D. I. Mendeleyev Institute for Metrology

Reference Materials in Measurement and Technology

Proceedings of the Fifth International Scientific Conference RMMT 2022

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Editors

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Certified Reference Material of Tylosin Tartrate

Anastasia Yu. Gulyaeva, Alexei Y. Khrushchev, Svetlana V. Kolyachkina, Victoria B. Muravieva, and Svetlana V. Antonova

Abstract Antibiotics are widely used to combat various infectious diseases in animals. The uncontrolled use of antibiotics leads to antibiotic residues in food products that are easily transmitted to humans through food chains. The quality of medicinal products and the food safety of animal products is controlled by determining the content of drug residues using reference materials (RMs). In this study, we develop a certified RM (CRM) for the composition of tylosin tartrate. The methodology involved selection of a procedure for preparing the initial RM and methods for measuring the content of tylosin tartrate. As a result, HPLC was used for quantitative determination of the certified value of the measured quantity (mass fraction of tylosin). In accordance with RMG 93-2015 and GOST ISO Guide 35-2015, the standard uncertainty of the RM certified value from the characterization method, heterogeneity and instability of the material was estimated. The expanded uncertainty of the certified value was calculated. A new CRM GSO 11632-2020 for the composition of tylosin tartrate was approved. The CRM can be used for certifying

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measurement methods and controlling the measurement results of the mass fraction of tylosin in veterinary medicinal products, food, and animal feed.

Keywords Tylosin · Reference material · Medicinal product · Veterinary medicine · Food safety · Customs union

Abbreviations Used

CRM	Certified reference material
HPLC	High performance liquid chromatography.

Introduction

In the contemporary world, the topic of the use of antibiotics for the treatment of infectious diseases in medicine and veterinary practice, along with the use of growth stimulants in animal husbandry, is particularly acute [1–5].

For the widespread use of antibiotics in the industry, such requirements are imposed as ease of use, rapid absorption and long-term elimination, high therapeutic efficacy, stability of finished dosage forms, and low cost. The listed parameters correspond to widely-used drugs based on tylosin [6, 7]. According to the information provided on the website of the State Register of Medicines for Veterinary Use in the territory of the Customs Union countries (Russian Federation, Republic of Belarus and Republic of Kazakhstan),[1] as of September 2022, more than 150 drugs containing tylosin and its salts have been registered [1].

Tylosin has a broad therapeutic effect and is well tolerated in all animal and poultry species. In medicines for veterinary use, both tylosin in the form of a base and its salt, tylosin tartrate, are used as an active substance. The preparations are produced in the form of powder, microgranules and solutions. It is administered with drinking water, in the form of a mixture with feed, via aerosol treatments and incubation egg treatments, as well as in the form of injectable and oral solutions. Non-compliance with veterinary requirements for the use of such drugs can lead to the manifestation of toxic and allergic reactions in animals. It is also worth noting that the use of antibiotics in animal husbandry in violation of the requirements of the instructions

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[1] State Register of Medicines for Veterinary Use (2022) Available via Galen. https://galen.vetrf.ru/#/registry/pharm/registry?page=1. Accessed 15 July 2022 (In Russ.).

Veterinary drugs Republic of Belarus. Available via vetsnab.info. https://vetsnab.info/veterinarnye-preparaty/. Accessed 15 July 2022 (In Russ.).

State register of veterinary drugs and feed additives of the Republic of Kazakhstan. Available via Ministry of Agriculture of the Republic of Kazakhstan. https://www.gov.kz/memleket/entities/moa/documents/details/471966?lang=ru. Accessed 15 July 2022 (In Russ.).

for their use may lead to the accumulation of residues in foods such as milk, eggs, meat and offal, which poses a risk to humans when eating these products [8, 9]. One of the main adverse reactions when using such products of animal origin in food is the development of resistance in microorganisms to antimicrobial drugs, which can lead to the appearance of bacteria of high virulence and reduce the effectiveness of measures for the treatment of infectious diseases of humans and animals [3, 8, 10, 11]. A number of authors have noted that residual traces of tylosin can result in allergic reactions or dysbiosis, as well as having carcinogenic, mutagenic and hepatotoxic effects [12, 13], which can cause complex, incurable and long-term infections in humans, leading to higher costs [3, 4, 8–10]. As a result of these undesirable effects, as well as in connection with the circulation of substandard and counterfeit medicines, quality control of medicines in civil circulation is carried out in the territory of the Russian Federation along with the monitoring of food safety [1, 14–20].

According to the requirements of the Technical Regulations of the Customs Union TR CU 034/2013 and TR CU 021/2011 [21, 22], the monitoring of the content of tylosin residues in order to establish veterinary safety is subject to meat and offal of all types of productive animals, as well as milk and eggs. The current legislation of the Customs Union establishes the following maximum permissible levels of residual amounts of tylosin in animal products: milk and eggs—prohibited (at the sensitivity level of the method definition); meat, liver, kidneys, raw fat—no more than 0.1 mg/kg.

In order to determine tylosin levels, microbiological and physicochemical methods of analysis are used, including planar chromatography, high-performance liquid chromatography, capillary electrophoresis, and voltammetry [23–27]. All of the listed measurement methods, as a rule, include the use of a reference material (RM) of an approved type. Thus, RMs are an important component for ensuring the unity, comparability and traceability of measurement results necessary to guarantee the quality of medicines, including monitoring the accuracy of measurement results and confirming the authenticity of the substance under study, thus ensuring the establishment of metrological characteristics that meet the requirements of 102-FZ [28–30].

