

# Certification and uncertainty evaluation of flavonoids certified reference materials

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## ABSTRACT

Flavonoids are the most widely used in the field of the food, medicine, and cosmetic due to their pharmacological functions and biological activities, such as anti-tumor, anti-inflammatory and antioxidation. This work described the assignment of purity values to 3 flavonoids certified reference materials (CRMs) including baicalein, 5,7-dihydroxy flavones and naringenine which were developed in this study according to the ISO Guides 34 and 35. The qualitative analysis was performed by liquid chromatography-mass spectrometry (LC-MS) and infrared spectroscopy (IR). The CRMs' purity values were assigned based on the weighted average of quantitative nuclear magnetic resonance method and mass balance approach with high resolution liquid chromatography-VWD. All the three CRMs with following value mass fractions: baicalein at a certified purity  $P \pm U$  ( $k = 2$ ) of  $98.8\% \pm 0.8\%$ ; 5,7-dihydroxy flavones of  $99.1\% \pm 0.7\%$  and naringenine of  $99.5\% \pm 1.0\%$  respectively. The homogeneity of the CRMs was determined by an in-house validated liquid chromatographic method. Potential degradation during storage was also investigated and a shelf-life based on this value was established.

**Keywords:** Flavonoids; Certified Reference Material (CRM); Quantitative Nuclear Magnetic Resonance (QNMR); Mass balance approach; Uncertainty

## 1. INTRODUCTION

A certified reference material (CRM) is a material or substance whose one or more property values are sufficiently homogeneous, stable, and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to ma-

terials [1].

Flavonoids are increasingly in demand in the field of the food, medicine, and cosmetic [2] due to their pharmacological functions and biological activities, such as anti-tumor, anti-inflammatory and antioxidation [3-5]. The qualities of flavonoids products will have analytical challenges. The flavonoids CRMs have been determined by at least two independent analytical methods. The flavonoids CRMs could be used to ensure the accuracy, traceability and comparability of the related products test results.

This paper describes the development of three flavonoids CRMs including baicalein, 5,7-dihydroxy flavones and naringenine. Qualitative analysis was carried out by infrared spectrometry (IR) and liquid chromatography-mass spectrometry (LC-MS). The moisture content and inorganic elements content were determined by Karl Fischer titration, inductively coupled plasma mass spectrometry (ICP-MS), respectively. Homogeneity study was evaluated by F-test and *t*-test both for between vials and within vial. The uncertainties of CRMs were evaluated extensively.

## 2. EXPERIMENTAL

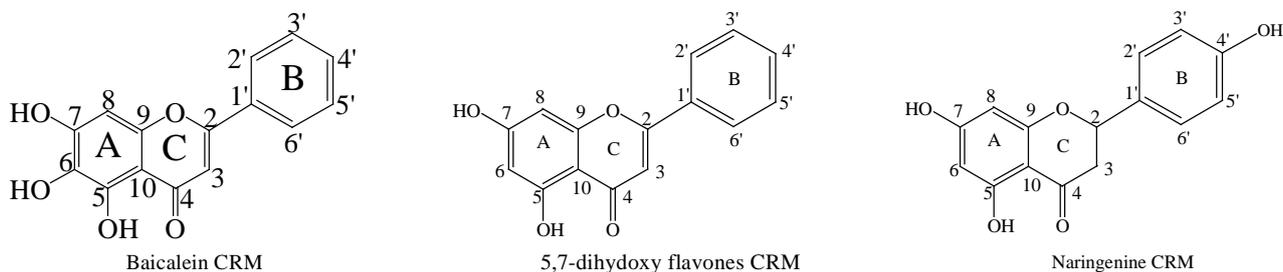
### 2.1. Materials

The chemical structure of flavonoid compounds studied including baicalein, 5,7-dihydroxy flavones and naringenine were shown in **Figure 1**, all were purchased from Sigma-Aldrich (USA). Benzoic acid certified reference material ( $99.9\% \pm 0.1\%$  purity) used as QNMR internal standard was obtained from NIST, China.

