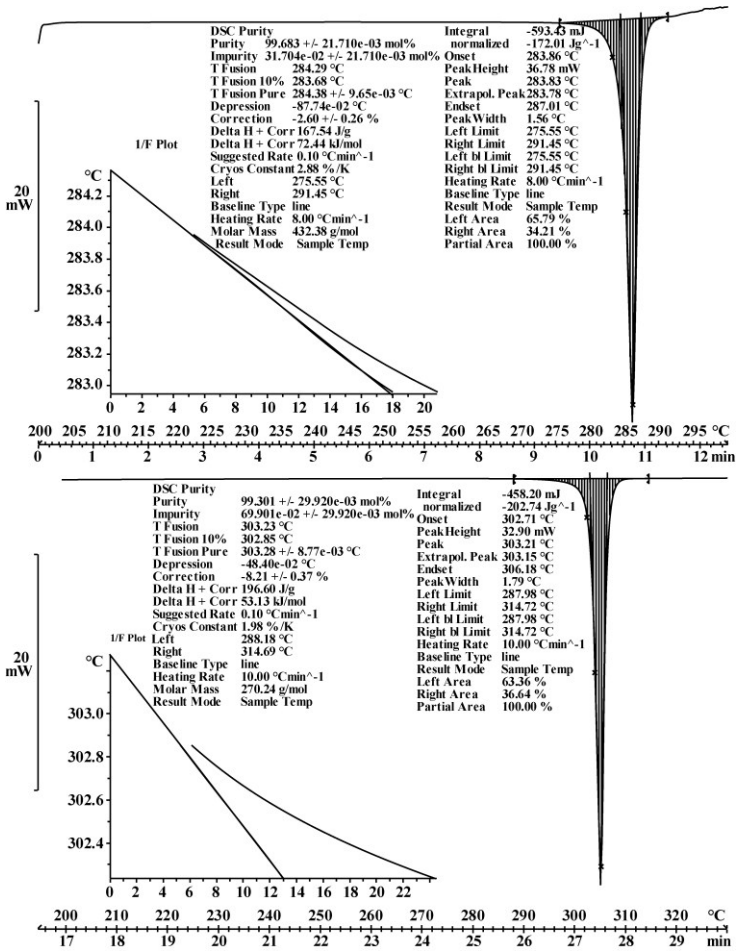

Fig. 2. The graph and purity result of genistin and genistein CRM by DSC.

Purity determination by Differential scanning calorimetry method

The mean values of purity determined by DSC were listed in Table 2. Fig. 2 showed the typical DSC curve of genistin and genistein CRM.

Several factors, which could affect the results of purity determination, were identified as the possible sources of uncertainty in DSC measurement and shown in Fig. 3. Uncertainty of purity should be equal to uncertainty impurity determined by DSC deduced from Eq. (1). Therefore, uncertainty of purity could be calculated by Eq. (11). Table 2 shows the results of purity determination and uncertainty evaluation by DSC.