To date, commercial tylosin RM products are defined by the following standards: Tylosin United States Pharmacopeia Reference Standard No. 1703805; Tylosin British Pharmacopoeia Reference Standard No. BP349; Tylosin European Pharmacopoeia (EP) Reference Standard No. T2880000; Tylosin HPC Standards GmbH No. 674913 (Germany)[2] [31]. However, the use of these RMs in Russia is currently difficult due to logistical constraints.

The preliminary analysis by the authors of the information submitted to the Russian Federal Foundation for Ensuring the Uniformity of Measurements[3] as of

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[2] Tylosin (2022) Merck. https://www.sigmaaldrich.com/DE/en/substance/tylosin916101401690. Accessed 17 October 2022.

[3] Foundation for Ensuring the Uniformity of Measurements (2022). https://fgis.gost.ru/fundmetrology/registry/19. Accessed 17 October 2022 (In Russ.).

May 2022 indicates the absence of a sufficient number of approved RM types necessary for metrological support of measurements related to food safety, which are subject to technical regulations TR CU 034/2013, TR CU 021/2011, as well as those concerning the quality of medicines for veterinary use.

Attention is drawn to the problem of ensuring the nomenclature of RM in the field of healthcare and pharmaceutical industry in works [31–35].

Therefore, the purpose of this study is to develop a RM-approved type of composition of tylosin tartrate. The objectives of the study include the following: justification of the selection of a candidate substance for the role of RM; implementation of input control of the candidate material of RM; determination of the procedure for certification of RM; testing for type approval; establishment of metrological characteristics of RM.

Materials and Methods

Reagents, Solutions, and Materials

A highly purified pharmaceutical substance of tylosin tartrate with a quality certificate (produced by Sigma-Aldrich, Germany) was used as a candidate material for the RM.

The American Pharmacopoeia tylosin RM (reference spectrum)[4] was used as a basis for comparison within the measurement of the certified characteristic. To confirm the authenticity of the candidate material, the tylosin tartrate RM of the American Pharmacopoeia[5] was used.

The following reagents and consumables were used during the measurements: acetonitrile, ACS reagent $\geq$ 99.5%, H.H., CAS No. 75-05-8; sodium perchlorate, ACS reagent $\geq$ 98.0%, H.H., CAS No. 7601-89-0; concentrated hydrochloric acid 37%, H.H., CAS No. 7647-01-0; distilled water according to GOST 6709-96; a syringe filter with a pore size of 0.45 microns; a tip for dispensers of various volumes.

Instruments and Conditions

The samples were analyzed:

- by IR spectroscopy using an IR-Fourier spectrometer infrared model NicoletiS50 (Thermo Fisher Scientific, USA), with the following instrumental and software

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[4] Tylosin LOT F0C008 (2022) Available via. https://static.usp.org/pdf/EN/referenceStandards/certificates/1703805-F0C008.pdf. Accessed 17 October 2022.

[5] Tylosin Tartrate (2022) Available via. https://store.usp.org/product/1703850. Accessed 17 October 2022.

settings: summation of 16 measurements; DTGS KBr detector; beam splitter—KBr; signal gain—1; speed—0.6329; aperture—100; interpolation—level 2; apodization "N-B strong"; phase correction—Mertz [36, 37].

- by the HPLC method. Separation was carried out on a column Luna 5u C(18) 100A 250 * 4.60 mm in isocratic mode, using a mixture of 9.8% sodium perchlorate solution and acetonitrile as an eluent. Detection was carried out in the ultraviolet region of the spectrum at a wavelength of 290 nm. This wavelength was chosen in the course of experimental studies using the parameters of the developed technique as being the most optimal for obtaining a peak of sufficient intensity.

Sample Preparation of the RM Source Substance

In accordance with the general requirements of the State Pharmacopoeia of the Russian Federation [38] for pharmaceutical substances and foreign pharmacopoeias for the quality of the substance tylosin tartrate, a list of quality indicators for the input control of the RM was developed.

In this regard, the following basic requirements were determined for the organic substance selected for research as a candidate substance for the role of RM: appearance, solubility, color and transparency of the solution, mass fraction of tyramine, mass fraction of tylosin, mass loss during drying. Special attention was paid to determining the authenticity of the RM.

To confirm the authenticity of RM substances, physicochemical methods were used: IR spectroscopy in the middle range and the high-performance liquid chromatography (HPLC) method. The authenticity and quality of the substance were evaluated according to the requirements of the OFS.1.2.1.1.0002.15 and OFC.1.2.1.2.0005.15 of the State Pharmacopoeia of the Russian Federation [39, 40].

Sample Analysis by IR Spectroscopy

The analysis of the candidate RM was carried out by IR spectroscopy by programmatically comparing the spectral data of the test substance and RM Tylosin Tartrate No. 1703850 of the American Pharmacopoeia (reference spectrum) in the average IR range of 400–4000 cm^{-1} (Fig. 1).[6]

To assess the correspondence of the spectra, the positions and relative intensities of the main bands on the reference spectrum were compared with those of the candidate certified reference material. In addition, the OMNIC software (search standards classification analysis algorithm) was used to more accurately analyze the correspondence of spectra in the entire range according to the "wave number" and "signal

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[6] Tylosin Tartrate (2022) Available via. https://store.usp.org/product/1703850. Accessed 17 October 2022.