HPLC grade Acetonitrile and methanol were bought from Merck (HPLC, Germany). Dimethyl sulfoxide- $d_6$  solvent was purchased from Cambridge Laboratories (USA). Distilled deionized water ( $18.2 \text{ M}\Omega\text{-cm}$ ) was obtained with Milli-Q water system and filtered through  $0.22 \mu\text{m}$  membrane.

### 2.2. Instrument

Infrared spectra were collected with FT-IR spectrometer



**Figure 1.** Structure of the studied flavonoids CRMs.

Thermo Nicolet IS10 spectrophotometers. All the flavonoids reference material solutions, diluted to concentration of  $10 \mu\text{g}\cdot\text{mL}^{-1}$  in methanol for LC/MS Analysis were quantified at flow injection analysis mode with an Agilent 6410 triple quad system with electro-spray ionization source (ESI). The QNMR measurements were performed with a Bruker AV600 spectrometer (Bruker, Billerica, MA). The Agilent 7500CE ICP-MS was used for the analysis of trace metal residues in all the samples. A Mettler Toledo DL39 Karl Fischer titrator was used for water measurement of all the samples.

## 2.3. Qualitative Analysis Methods

### 2.3.1. LC-MS Method

The flavonoids CRMs in methanol with concentration of  $10 \mu\text{g}\cdot\text{mL}^{-1}$  were used to LC-MS analysis. The ion spray voltage was set at 4.0 kV. Drying gas temperature was maintained at  $350^\circ\text{C}$  with flow rate of  $9.0 \text{ mL}\cdot\text{min}^{-1}$ . Nebulizer pressure was set at 40 psi. The fragmentor energy was set at 135 V. The collision energy respectively were optimized at 30 V for baicalein and 5,7-dihydroxy flavones, and 16 V for naringenine. The analysis the mass spectrum peak molecular weight in an ESI positive ion mode at specified MS scan range of 60 - 300.

### 2.3.2. IR Method

Infrared spectra were collected with FT-IR spectrometer Thermo Nicolet IS10 spectrophotometers via KBr pellets technique. Specimens were ground with dried spectroscopic grade KBr powder and the mixture was compressed to pellets for FT-IR measurements. The sample to KBr mass ratio was optimized as 1:100. All the spectra were collected in the  $4000 - 400 \text{ cm}^{-1}$  range at  $4 \text{ cm}^{-1}$  resolution.

## 2.4. Quantitative Analysis Methods

### 2.4.1. Mass Balance Approach

The flavonoids were quantified by HPLC equipped with an ZORBAX Eclipse XDB-C<sub>18</sub>(460 mm × 250 mm × 5 μm, Agilent ) column with VWD detector. The chromatography conditions are detailed in **Table 1**. The purity values of flavonoids CRMs were calculated using

the peak area normalization method. Following subtract the moisture and inorganic elements impurities which not measured by HPLC from 100%, as follows [6,7]:

$$\text{Purity} = (1 - P_m - P_a) * P_{\text{HPLC}} \quad (1)$$

Where,  $P_{\text{HPLC}}$  is the percentage area of main compound acquired by HPLC-VWD,  $P_m$  is the moisture content measured by Karl Fisher method, and  $P_a$  is the inorganic elements content measured by ICP-MS.

