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Research on a Certified Reference Material of Rhodamine B for Purity

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Abstract

A certified reference material for purity of Rhodamine B was developed through qualitative analysis, quantitative analysis, homogeneity test, stability test and uncertainty evaluation. Raw material of Rhodamine B purchased by business was recrystallized for preparation of candidate reference material. The purified was dried, then bottled in dark glass vials. Stratified random selection was applied to select a number of vials for homogeneity and stability studies, which revealed that the prepared reference material was homogeneous and sufficiently stable. The chemical composition of Rhodamine B sample was determined by infrared spectroscopy and nuclear magnetic resonance. A cooperative certification was conducted with eight qualified laboratories. The certified value was studied by mass balance method (including analysis of liquid chromatography with diode array detector, water content, ash content and residual solvents analysis). The certified value of the reference material for purity was found to be 99.0% with a relative expanded uncertainty of 1.0% ($k=2$), which made the candidate reference material a very useful calibrant in food and cosmetic analysis.

Keywords: Rhodamine B, Reference Material, Uncertainty, Quantify

1. Introduction

At present, the issue of food safety has become the focus of the whole society¹. Many food safety issues are related to the improper use of food additives, such as over-use or illegal use of food additives and excessive use of additives.². Food additives are artificial synthetic or natural substances added to food for the purpose of improving the color, smell or taste of food, as well as for the needs of anti-corrosion and processing technology. With the development of experimental techniques for food coloring safety, some synthetic pigments have been found to have carcinogenic effects and can induce chromosomal mutations. In recent years, China witnessed a string of food safety scares including Sudan red, melamine, dyeing steamed bread, poison bean sprouts and poison ginger.

Rhodamine B, also known as Rose Bengal B, is a peach red synthetic dyes. It has been found that Rhodamine B could be carcinogenic and cause subcutaneous tissue sarcoma, thus products containing it must contain a warning on its label. Cases of economically motivated adulteration, where it has been illegally used to impart a red color to chili powder, have come to the attention of food safety regulators³. In China, the government has explicitly banned the use of Rhodamine B in any food.

Certified Reference Materials (CRMs) are "controls" or standards used to check the quality and metrological traceability of products, to validate analytical measurement methods, or for the calibration of instruments. A certified reference material is a particular form of measurement standard. In the field of analysis and testing, CRMs play an important role in the research of qualitative and quantitative methods and also in error determination⁴⁻⁶. At present, most of the industries in China use Rhodamine B standards imported from abroad, such as the purity reference materials from Dr. Ehrenstorfer GmbH in Germany or the solution standards from Accustandard[®] in the United States. However, these standards are usually expensive for developing countries and have a long purchasing cycle⁷⁻⁸. No published research work on the certification process of pure Rhodamine B Reference Materials (RM) is available in the literature, and only CRM certificates issued by the producers can be obtained. Therefore, based on needs of food safety and the protection of national food safety traceability system, the development of Rhodamine B certified reference materials will fill these gaps, thus providing important technical support for food safety inspection, supervision and management⁹⁻¹³.

In this work, we report for the first time, a full scientific process of the purification, qualitative analysis, homogeneity test, stability test and uncertainty evaluation and certification of Rhodamine B RM. The certified value was studied by mass balance method, which including analysis of liquid chromatography with diode array detector, water content and ash content. The purity of the main component of Rhodamine B purity reference material was characterized by the cooperation of eight laboratories though the liquid chromatography area normalization method with diode-array detection (LC-DAD). Data from these methods was combined to produce the certified value and uncertainty.

2 Materials and Methods

2.1 Major instruments and reagents

Liquid Chromatography with Diode-Array Detection (LC-DAD): LC 2695 -2998, Waters; Fourier Transform Infrared spectroscopy (FTIR): Tensor-37, Bruker; Nuclear Magnetic Resonance: DPX-300, Bruker; Ultrapure water: produced by Milli-Q Advantage A10, Millipore, resistance:18.2 M Ω ; Water content: measure by Karl-FisherTitrator,T50, Mettler-Toledo; Residual solvents: Gas Chromatograph (FID detector, Headspace sampler), GC2010,SHIMADZU. Ash content: Muffle furnace, P330, Nabertherm.

Rhodamine B raw materials: AR, purity>95%; Methanol: chromatographic grade, Merck. The other reagents used in the experiment were all analytical grade.