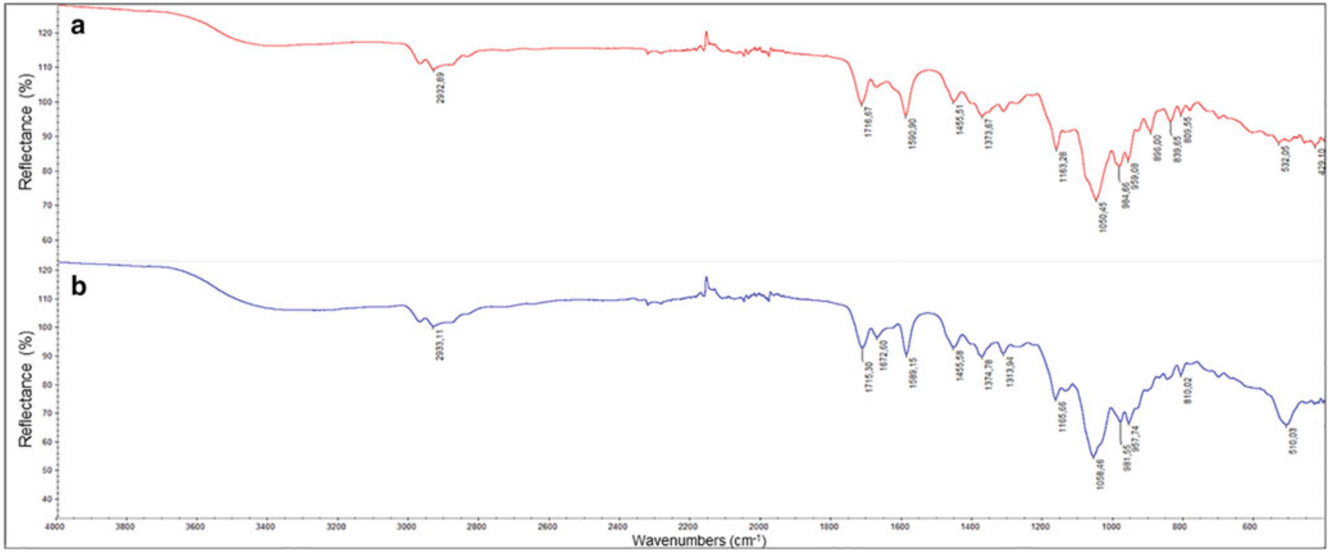

Fig. 1 IR spectra of the reference spectrum of the candidate reference material of tylosin tartrate (**a**) and reference material of tylosin tartrate No. 1703850 of the American Pharmacopoeia (**b**)

intensity" characteristics and calculate the total correlation coefficient (R) as a function of data variance on a scale from 0 to 100% (a coefficient value of 100 indicates full compliance) [41].

In the course of the IR spectroscopy studies, the correlation coefficient between the spectrum of the test sample and the reference spectrum was 96.2%, which indicates its identity in structure and chemical composition to the reference spectrum (R > 0.95%).

HPLC Sample Analysis

The RM candidate material was analyzed by HPLC with spectrophotometric detection in the ultraviolet region of the spectrum at a wavelength of 290 nm by comparing the retention time of the main peaks of the test substance and RM Tylosin Tartrate No. 1703850 of the American Pharmacopoeia.

During the confirmation of the authenticity of the material by HPLC, it was found that the retention time of the main peaks of the material coincided with the retention time of the main peaks of the basis for comparison (Figs. 2 and 3).

After the input control was carried out and the compliance of the candidate RM with the stated requirements was established, the feedstock was packaged at (50 ± 10) mg in sealed glass transparent ampoules for medicines for conducting studies to determine the metrological characteristics of the RM.

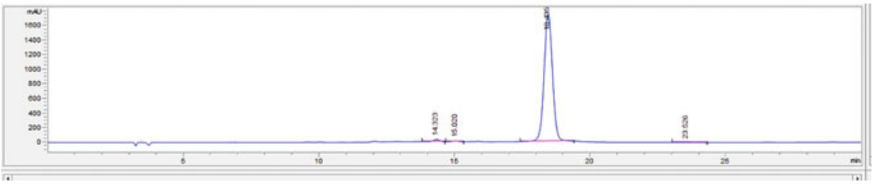

Fig. 2 Chromatogram of reference base: the reference material of tylosin tartrate No. 1703850 American Pharmacopeia

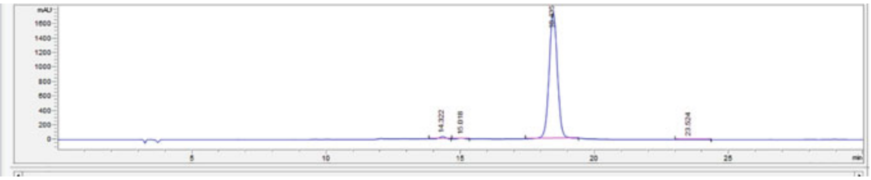

Fig. 3 Chromatogram of the candidate reference material for the composition of tylosin tartrate

Establishment of Metrological Characteristics of the Candidate RM

Taking into account the requirements of RMG 93-2015 [42] and GOST 8.315-2019 [43], a method was chosen for the RM characterization using a certified measurement technique based on applying an external standard. In this connection, as part of the planned work, MU A-1/065 Methodology for Measuring the Content of Tylosin in the Tylosin Tartrate Substance by High-Performance Liquid Chromatography[7] was developed and certified. The technique is based on the application of the HPLC method with spectrophotometric detection in the ultraviolet region of the spectrum at a wavelength of 290 nm. The study of the homogeneity and stability of the RM was carried out using a certified method of measuring the mass fraction of tylosin (MU A-1/065) in accordance with the provisions of RMG 93-2015.