### 2.4.2. QNMR Method

The QNMR method is based on the directly proportional relationship between the signal response (integrated signal area,  $I_x$ ) and the number of nuclei generating the corresponding resonance line, which is represented by **Eq.1** as [8].

$$P_x = \frac{m_x}{m_s} = \frac{I_x}{I_{std}} \cdot \frac{N_{std}}{N_x} \cdot \frac{M_x}{M_{std}} \cdot \frac{m_{std}}{m_s} \cdot P_{std} \quad (2)$$

Where  $P_x$  is purity of analyte;  $I_x$  and  $I_{std}$  are the integrated signal areas of analyte and internal standard, respectively;  $N_x$  and  $N_{std}$  are the spin numbers of the analyte and internal standard, respectively;  $M_x$  and  $M_{std}$  are molar mass of analyte and internal standard, respectively;  $m$  and  $m_{std}$  are the mass of analyte and internal standard, respectively;  $P_{std}$  is the purity of the internal standard.

The QNMR measurements were performed with a Bruker AV600 spectrometer (Bruker, Billerica, MA). Spectra were run with the following optimized parameters: probe size, 5 mm; probe temperature, was  $23.0^\circ\text{C}$ ; excitation pulse angle,  $45^\circ$ ; 32 K time domain points; 16 K spectral data points; pulse delay, 4.15 μs; relaxation delay, 32 s and number of scans, 32. A sample solution containing about 10 mg benzoic acid, 70 mg of carbohydrate samples diluted in 0.5 mL DMSO was analyzed by QNMR. The total mass of analyte in the sample solution was determined using **Eq.1**.

## 2.5. Homogeneity Study and Stability Study

According to the Technical Norm of Primary Reference Material of China [9], the homogeneity study and the stability study were carried out by HPLC-VWD method. 15 bottles, were randomly selected and assayed for

**Table 1.** The chromatography conditions of the studied flavonoids CRMs.

CRMs	Mobile phase (Acetonitrile:0.1% phosphoric acid)	Wavelength (nm)	Injection volume ( $\mu$ L)	Flow (mL/min)	Concentration (mg/mL)
Baicalein	35:65	275	5	1	0.5
5,7-dihydroxy flavones	43:57	268	5	1	0.3
Naringenine	35:65	288	2	1	1

homogeneity between bottles, and 7 portions from each bottle were assayed for homogeneity within bottle. The results were examined via *F*-test and *t*-test. In *F*-test, standard deviations were used to examine whether the deviation between bottles was significantly greater than the deviation within bottle. In *t*-test, means were used to examine whether the mean between bottles was significantly different from that of within bottle [10].

A stability study monitored a significant change in value of the flavonoids CRMs was performed over one year.

A study of long-term stability for storage at 4°C was carried out by HPLC-VWD method [11] in which material was determined at 1, 3, 6 and 12 months. For each condition, two solutions were prepared and analyzed in duplicate. A comparison was made between the purity determined by HPLC-VWD for different storage time. A freshly prepared control sample was analyzed at each time. All the samples were randomized for analysis.

## 2.6. Uncertainty Evaluation

The uncertainty evaluation for the flavonoids CRMs derived from the certified value, homogeneity and stability study. The expanded uncertainty was calculated as follows:

$$U = k \cdot U_c = k \cdot \sqrt{u_v^2 + u_h^2 + u_s^2} \quad (3)$$

where, *k* is the coverage factor, *U<sub>c</sub>* is the combined uncertainty of the sample, *u<sub>v</sub>*, *u<sub>h</sub>*, *u<sub>s</sub>* are the uncertainty components of the assigned value, homogeneity and stability study.

## 3. RESULTS AND DISCUSSION

### 3.1. Qualitative Analysis Methods

#### 3.1.1. LC-MS Method

The experiment obtained the molecular weight of the flavonoids CRMs by full scan mass spectra. The results indicted the molecular ion peak (*M* + *H*)<sup>+</sup> of mass charge ratio *m/z* of baicalein and 5,7-dihydroxy flavones was the molecular weight plus the atomic weight of hydrogen (1.0), the molecular ion peak (*M* - *H*)<sup>-</sup> of mass charge ratio *m/z* of naringenine was the molecular weight subtract the atomic weight of hydrogen (1.0). Ion fragments by product ion scan are detailed in **Figure 2**. The results showed the flavonoids mainly fractured in 1,2 C-Ochem-

ical bond and 3,4 C-C chemical bond.