2.2 Preparation of Rhodamine B Standard Samples

15 g of Rhodamine B was added in 100 mL methanol-water mixed solvent (volume ratio 5:1), and heated at 80 °C to dissolve. With stirring, 20 mL methanol-water mixed solvent (volume ratio 1:1) was added for 3 times, and the solution was kept at 80 °C for 20 min and then recrystallized at 4 °C for 5 h. After filtered, dried, milled and sieved, the candidate reference material was obtained. Repeat these process for 6 ~ 9 times. The purified sample was packed in totally 400 brown glass vials, which contained 200 mg reference materials. Packaged samples were stored at 4 °C.

The packed Rhodamine B reference materials were selected randomly, accurately weighed 100 mg, diluted with methanol in 100 mL of volumetric flask to give a concentration of 1.0 mg·mL⁻¹.

2.3 Characterization Analysis

The main components of Rhodamine B reference material were characterized by infrared spectroscopy and nuclear magnetic resonance spectroscopy.

FTIR: The sample was prepared by potassium bromide (KBr) solid tableting method and put on the test. Sample gain was 1.0, using DTGS KBr detector and KBr beam splitter, the infrared spectrum scanning range is (4000 ~ 400) cm⁻¹, the sample was scanned 8 times as the

same as the background.

Nuclear Magnetic Resonance: The sample was determined by nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$). Deuterated chloroform was chosen as solvent, using Bruker DRX-300 NMR. The optimized relaxation time was 30 s and the number of scans was 32.

2.4 Purity analysis

The purity of Rhodamine B reference material was determined by mass balance (MB) method, which was based on area normalization methods of liquid chromatography, water analysis, ash analysis and residual solvents analysis¹⁴⁻¹⁵.

Liquid Chromatography: Diode-array detection (DAD), C18 Column, 5.0 μm , 4.6 mm \times 250 mm; mobile phase: methanol; ultraviolet detector wavelength: 545 nm; flow rate: 1.0 mL/min; injection volume: 10 μL ; column temperature: 35 $^\circ\text{C}$.

Residual solvents analysis: The sample was dispersed in 10 g of N-Ndimethylacetamide in a sealed headspace vial. The vials were heated and agitated to establish equilibrium between the liquid and vapor ensuring saturation of the headspace with the residual solvents present in the liquid phase. Static headspace(HS) section: The equilibrium temperature was 80 $^\circ\text{C}$, the loop temperature was 90 $^\circ\text{C}$, the transfer line temperature was 110 $^\circ\text{C}$, and the equilibrium time was 30 min, pressurization time was 2 min; GC-FID part: Agilent DB-6, column, 30 m \times 0.32 mm \times 0.50 mm; carrier gas flow rate was 1 mL/min, split ratio was 10: 1, headspace injection; inlet temperature was 200 $^\circ\text{C}$; starting column temperature was 40 $^\circ\text{C}$, hold for 15 min, program temperature to 150 $^\circ\text{C}$ at 10 $^\circ\text{C}$ / min, hold for 10 min. The detector temperature was 250 $^\circ\text{C}$.

Water analysis: The water content was determined using the Karl Fischer (KF) Coulometric method¹⁶. The sample volume was 2 mg; the detection ambient temperature was 20 $^\circ\text{C}$, the humidity was 40%; the polarized electrode was DM143-5C; the polarization current was 5.0 A; stirring speed efficiency was 45%; control end point was 100.0 mV; electrolysis electrode current was automatic end, with 3.0 g/min drift relative termination. The average of six repeated experiments was taken as the experimental result.

Ash analysis: The ash content of pure raw materials was measured by high-temperature

ignition, and the appropriate amount of sample was accurately weighed, approximately 1 g, accurate to 0.01 mg, and was burned to a constant weight in a muffle furnace at (650 ± 50) °C. As a result, measurements were repeated six times and the average was taken as the experimental result.

3 Results and Discussion

3.1 Characterization Analysis

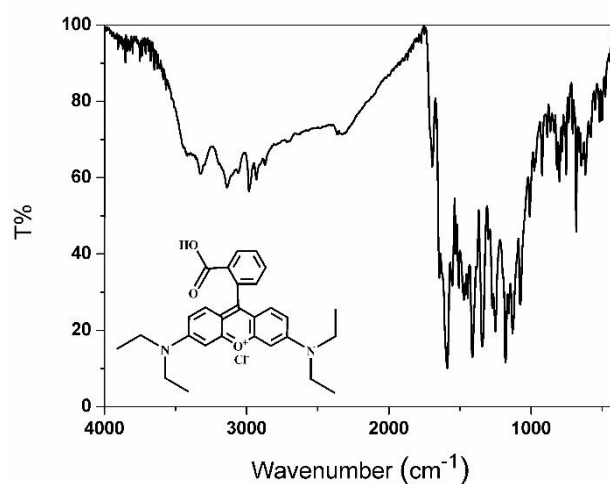


Figure 1. Fourier transform infrared spectrum of Rhodamine B purity reference material