Results and Discussion

There are several methods for determining the certified value of RM, for example: the method of direct determination of quantitative content (titration method), the indirect method (mass balance method) and the external standard method. Tylosin consists of four main closely related compounds, the ratio between which may vary. Therefore, we assumed that the use of the non-aqueous titration method cannot guarantee reliable results. At the same time, the American and European Pharmacopoeias suggest using a microbiological method for the quantitative determination of tylosin. This method is based on the ability of tylosin to inhibit the growth of microorganisms. In this case, the determinations are carried out by diffusion into agar on a dense nutrient medium by comparing the sizes of growth inhibition zones of test cultures of microorganisms. However, a characteristic feature of the method of diffusion into agar is its labor intensity and long time duration. Here, the measurement results depend on many factors, including the properties of test microorganisms, nutrient media, the designation of the antibiotic, as well as the skills of the employee conducting the research [44].

In addition to the microbiological method of diffusion into agar, along with the generally accepted pharmacopeial method, an instrumental HPLC method can be used to determine the content of tylosin in the RM [36, 45].

Thus, in order to confirm the certified value (mass fraction of tylosin), it was decided to combine the microbiological method of diffusion into agar with the HPLC method.

The method of measuring the content of tylosin in the tylosin tartrate substance using the microbiological method is based on the ability of the tylosin molecule

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[7] MU A-1/063 Method for Measuring the Content of Tylosin in the Tylosin Tartrate Substance by the Microbiological Method (2020). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/1010594. Accessed 7 October 2022 (In Russ.).

to diffuse in agar media and form suppression zones in which the used strain of Micrococcus luteus ATCC 9341, which is sensitive to the antibiotic, does not grow. The determinations are carried out by diffusion into agar on a dense nutrient medium by comparing the sizes of growth inhibition zones of test cultures of microorganisms.

The method for measuring the content of tylosin in the tylosin tartrate substance by HPLC (MU A-1/065) is based on the application of the HPLC method with spectrophotometric detection in the ultraviolet region of the spectrum at a wavelength of 290 nm.

The respective metrological characteristics of the methods for measuring the mass fraction of tylosin by the microbiological method of diffusion into agar and the HPLC method are presented in Tables 1 and 2.

The results of the certification of methods and the establishment of metrological characteristics (Tables 1 and 2) indicate that the method for determining the basic substance by HPLC has a higher accuracy compared to the method based on the microbiological method. At the same time, according to the results of the measurements of the tylosin content by both methods, a convergence of the results within the error of the methods was noted.

Taking into account the fact that the measurement of the content of tylosin in the substance of tylosin tartrate by HPLC has lower labor costs, as well as higher accuracy of determination, it was chosen for further work on the development of the tylosin RM. The HPLC method was used to evaluate the certified value of the mass fraction of tylosin in the RM, as well as to study its stability and uniformity.

Table 1 Metrological characteristics of the method for measuring the content of tylosin in the substance of tylosin tartrate by high performance liquid chromatography

Measured value	Tylosin content measuring range, mcg/mg	Relative extended uncertainty with confidence probability P = 0.95, Up*, %
Mass fraction of tylosin	From 800 to 1100 inclusive	3.2

* Corresponds to the boundaries of the relative error with a confidence level of P = 0.95

Table 2 Metrological characteristics of the method for measuring the content of tylosin in the substance of tylosin tartrate by the microbiological method

Measured value	Tylosin content measuring range, mcg/mg	Relative extended uncertainty with confidence probability P = 0.95, Up*, %
Mass fraction of tylosin	From 800 to 1100 inclusive	6

* Corresponds to the boundaries of the relative error with a confidence level of P = 0.95

Table 3 Results of determining the certified value of the certified reference material for the composition of tylosin tartrate; standard uncertainty from the method of characterizing the certified value of the certified reference material

Certified characteristic	Certified value of the RM $\hat{A}$, mcg/mg	Standard uncertainty from the characterization method $\hat{A}$ of the RM, u_{char}
Mass fraction of tylosin, mcg/mg	968.9	15.78 mcg/mg

Determination of the Certified Value of the RM and Evaluation of the Standard Uncertainty from the Method of Determining the Certified Value

The certified characteristic for determining the certified value of the RM of the composition of tylosin tartrate is the mass fraction of tylosin, mcg/mg.

In order to determine the certified RM value in randomly selected samples of the material, the mass fraction of tylosin was measured according to the developed and certified MU A-1/065 method at various levels of intermediate precision, as described above.

The evaluation of the results obtained to establish the certified value and the standard uncertainty from the method of determining the certified value of the RM was carried out in accordance with RMG 93-2015. The obtained value of the certified RM characteristic and standard uncertainty from the method of determining the certified RM value are shown in Table 3.

The obtained results were further applied to the evaluation of the extended uncertainty of the certified value of the RM of the composition of tylosin tartrate.

RM Uniformity Study

The study of the uniformity of the RM was carried out simultaneously with the determination of the certified value. Considering that the candidate RM is a dispersed material, the homogeneity study was performed by repeated measurements of the certified value in several samples randomly selected from the entire volume of the RM. Thus, ampoules with the material ($N = 12$) were taken from the entire prepared batch, from which the necessary amount was then taken by weighing for measurements ($J = 2$) under repeatability conditions.

Operations with samples and measurements of the mass fraction of tylosin were carried out in accordance with the measurement procedure MU A-1/065 described above.