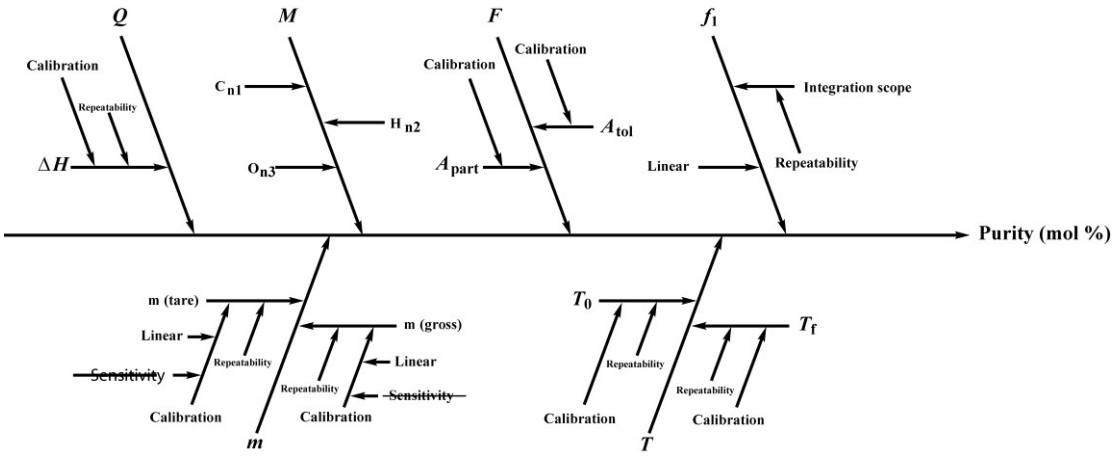

Fig. 3. Cause and effect diagram showing the possible sources of uncertainty in DSC.

Purity determination by Coulometric titrimetry method

The mean values of purity determined by CT were listed in Table 2. Fig. 4 shows the principle of CT of the genistin and genistein, i.e., substitution reactions of bromine to a hydrogen atom in the genistin and genistein structure.

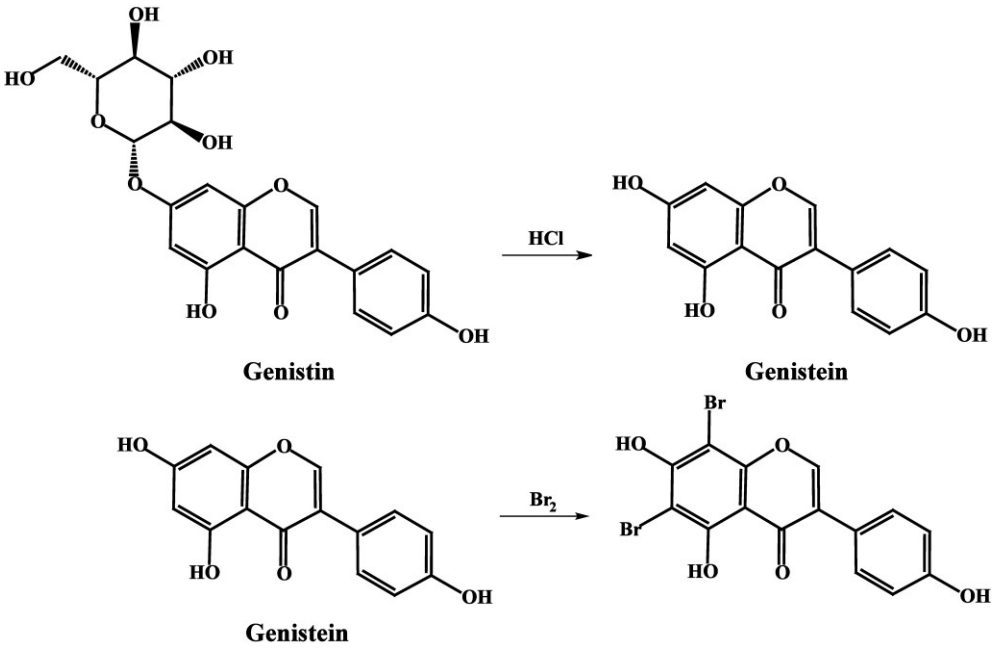

Fig. 4. The substitution reaction formula of genistin, genistein with bromine.

Several factors, which could affect the results of purity determination, were identified as the possible sources of uncertainty in CT measurement and shown in Fig. 5. Therefore, uncertainty of CT could be calculated by Eq. (12). Table 2 shows the results of purity determination and uncertainty evaluation by CT.