#### 3.1.2. IR Method

The Infrared spectrum of the flavonoids CRMs were shown in **Figure 3**. The results revealed the peaks at 3090, 3097, and 3114  $\text{cm}^{-1}$  belong to hydroxide peaks, 1660, 1653, and 1630  $\text{cm}^{-1}$  belong to associated carbonyl peaks, 1449 ~ 1618  $\text{cm}^{-1}$  belong to benzene ring skeleton of C = C peaks of the flavonoids CRMs. The Infrared spectrums of the flavonoids CRMs were consistent with those spectrums from literature [13-15] and can be used for quantification.

### 3.2. Quantitative Analysis Methods

#### 3.2.1. Mass Balance Approach

The HPLC chromatogram of the flavonoids CRMs were shown in **Figure 4**. The results of the moisture content and the inorganic elements contents were listed in **Table 2**, as indicated, The water content for Baicalein, 5,7-dihydroxy flavones, and naringenine were 0.538%, 0.150%, 0.098% and 0.115%, respectively, all were less than 0.1%, while the inorganic content all warless than 0.00002% at the studied conditions.

#### 3.2.2. QNMR Method

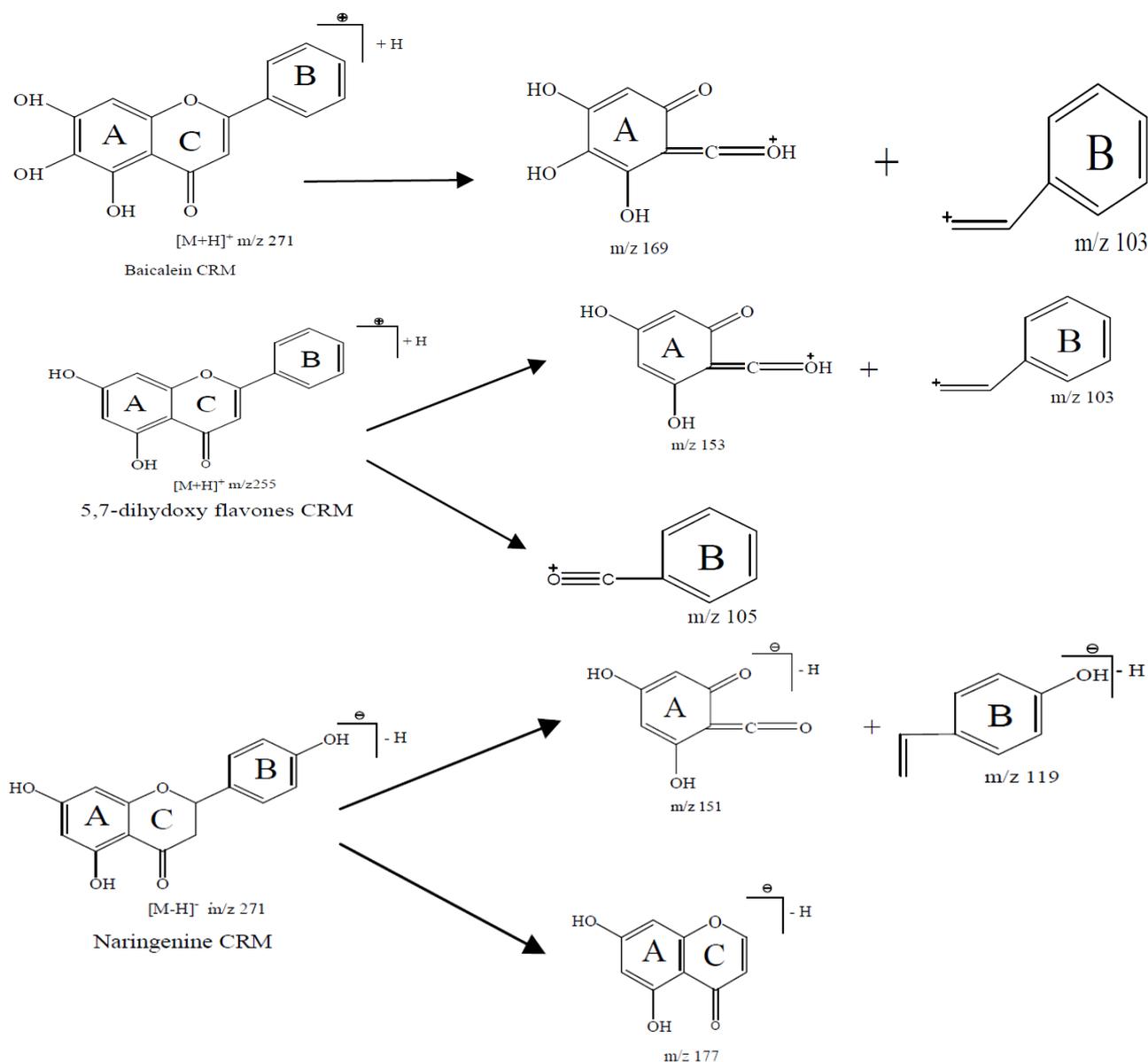
QNMR was also used to determinate purity values flavonoids CRMs. The NMR spectrums were shown in **Figure 5**. The C<sub>3</sub>-H peak areas of baicalein, C<sub>8</sub>-H peak areas of 5,7-dihydroxy flavones, C<sub>4</sub>-OH peak areas of naringenine, C<sub>2,2</sub>-2H peak areas of the benzoic acid internal standard were selected for integration.