Processing of the obtained results was carried out according to the scheme of one-factor analysis of variance in accordance with the provisions of RMG 93-2015. The

Table 4 Results of assessing the heterogeneity of the certified reference material for the composition of tylosin tartrate

Certified characteristic	Results of intermediate calculations				Standard uncertainty from the heterogeneity of the RM, u_h, mcg/mg (absolute)
	SS_e	SS_h	$\overline{SS}_e$	$\overline{SS}_h$	
Mass fraction of tylosin, mcg/mg	203.50	1838.46	16.96	167.13	8.67

SS_e sum of the squares of the deviations of the measurement results from the average values of each sample

SS_h sum of squared deviations of the average results for each sample from the arithmetic mean of all measurement results

$\overline{SS}_e$ square of the standard deviation of the average sample results

$\overline{SS}_h$ square of the standard deviation of the average results between samples

results of the evaluation of the heterogeneity of the material with the composition of tylosin tartrate are given in Table 4.

The obtained results were used in the evaluation of the expanded uncertainty of the certified value of the RM.

RM Stability Study

For the stability study, which aimed to determine the shelf life and storage conditions of the RM, as well as the standard uncertainty from the instability of the RM, an isochronous stability study method referred to as the "accelerated aging method" was used, taking into account the provisions of RMG 93-2015 and GOST ISO Guide 35-2015 [46]. The principle of this method consists in increasing the impact of factors that cause the trend of the certified characteristic over a period of time much shorter than the shelf life of the RM.

To calculate the aging time, the Van't Hoff rule was used for slow reactions: when heated at 10 °C, the reaction rate increases 2–4 times in accordance with RMG 93-2015.

To study long-term and short-term stability, a pre-calculated number of the required RM specimens were randomly selected and established at low and elevated temperatures.

The following conditions are chosen as the intended storage conditions: the RM is stored in sealed ampoules, separately from food and feed, in a dry place protected from light at a temperature of $+4$ to -18 °C.

To study stability, 13 selected specimens were established at low and elevated temperatures to study long-term stability.

The duration of the stability study was calculated based on the estimated shelf life of the RM (4 years) and fixed storage temperature values. The duration of the stability study τ was estimated by the formula:

$$\tau = \frac{T}{2^{\frac{t_1 - t_0}{10}}}, \tag{1}$$

where

T is the estimated shelf life of the RM sample;
$t_0, t1$ is the storage temperature of the RM and the storage temperature of RM during accelerated aging, respectively.

The period of stability study at the temperature of the intended storage conditions (+ 4 °C) and elevated temperature (+ 43 °C) was 96 days. Measurements were carried out at regular intervals once every twelve days.

The results of assessing the long-term stability of the RM are presented in Table 5.

To assess the effect of low temperatures, 5 RM samples were selected and placed in the freezer of a household refrigerator at a temperature of −18 °C. Measurements of the values of the certified characteristic were carried out once every five days.

All measurements were carried out under repeatability conditions according to the measurement method MU A-1/065 to establish a certified value in accordance with the above-described parameters of chromatographic analysis.

The results were processed in accordance with GOST ISO Guide 35-2015.

To calculate the standard deviation associated with the slope of the graphical dependence of the mass fraction of tylosin on time, the following formula was used:

$$s(b_1) = \frac{s}{\sqrt{\sum_{i=1}^{n} \left(X_i - \overline{X}\right)^2}}, \tag{2}$$

where

s is the standard deviation of the points from the line;
$s(b_1)$ is the standard deviation of b_1;

Table 5 Results of assessing the long-term stability of the certified reference material for the composition of tylosin tartrate

Time period, days	Estimated shelf life, T, days	Mass fraction of tylosin, mcg/mg	Standard uncertainty from instability of the RM, u_{stab}, mcg/mg
12	178.8	973	3.41
24	357.6	965	
36	536.4	968	
48	715.2	971	
60	894.0	968	
72	1072.8	969	
84	1251.6	968	
96	1430.4	976	

X_j is the number of days during natural storage, corresponding to days during "accelerated" aging, days;
$\overline{X}$ is the average number of days in natural storage, days.

The inequality was used to estimate the slope level:

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

where

$t_{0.95,\mathrm{n}-2}$ is Student's t-test (*t*-criterion) for *n* is 2 degrees of freedom and $p = 0.95$ (95% confidence level);
$s(b_1)$ is the standard deviation of b_1;
b_1 is the slope of the graphical dependency.

The calculation results are summarized in Table 6.

The obtained data, which indicate the fulfillment of the inequality according to formula 3, allow us to conclude that the slopes are insignificant; consequently, that the instability of the RM is not observed.

Based on the results of the stability control studies and tests to assess the effect of a reduced temperature of −18 °C, the shelf life of the tylosin tartrate RM is 4 years when stored at temperatures from +4 to −18 °C.

To establish the metrological characteristics of RM, the standard uncertainty from instability u_{stab} at time t was estimated by the formula:

$$\mathrm{u}_{stab} = \mathrm{s(b_1)} \cdot \mathrm{T} = 3.41\ \mathrm{mcg/mg}, \tag{4}$$

where

$\mathrm{s(b_1)}$ is the standard deviation of $\mathrm{b_1}$;
T is the estimated shelf life of the RM, days.

The obtained value $\mathrm{u_{stab}}$ was taken into account when determining the extended uncertainty of the certified value of the RM.