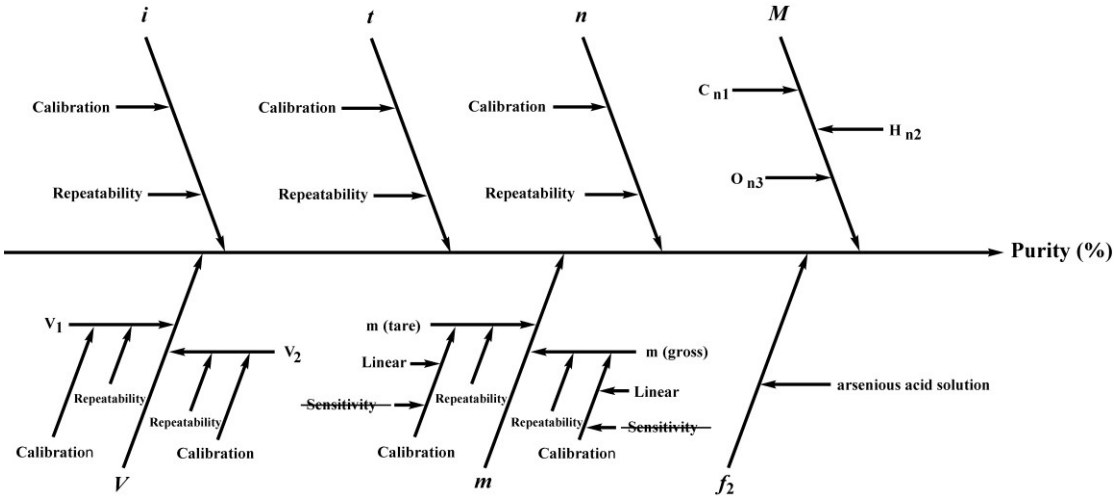

Fig. 5. Cause and effect diagram showing the possible sources of uncertainty in CT.

Value assignment

Although the purity determined by DSC was mole percentage (mol %), for the high purity sample (> 99.0 %), its value was approximately equal to mass percent (%) in this work. In this case, the purities of CRMs, as obtained by DSC and CT methods, were compared with each other via the *t*-test, which indicated that there was no significant difference between the results. Therefore, the certified values of CRMs could be calculated as Eq. (13) and the uncertainty of value assignments (u_p) could be calculated as Eq. (14). Table 2 shows the results of value assignment.

u_{CRM} and U_{CRM} estimation

The combined standard uncertainties (u_{CRM}) and expanded uncertainties (U_{CRM}) for the CRMs were calculated according to Eq. (15) and (16), respectively. Table 2 shows the results of u_{CRM} and U_{CRM} estimation.

Results of CRM analysis

The purities of the genistin and genistein CRMs were therefore found to be 99.7% ± 0.3% and 99.3% ± 0.5% (k = 2, P=95%), respectively, which showed in Table 2 as results of CRM analysis.

Cross-checked result

The mean values of purity determined by MB were listed in Table 2. The results showed the purities determined by MB fell within the interval of results of CRM analysis. Fig. 6 showed the typical chromatography of genistin and genistein CRMs.