#### 3.2.3. Purity Values of the Flavonoids CRMs

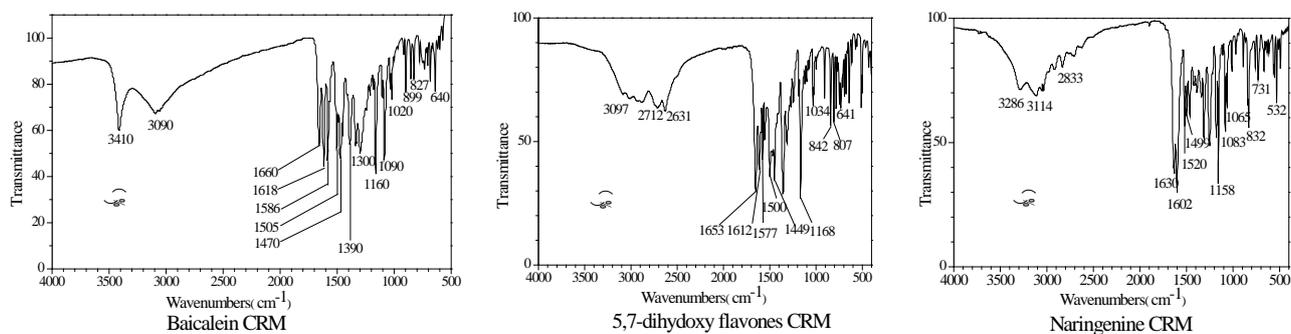
The purity values of flavonoids CRMs were calculated with the average of mass balance approach and QNMR method. The results are listed in **Table 2**.

### 3.3. Homogeneity Study and Stability Study

The *F*-Test and *t*-test results of all the studied were summarized in **Table 3**. No significant differences were found for all the studied compounds in homogeneity test as the calculated *F*-values were less than the critical *F*-values (**Table 3**). Although a slight difference was observed, this was attributed to the variation of the analytical method.