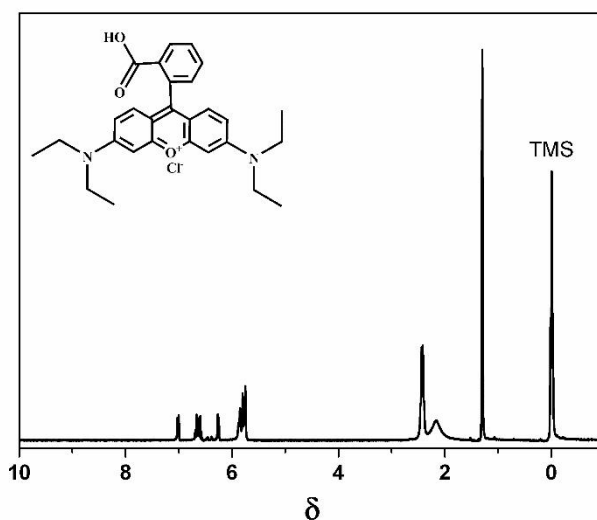


Figure 2. ¹H Nuclear Magnetic Resonance Spectrograph of Rhodamine B Purity Standard Material

The main component of Rhodamine B reference material was qualitatively analyzed by

infrared spectroscopy and nuclear magnetic resonance spectroscopy. Figures 1 and 2 were the IR spectrum and ¹H-NMR spectrum of the main components. Compare with the data of Rhodamine B pure substance in Spectral Database for Organic Compounds (SDBS), the results of IR and ¹H-NMR of Rhodamine B reference material were completely in conformity with the data in SDBS, that suggested that the main component was highly purified Rhodamine B.

3.2 Homogeneity Analysis

Table 1. The Homogeneity Analysis Results of Rhodamine B Purity Reference Material Uniformity

Bottle No.	Purity/%	Average / %
1	98.29, 99.45, 99.24	98.99
2	99.18, 99.13, 99.10	99.14
3	99.21, 99.24, 99.11	99.19
4	99.18, 99.13, 99.61	99.31
5	99.17, 99.24, 99.17	99.19
6	99.25, 99.23, 99.25	99.24
7	99.25, 99.49, 99.15	99.30
8	99.46, 99.14, 99.40	99.30
9	99.47, 99.20, 99.42	99.36
10	99.19, 99.14, 99.32	99.22
11	99.20, 99.25, 99.36	99.27
12	99.30, 99.19, 99.22	99.24
13	99.24, 99.20, 99.19	99.21
14	99.14, 99.31, 99.30	99.25
15	99.12, 99.23, 99.09	99.15

The homogeneity of reference material was an important indicator for measuring the performance of reference material, and it also played an important part in the accurate and identical dissemination of a value of quantity¹⁷. The variance analysis method was adopted to statistically test sample homogeneity. 15 bottles of Rhodamine B purity samples were randomly selected from 400 bottles of Rhodamine B purity samples respectively according to the numbers to evaluate between-bottle homogeneity. About 10 mg of the sample was precisely weighed and formulated into a solution. The purity was determined by liquid chromatography - area normalization method. Each bottle was injected 3 times to evaluate within-bottle homogeneity. The average value of each measurement was used as the result of homogeneity evaluation and perform an analysis of variance. The results are listed in Table 1 and Table 2. Dixon criterion and the Grubbs criterion were used to analyze these data and found no suspicious values. Within-bottle homogeneity (intergroup) dictated the minimum sample intake, for which the established uncertainty was still valid. Then the between-bottle homogeneity (intragroup) dealt with the bottle-to-bottle variation. When between-bottle variation should be quantified, the two effects can be separated by analysis of variance (ANOVA) with the intergroup homogeneity and intragroup homogeneity.