Table 6 Results of studying the stability of the certified reference material for the composition of tylosin tartrate and assessing the effect of low temperatures

Indicator	Stability study under conditions of "accelerated aging" at elevated temperature (at + 43 °C)	Studies of the effect of low temperatures on the RM (at − 18 °C)
$s(b_1)$	0.00239	0.004887
Student's t-test (t-criterion) for (n − 2), $\mathrm{t_{0.95,\ n2}}$	2.365, with n = 7	2.776, with n = 4
$\|b1\| < \mathrm{t_{0.95,\ n\text{-}2}} \cdot \mathrm{s(b1)}$	|0.0020| < 0.00565	|0.001374| < 0.013567

Table 7 Normalized metrological characteristics of the certified reference material for the composition of tylosin tartrate

Certified characteristic	Interval of permissible certified values of the reference material, mcg/mg	Permissible values of relative extended uncertainty at k = 2, %
Mass fraction of tylosin	From 800 to 1100 inclusive	6

RM Metrological Characteristics

The extended uncertainty of the certified value of the RM composition of tylosin tartrate was calculated according to RMG 93-2015 according to the formula:

$$U(\widehat{A}) = 2 \cdot \sqrt{u_{char}^2 + u_h^2 + u_{stab}^2}, \tag{5}$$

where

$U(\hat{A})$ is the expanded uncertainty where coefficient of the scope $k = 2$;
u_{char} is the standard uncertainty from the method of establishing the certified value of the standard RM;
u_h is the standard uncertainty of heterogeneity of the RM;
u_{stab} is the standard uncertainty of instability of the RM.

The normalized metrological characteristics of the developed RM are given in Table 7.

As part of the tests for the approval of the RM type, the traceability of the measurement results to the "mass fraction" unit of magnitude reproduced by GET 208-2019[8] was ensured by conducting confirmatory measurements of the mass fraction of the main substance on the GWET 208-1-2016 state secondary standard of mass fraction units and mass (molar) concentrations of organic components in liquid and solid substances and materials based on gas and liquid chromatography [42].

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[8] GET 208-2019 State primary standard of units of mass (molar) fraction and mass (molar) concentration of organic components in liquid and solid substances and materials based on liquid and gas chromatography-mass spectrometry with isotope dilution and gravimetry: D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1382717. Accessed 17 October 2022 (In Russ.).

Conclusion

Based on the results of the study, the GSO 11632-2020 tylosin tartrate approved composition pattern[9] was developed. The RM material is a substance of tylosin tartrate, a hygroscopic powder from white to slightly yellow; the material is packaged in (50 ± 10) mg in sealed glass ampoules equipped with a label. Normalized metrological characteristics: mass fraction of tylosin—800–1100 mcg/mg; relative extended uncertainty at $k = 2$; $P = 0.95$ equal to 6%. The RM is stored separately from food and feed in sealed ampoules in a dry, light-protected place at a temperature from plus 4 °C to minus 18 °C; shelf life −4 years.

To achieve this goal, the following tasks have been solved: the input control of the material has been carried out; the procedure for certification of the RM has been determined; tests have been carried out for type approval and the metrological characteristics of the RM have been established.

To establish the content of the certified RM value, two methods were used: the microbiological method of diffusion into agar and the HPLC method. However, since the method of quantitative determination of the certified value of the measured value (mass fraction of tylosin) by HPLC has a higher accuracy compared to the method based on the microbiological method, the measurements were carried out by HPLC. At the same time, according to the results of the measurements of the tylosin content by both methods, a convergence of the results within the error of the methods was noted. In accordance with RMG 93-2015 and GOST ISO Guide 35-2015, the standard uncertainty of the certified value of the RM was estimated from the method of characterization, as well as from the heterogeneity and instability of the material. The expanded uncertainty of the certified value has been calculated. The traceability of the measurement results to the “mass fraction” unit of magnitude reproduced by GET 208-2019 has been established by conducting confirmatory measurements of the mass fraction of the main substance at GWET 208-1-2016.

The theoretical significance of the conducted research lies in the fact that the results of the research part can be used for metrological support of measurements related to food safety, which are subject to technical regulations TR CU 034/2013 and TR CU 021/2011, as well as the quality of medicines for veterinary use.

The practical significance of the obtained results consists in the possibility to expand the possibility of certification of measurement methods and control of the accuracy of measurement results of the mass fraction of tylosin in veterinary medicines, in food and food raw materials, animal feed, as well as various environmental facilities. In addition, the RM can be used to determine the authenticity of tylosin in medicines and establish the sensitivity of pathogenic microorganisms to tylosin.

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[9] GSO 11632-2020 Reference materials of the composition of tylosin tartrate. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1384760. Accessed 17 October 2022 (in Russ.).

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Contribution of the Authors Gulyaeva A. Yu.—general research management, development of the research concept, collection and analysis of experimental data, collection of literature data, preparation of documents on RM tests for type approval, writing a draft of the article; Khrushchev A. Y.—acquisition and analysis of experimental data on IR spectroscopy, revision of the text; Kolyachkina S. V.—conducting research, developing methods, experimental data acquisition; Muravieva V. B.—conducting research, developing methods, experimental data acquisition; Antonova S.V.—collection of literary data, revision of the text.