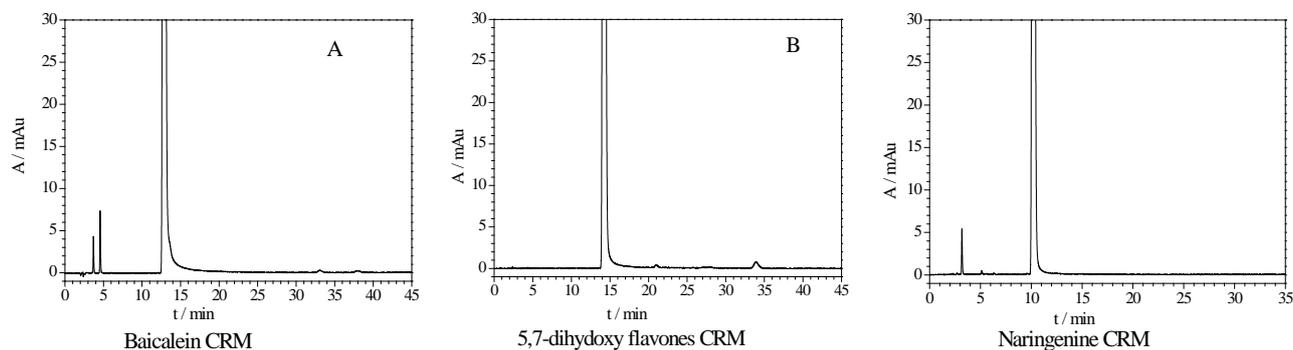
**Figure 2.** Main fragmentation schemes of the flavonoids CRMs.



**Figure 3.** The infrared spectra of the studied flavonoids CRMs.

The flavonoids CRMs stored at  $4^{\circ}C$  were used to test stability. The testing data of stability study are showed in

**Table 4.** The results in different times were consistent with the standard value through the *t*-test analysis. There



**Figure 4.** Purity determination HPLC chromatogram of the flavonoids CRMs.

**Table 2.** The purity values results of flavonoids CRMs.

CRMs	HPLC (%)	QNMR (%)	Average (%)
Baicalein	98.78	98.88	98.8
5,7-dihydroxy flavones	99.40	98.84	99.1
Naringenine	99.47	99.46	99.5

were no obvious changes in purity values monitored of the flavonoids CRMs over one year. The results mean that they were stable.

### 3.4. Uncertainty Evaluation

The uncertainty for the flavonoids CRMs is composed of the uncertainty from the certified value, homogeneity and stability study.

#### 3.4.1. Uncertainty of Certified Value

1) Uncertainty from HPLC measurement [16].

a)  $u_1$  (type A uncertainty) is the uncertainty from repeatability of HPLC measurement.

b)  $u_2$  (type B uncertainty) is the uncertainty from different responses from flavonoids CRMs and impurities at different wavelengths of HPLC measurement.

The combined uncertainty is as follow:

$$u_{\text{HPLC}} = \sqrt{u_1^2 + u_2^2}$$

2) Uncertainty of the QNMR measurement

a)  $u_3$  (type A uncertainty) is the uncertainty from repeatability of QNMR measurement.

b)  $u_4$  is the uncertainty from relative molar masses of flavonoids CRMs and benzoic acid internal standard CRM. The value of  $u_4$  calculated is less than 0.1%. In this case,  $u_4$  is ignored.

c)  $u_5$  is the uncertainty from benzoic acid CRM. The uncertainty of benzoic acid CRM was 0.1%, according to the uniform distribution,  $u_5 = 0.1\%/3^{1/2} = 0.06\%$ .

d)  $u_6$  is the uncertainty from balance weighing. The balance (Max = 2.1 g, d = 0.1  $\mu\text{g}$ ) was used to weigh the flavonoids CRMs and benzoic acid internal standard CRM. Balance changing and test error estimate for the 0.5  $\mu\text{g}$ , therefore  $u_6$  is estimated 0.01%.