Table 2. Variance Analysis of Rhodamine B Purity Reference Material

Homogeneity	SS ^a	df ^b	MS ^c	F ^d	F crit ^e
Intergroup	0.347	14	0.025	0.658	2.037
Intragroup	1.147	30	0.038		
Totally	1.494	44			

^aSS: Stdev Square; ^bdf: degree freedom; ^cMS: Mean Square; ^dF: Factor; ^eFcrit: Factor Critical.

$$F_{\alpha}(v_1, v_2) = 2.037, \alpha = 0.05 \quad (1)$$

$$F = \frac{S_1^2}{S_2^2} = \frac{0.025}{0.038} = 0.658 \quad (2)$$

v_1 : intergroup degree freedom; v_2 : intragroup degree freedom; α : significant level; $F_{\alpha}(v_1, v_2)$: Factor Critical with v_1 and v_2 ; S_1^2 : Mean Square of intergroup; S_2^2 : Mean Square of intragroup

$F < F_{\alpha}$ suggested that there was no significant difference between the samples.

3.3 Stability Analysis

Table 3. Long-term Stability Test of Rhodamine B Purity Reference Material

Time / month	Purity / %
1	99.25
3	99.17
6	99.56
12	99.74
18	99.33
24	99.88
30	99.50

According to the technical standard of reference materials¹⁸, the stability of the purity reference material of Rhodamine B was tested for 30 months of long-term stability (stored at 4 °C) (Table 3) and 7 days of short-term stability (stored in a simulated 60 °C thermostat) (Table 4). Long-term stability referred to the stability of reference material properties over time.

A linear model was used as an empirical model to analyze the long-term stability data of Rhodamine B and calculated the slope b_1 . From this model, we obtained:

$$|b_1| < t_{0.95, n-2} \cdot s(b_1) \quad (3)$$

b_1 : slope; $t_{0.95, n-2}$: t factor with 95% confidence level, degree of n-2; $s(b_1)$: standard deviation of b_1 .

That meant the slope was not significant. It indicated that a clear trend of change magnitude of the Rhodamine B purity reference material does not occur within 30 months.

Short-term stability referred to the stability of the reference material during transport under transportation conditions (transportation stability). In order to explore the influence of

transportation conditions on the stability of the reference materials, and to take into consideration the high temperature and wet weather such as in the south of China, we simulated the transport conditions and randomly selected 6 bottles of samples under transport conditions (60 ± 5) °C and normal storage conditions (4 ± 1) °C for one week. Table 4 showed the effect of two different conditions on the stability of Rhodamine B purity reference material.

Table 4. Test data and analysis of variance of short-term stability of Rhodamine B purity reference material

Time / Day	Purity under 4 °C	Purity under 60 °C
1	99.45	99.56
2	99.43	99.49
3	99.51	99.32
5	99.32	99.28
7	99.23	99.31

Similarly, regression analysis was performed on short-term stability data, and the slope and standard deviation were calculated with formula 3.

Compare with the purity of Rhodamine B purity reference material under two different conditions, the difference of purity in different days were all less than the uncertainty. It was found that both the storage conditions and the transport conditions could well ensure the stability of the reference material.

3.4 Characterization of Rhodamine B purity reference material

3.4.1 Collaborative Characterization

According to the requirements of “Technical Norm of Primary Reference Material” in the Chinese National Calibration procedure (JJG 1006-94)¹⁹, the purity of the main component of Rhodamine B purity reference material was characterized by the cooperation of eight laboratories though the liquid chromatography area normalization method. The purity results

of the eight laboratory were shown in Table 5. Grubbs criterion and Dixon criterion were used to analyze these data and found no suspicious values.

Table 5. Characterization of Rhodamine B purity reference material in eight laboratories

Laboratory No.	Purity /%	Average /%
1	98.19, 99.31, 99.19	99.23
2	99.90, 99.85, 99.89	99.88
3	99.21, 99.25, 99.15	99.20
4	99.29, 99.28, 99.28	99.28
5	99.47, 99.43, 99.43	99.44
6	99.95, 99.88, 99.84	99.89
7	99.64, 99.65, 99.68	99.66
8	99.49, 99.43, 99.39	99.44

Grubbs method: A certain value of the set of data, which had the largest absolute residual, was suspected to be the suspicious if it satisfied the formula 5. The opposite was not.

$$\frac{|x_d - \bar{x}|}{s} \geq G(\alpha, n) \quad (5)$$

x_d : suspicious value with maximum residual; \bar{x} : average value; s : standard deviation; $G(\alpha, n)$: Grubbs threshold associated with significant level and repeated observation.

Based on the experimental data, the sixth with largest residual was suspected to be suspicious, and the suspicious value analysis was performed.

$$\frac{|x_6 - \bar{x}|}{s} < G(0.05, 8) \quad (6)$$

The result shows that the sixth was not a suspicious value and was retained.