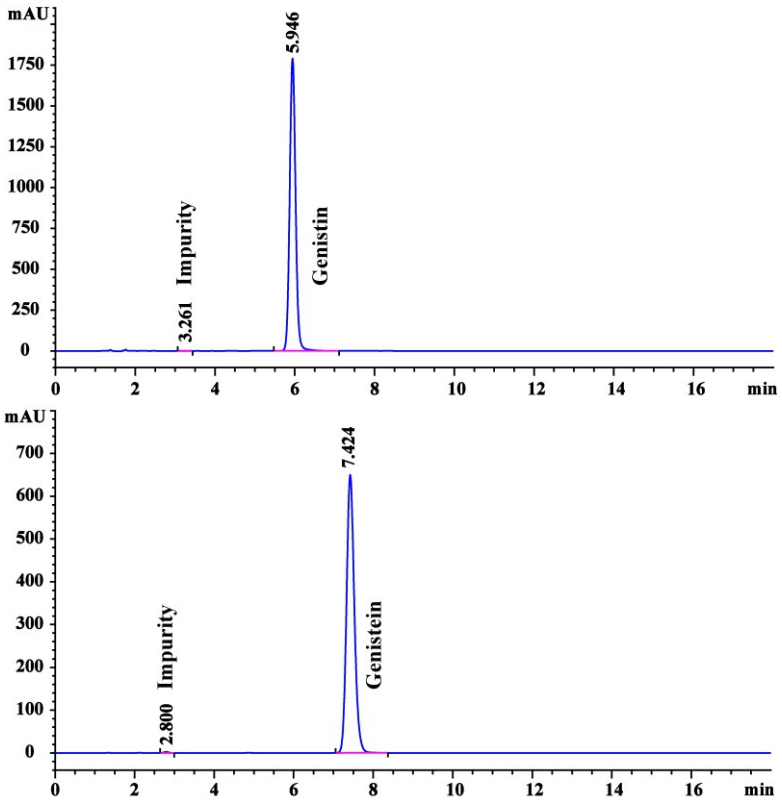

Fig. 6. The chromatography of genistin and genistein CRM.

Conclusion

Two new certified reference materials for genistin and genistein were developed to fulfill the strong demand for CRMs of analysis of soy isoflavones in relative products, since only a few were available internationally. Two different methods were used to determine the purity and the purities of the genistin and genistein CRM were found to be 99.7% and 99.3% with expanded uncertainties of 0.3% and 0.5 % (k = 2) , respectively. The developed CRMs were stable for at least one year. The new CRMs of genistin and genistein have been approved by the national administrative committee for CRM's as GBW 09558 and GBW 09559.

Acknowledgements

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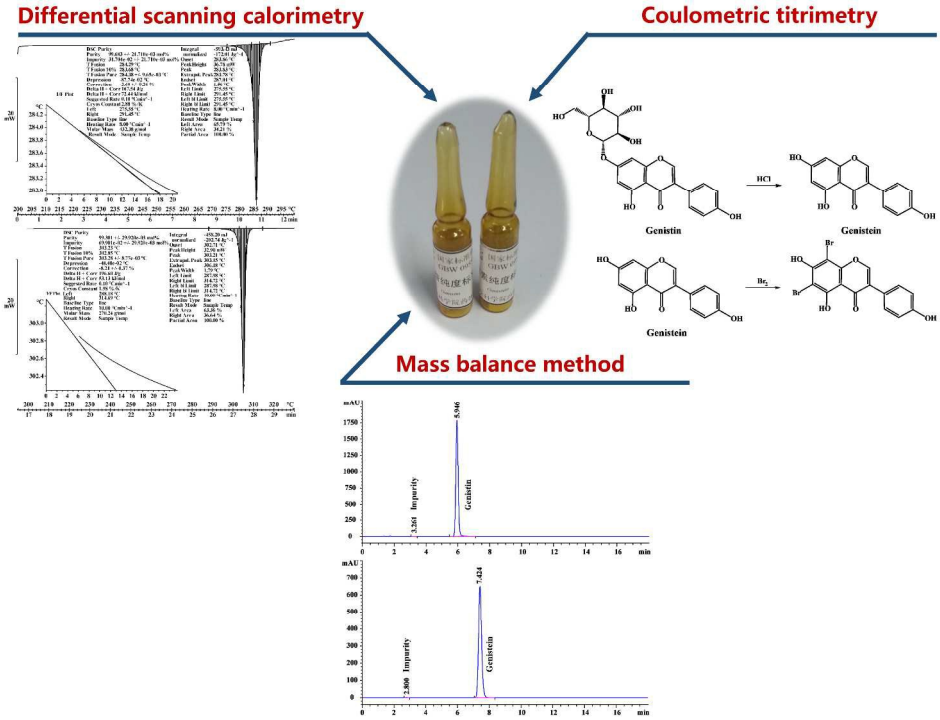

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