The combined uncertainty is as follow:

$$u_{\text{QNMR}} = \sqrt{u_3^2 + u_4^2 + u_5^2 + u_6^2}$$

#### 3.4.2. The Combined Uncertainty

The combined uncertainty for the flavonoids CRMs is composed of the uncertainty from the certified value, homogeneity and stability study. The results of homogeneity and stability study completely conformed to the requirement of homogeneity and stability in the regulation of the CRMs. The homogeneity and stability of the flavonoids CRMs both are good. Therefore the uncertainty of homogeneity ( $u_h$ ) and stability ( $u_s$ ) is neglected.

$$\begin{aligned} U_C &= \sqrt{u_v^2 + u_h^2 + u_s^2} = \sqrt{u_{\text{HPLC}}^2 + u_{\text{QNMR}}^2 + u_h^2 + u_s^2} \\ &= \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + u_5^2 + u_6^2 + u_h^2 + u_s^2} \\ &\approx \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + u_5^2 + u_6^2} \end{aligned}$$

#### 3.4.3. The Expanded Uncertainty

The expanded uncertainty ( $U$ ) for this flavonoids CRMs was calculated by multiplying the combined uncertainty by a coverage factor of  $k = 2$ . The uncertainty evaluation for flavonoids CRMs were listed in **Table 5**.

## 4. CONCLUSION

The three flavonoids CRMs were developed with the procedures of sample preparation, certified value, homogeneity study, and stability study and uncertainty evaluation. The three flavonoids CRMs can not only meet the requirement of quantitative detection of food, medicine and related materials, but also provide a more quickly and accurately experimental methods for the research and development of related products.

## 5. ACKNOWLEDGEMENTS

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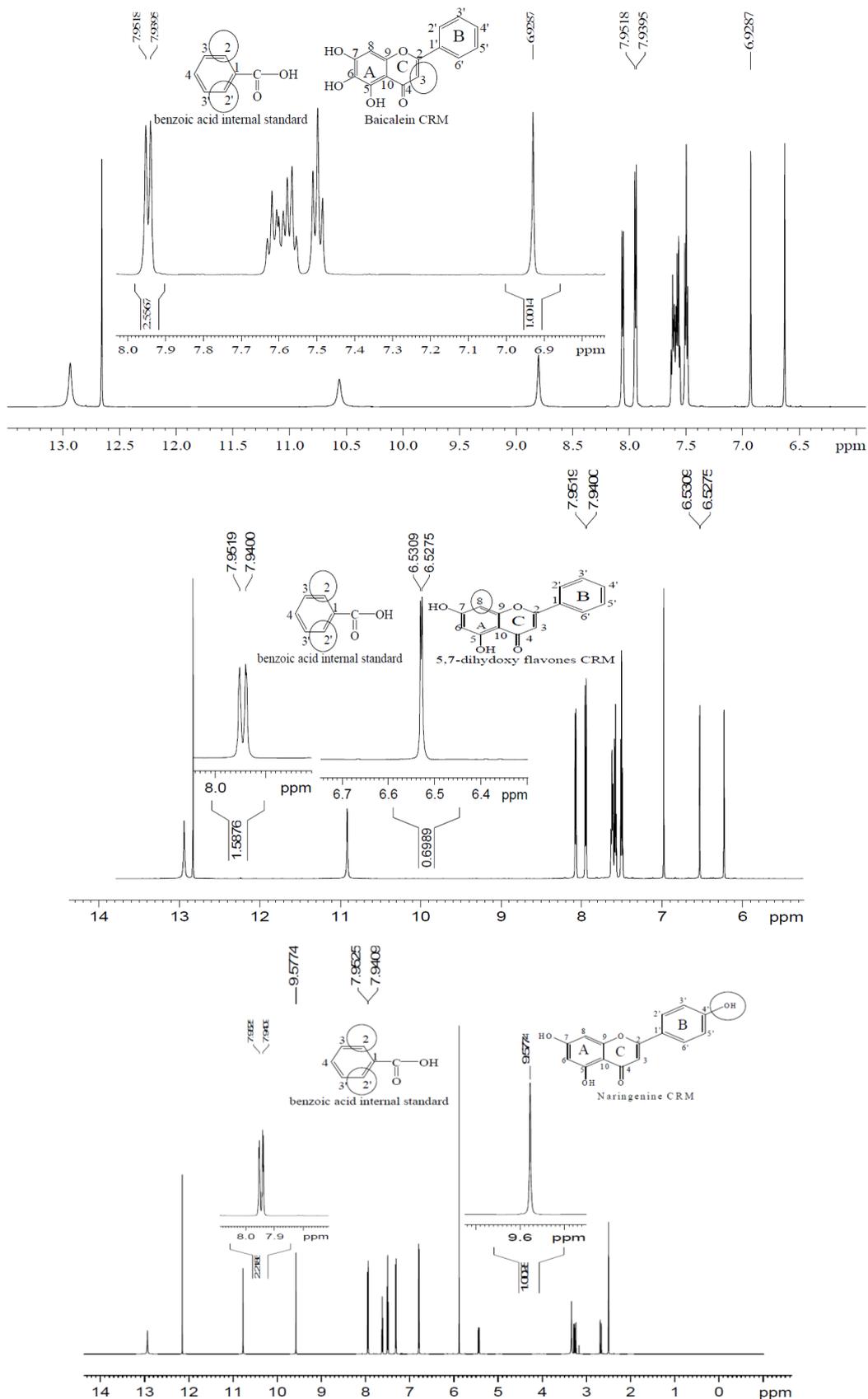