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Virus-Like Particles: Properties and Characteristics of Reference Materials

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Abstract Virus-like particles (VLPs) have long attracted the attention of biologists and metrologists due to their dual nature. Despite their similarity to viruses, VLPs lack a virus DNA or RNA, thereby posing no threat of infection. The development of methods for the creation and standardization of VLPs, as well as for monitoring their characteristics, represents an important problem in the field of biotechnology and molecular biology. In this work, we aim to substantiate the requirements, methods, and instruments used to ensure the traceability of VLP-based certified reference materials (CRMs). The following methods for characterization of biological and physicochemical properties of such CRMs were considered: mass balance; high-performance liquid chromatography-mass spectrometry (HPLC-MS); gas chromatography-mass spectrometry (GC-MS); Karl Fischer coulometric titration; ion chromatography; inductively coupled plasma mass spectrometry; and isotope dilution. It was established that VLP-based CRMs should meet the following requirements: the presence of molecular patterns common to many pathogenic biological agents (PBAs) but absent in the organism; traceability to SI units; the absence of own genetic material; the presence only of inactivated homogenous strains when used for vaccine delivery. VLP-based CRMs facilitate metrological support in the field of medicine and vaccine production.

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Introduction

Virus-like particles (VLPs) represent an important object in the field of vaccine production and vaccine application, which makes research into the properties and characteristics of VLPs highly promising.

VLPs are specifically-engineered nanostructures that resemble viruses, thus being capable of penetrating the target body cells (those intended to be penetrated). VLPs contain no own genetic material, i.e., neither DNA nor RNA, which is their significant and unique advantage. As a result, VLPs can efficiently deliver medicines to the target molecules without posing a threat of infection.

VLPs are widely used to deliver drugs or vaccines not only to human [1], but also to animal [2] and plant cells [3]. These particles can be designed to perform both transport and antibacterial functions [4]. Acting as pre-concentrated antimicrobial agents that cause a rapid and local response, VLPs destroy the cell membrane by creating new, non-cellular pores at precise landing points on the membrane. This approach makes it possible to create virus-like frameworks with biologically regulated properties.

Vaccine delivery is an important function of VLPs [5, 6]. Their inherent properties, such as multimeric antigens the size of viruses, highly organized and repetitive structure, and the absence of infectivity, make VLPs suitable for inducing safe and effective humoral and cellular immune responses. VLP-based vaccines have been approved for treating hepatitis B virus and human papillomavirus, as well as for veterinary use [2] for the prevention of porcine circovirus infection. Consequently, the structural characterization of VLPs is an important requirement for triggering an immune response in the body.

Standardization of VLP properties, their creation methods, and approaches to monitoring their characteristics represent an important task in the field of biotechnology and molecular biology. Metrological traceability is the most important tool for ensuring the comparability and reliability of measurement results in laboratory medicine. In turn, metrological traceability is ensured by certified reference materials (CRMs), which allow the reproducibility and traceability of delivery systems to be determined in correlation with their physicochemical and biological properties.

In this work, we aim to substantiate the requirements, methods, and instruments used to ensure the traceability of VLP-based certified reference materials in Russian metrology.

Analytical Review

Virus-like particles contain no RNA or DNA of a pathogenic infectious agent, are non-infectious and structurally native, immunologically relevant viral antigens or proteins recognized by the immune system. VLPs morphologically resemble their living virus counterparts and are easily recognized and processed by the antigen-presenting cells of the immune system. In other words, due to their affinity to living viruses, the introduction of VLPs into the body triggers its immune response.

VLPs are good candidates for vaccine development due to their ability to induce a protective immune response. The human immune system has evolved to recognize the non-infectious self and the infectious non-self. The body has learned to elicit a strong immune response against microbial infections, at the same time as not responding to its own molecules. The human immune system consists of innate (nonspecific) and adaptive (specific) types [7, 8]. Innate immunity represents the first line of defense of the host against microbial infections. After the pathogen crosses the physical barriers of the host, such as the mucous membranes of the nose, mouth, eyes or skin, the immune system initiates an innate immune response within minutes. The cells of the innate immune system can recognize the common characteristics of a pathogenic biological agent (PBA) and trigger a response. The PBA-related structure, size, shape (e.g., icosahedron), molecular outer shell composition or so-called molecular patterns are molecules or properties (patterns) common to many PBAs, usually absent in humans but present in viral infection molecules. Molecular patterns, the ordered structure of the pathogen molecule, and the surface configuration can be recognized by pattern recognition receptors found in the body cells, which in turn leads to increased immunogenicity. In such a manner, innate immune perception mechanisms are triggered, which in turn stimulates antigen creation.

Standardization of VLP properties and methods of their determination presents a real challenge for metrologists [1]. Therefore, the development of methods and instruments for metrological support of VLP characteristics is a relevant research task.

Virus-like gene delivery systems represent a rapidly developing field of gene therapy. However, the lack of validated measurement techniques and reference materials of approved types (CRMs), which are used to deliver the necessary material into cells, justifies the need to develop appropriate CRMs and methods for comparative analysis of the characteristics of both synthetic and virus-derived gene delivery systems. The task of harmonizing the approaches to their characterization and test programs seems relevant.

A review of the studies performed in this field shows that the problem of VLP standardization is increasingly attracting research attention. There are CRMs traceable to SI units, which are used to determine the reproducibility and traceability of delivery systems in correlation with their physicochemical and biological properties. For example, a CRM based on high-purity nucleic acids was previously developed in [9]. This well-characterized CRM provides an effective tool for assessing the accuracy of next-generation sequencing (NGS) assays, thus facilitating the quantification of total nucleic acids. However, this CRM is not homogeneous, and its components are not fully characterized, which can lead to variations in measurement results.