Dixon method: Arrange data from small to large, if $r_{11} > r'_{11}$, $r_{11} > D(0.05, 8)$, x_n was the suspicious; $r'_{11} > r_{11}$, $r'_{11} > D(0.05, 8)$, x_1 was the suspicious, when $n=8\sim 11$. $D(\alpha, n)$: Dixon threshold associated

with significant level and repeated observation.

$$r_{11} = \frac{x_n - x_{n-1}}{x_n - x_2} = \frac{x_8 - x_7}{x_8 - x_2} = 0.015 \quad (7)$$

$$r'_{11} = \frac{x_2 - x_1}{x_{n-1} - x_1} = \frac{x_2 - x_1}{x_7 - x_1} = 0.044 \quad (8)$$

$$D(0.05, 8) = 0.608 \quad (9)$$

The result showed that there was not suspicious value and all of data were retained.

The total average purity result of eight laboratories was used as the result of the purity value of Rhodamine B purity reference material.

The purity value was as follows:

$$W_{LC} = \frac{1}{8} \sum_{i=1}^p \overline{W}_i = 99.50 \% \quad (9)$$

W_{LC} : the average purity of the liquid chromatography in eight laboratories.

The purity of Rhodamine B purity reference material was normalized by liquid chromatography area normalization method. However, the results did not include the water, residual solvent content and ash of the reference materials, so it was necessary to test the water and, residual solvent content and ash.

3.4.2 Determination of Water Content

The Rhodamine B water content tests were performed on a Karl Fischer water analyzer, which was measured with six times. The average value 0.17% was used as the water content: W_{water} .

3.4.3 Determination of Residual Solvent Content

The solvent residue of Rhodamine B was determined by headspace-gas chromatography, and no solvent was detected in the experiment. Therefore, the solvent residue in Rhodamine B was negligible.

3.4.4 Determination of Ash Content

The ash content of Rhodamine B purity reference material was measured after

high-temperature ignition and then weighted in high-precision balance. The appropriate amount of sample was weighed and ignited to a constant weight in a muffle furnace at (650 ± 50) °C. The result was weighed and measured by six times. The average value 0.34% was used as the ash content W_{ash} .

3.4.5 Final Result of the Purity Value

The content of water and ash was taken into consideration^{6, 14, 20-21}, and the final result of the purity value of Rhodamine B purity reference material was:

$$W = W_{LC} \times (1 - W_{\text{water}} - W_{\text{residual}} - W_{\text{ash}}) = 99.0\% \quad (10)$$

w : final purity.

3.5 Evaluation of Uncertainty

Table 6 The uncertainty components of Rhodamine B purity reference material

Uncertainty components	Result / %
u_w	0.181
u_{bb}	0.057
u_{Its}	0.270

u_w : the uncertainty component of the characterizing of the purity; u_{bb} : the uncertainty component of the homogeneity analysis; u_{Its} : the uncertainty component of the stability analysis.

The total uncertainty value consisted of three parts^{15, 22-24}. The first part was the uncertainty value Rhodamine B experiments introduced from the characterization procedure (u_w); the second part was introduced from the heterogeneity of sample (u_{bb}); the third part was introduced from the instability of sample (u_{Its}). According to the experimental results, the characteristic standard value and uncertainty component of Rhodamine B standard substance were calculated and shown on Table 6.

Thus the combined standard uncertainty was as following:

$$u_c = \sqrt{u_v^2 + u_{bb}^2 + u_{ts}^2} \quad (11)$$
$$= \sqrt{0.181\%^2 + 0.057\%^2 + 0.27\%^2} = 0.330\%$$

Extended uncertainty U_{95} was as following:

$$U_{95} = k \times u_c = 2 \times 0.330\% = 0.660\% \approx 1.0\% \quad (12)$$

Where 95 was the coverage probability 95%, and k was the confidence factor.

In summary, the purity value results of Rhodamine B reference materials could be expressed as:

$$W = (99.0 \pm 1.0)\%, k = 2 \quad (13)$$

The purity standard substance has been approved as a national second-class certified reference material by the State Administration of Quality Supervision, Inspection and Quarantine, the No. was GBW(E)100371, which could be used as a measurement and value traceability standard.

4. Conclusion

The development of Rhodamine B purity reference material has been investigated in homogeneity and stability, and a number of laboratories have taken part in the joint determination of the purity value. These reference materials have been used in the measurement of actual samples and the results have been satisfactory. The development of this series of reference materials can meet the needs of food, pharmaceuticals, daily chemicals, environmental protection and chemical product research and testing, and it is expected to be used in the calibration and analysis method to evaluate the related instruments in the future (chromatographs, fluorescence spectrophotometer or photometer for example).

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