Figure 5. The spectrums of the studied flavonoids CRMs.

**Table 3.** Homogeneity results for the contents, the within-bottle, and between-bottle and homogeneity test of flavonoids CRMs.

Homogeneity test		Baicalein CRM	5,7-dihydroxy flavones CRM	Naringenine CRM
$\bar{X}$	between-bottle ( $s_1, \%$ )	99.31 ± 0.018	99.58 ± 0.013	99.58 ± 0.015
	within-bottle ( $s_2, \%$ )	99.31 ± 0.011	99.58 ± 0.010	99.58 ± 0.010
F-test	$F = s_1^2 / s_2^2$	2.63	1.86	2.60
	$F_{0.05}(14,6)$	3.96	3.96	3.96
	$F < F_{0.05}$	Y	Y	Y
t-test	$t =  \bar{x}_1 - \bar{x}_2 $	0.0010%	0.0011%	0.0022%
	$t_a = 2 \left[ \left( \frac{s_1}{\sqrt{n_1}} \right)^2 + \left( \frac{s_2}{\sqrt{n_2}} \right)^2 \right]^{\frac{1}{2}}$	0.0064%	0.0049%	0.0053%
	$t \leq t_a$	Y	Y	Y

**Table 4.** The results of stability of flavonoids CRMs.

Time interval	Baicalein	5,7-dihydroxy flavones	Naringenine
Zero month	99.27	99.50	99.53
One month	99.37	99.56	99.57
Three months	99.31	99.54	99.58
Six months	99.31	99.52	99.50
Twelve months	99.35	99.46	99.55
Average (%)	99.32	99.52	99.55
RSD (%)	0.04	0.04	0.03

**Table 5.** The results of uncertainty evaluation of flavonoids CRMs.

Uncertainty	Baicalein CRM	5,7-dihydroxy flavones CRM	Naringenine CRM
$u_1/\%$	0.01	0.02	0.03
$u_2/\%$	0.04	0.04	0.01
$u_3/\%$	0.36	0.33	0.50
$u_4/\%$	0	0	0
$u_5/\%$	0.06	0.06	0.06
$u_6/\%$	0.01	0.01	0.01
$u_c/\%$	0.37	0.34	0.50
k	2	2	2
U/%	0.8	0.7	1.0
Purity value/%	98.8	99.1	99.5

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