Briones et al. [10] described the development of an RM for virus-like particles traceable to the International System of Units (SI). The RM major component is the C3-triskelion peptide, designed in the form of an icosahedron. It was established that the proteins and peptides in the composition of the RM are traceable to the International System of Units through the corresponding reference materials.

Taking into account the experience in the development of CRMs [9, 10], it can be assumed that commutative CRMs of approved types should be created in order to obtain highly-accurate and well-characterized VLPs.

Requirements for the Characteristics of CRMs for Virus-Like Particles

According to the conducted analytical review, the CRM material of virus-like particles must:

- have an affinity to live viruses;
- have a spiral, cylindrical, or icosahedron surface configuration, to be easily recognized by the immune system as alien, not inherent to the organism;
- have a repetitive structure;
- have the size of viruses;
- have molecular patterns common to many PBAs, but absent in human, animal, or plant organisms;
- be traceable to SI units;
- exhibit an antibacterial action when used as a virus-like scaffold with biologically regulated properties;
- not contain its own genetic material, i.e., DNA or RNA of pathogenic biological agents. In the case of vaccine delivery, it can only contain inactivated strains;
- be homogeneous.

The RM envelope material must contain a known, measured with a specified uncertainty, mass fraction of the main component. According to regulatory guidelines for advanced therapeutic drugs (e.g., 2009/120/EC [11]), such as gene therapy products, the RM must be relevant and specific for the products and substances used for comparison. Its physicochemical properties must be characterized and documented [10]. The requirements for the material include its accessibility; it must assemble into a virus-like particle in an understandable and predictable way, delivering the contents of the capsule into the body cells. For example, triskelion is synthetically available, predictably assembled into VLPs, which encapsulate and transfer genes into human cells and are devoid of the side effects typical of the virus [10].

Materials and Methods

The following methods and instruments for determining the certified characteristics (including qualitative properties) of VLP-based CRMs are proposed.

Determining the CRM Purity Mass Balance

Similar to any CRM of a biological nature, the purity of the proteins and/or peptides used as a VLP envelope must be initially confirmed. To this end, their comparison with the corresponding CRMs of proteins/peptides with established traceability to the International System of Units should be carried out.

The mass balance method can be considered appropriate for these purposes. Purity is understood as the mass fraction of the main component minus the mass fraction of all impurities in the material from 100%, according to RMG 55–2003 [12] by formula (1):

$$w_{mc} = 100\% - w_{rc} - w_{sol} - w_{inorg}, \tag{1}$$

where

w_{mc}	is the mass fraction of the main component in a sample, %;
w_{rc}	is the mass fraction of related compounds, %;
w_{sol}	is the mass fraction of water and organic solvents, %;
w_{inorg}	is the mass fraction of inorganic impurities, %.

The mass fractions of related compounds are determined by HPLC-MS, e.g., using an X500R QTOF mass spectrometer, AB Sciex Pte. Ltd, Singapore.

The mass fraction of water and organic solvents can be simultaneously calculated by determining the loss in weight during drying, based on the general pharmacopoeial article OFS.1.2.1.0010.15 [13]. However, the mass fraction of water and organic solvents can be determined by two separate methods. The mass fraction of water is determined by the Karl Fischer coulometric titration method, e.g., using an automatic Karl Fischer titrator CA-31, KF-3, and CA-310, Nittoseiko Analytech Co. Ltd. The mass fraction of residual organic solvents can be determined by mass spectrometry by passing a sample through a GC-MS gas chromatograph, e.g., using a Hexin GCMS 1000 gas chromatography mass spectrometer, Guangzhou Hexin Instrument Co., Ltd, China.

The mass fraction of inorganic impurities can be determined by ion chromatography using, e.g., a Dionex ICS-6000 ion chromatograph, Thermo Fisher Scientific Inc., USA, or using, e.g., an NexION 2000 inductively coupled plasma mass spectrometer, PerkinElmer, Inc. In addition, a sulfated ash test can be used, where the material placed in a crucible is heated on an electric hotplate with a closed heating element and a thermostat until charred. After cooling down, the residue is moistened with concentrated sulfuric acid and carefully heated until the sulfuric acid vapor is removed. The crucible is then placed in a muffle furnace and calcined at 600 °C until the residue has been completely reduced to ash. The percentage of the residue is equal to the total mass fraction of inorganic impurities.

Amino Acid Analysis with Isotopic Dilution

Amino acid analysis determines the purity of a material by measuring the mass fraction of amino acid residues that are constituent amino acids of the RM. The existing amino acid CRMs with established metrological traceability to the SI system should be used as the basis for comparison. The availability of these CRMs ensures accurate and traceable quantification of the material.

The use of two orthogonal methods for analyzing the characteristics of an RM will provide accurate values of its characteristics traceable to SI units.

Results and Discussion

The conducted research allows the following conclusions to be drawn. VLPs constitute the main type of materials for targeted drug delivery. VLPs satisfy the need for non-toxic and biologically differentiated activity, enabling efficient solutions for structural homogeneity by mimicking the envelope and architecture of viral capsids.

VLPs are promising candidates for vaccine development due to their properties:

- the size similar to that of viruses;
- a highly organized and repetitive structure;
- the absence of infectious activity.

These properties ensure the safety of VLPs and a predictable cellular immune response.

CRMs for VLPs traceable to SI units play a crucial role in VLP standardization. Such CRMs can be used to determine the reproducibility and traceability of delivery systems in correlation with their physicochemical and biological properties.

Since monomers are packed around the surface of a VLP capsid, forming a regular nanoscale grid, the VLP quantification is limited to the quantification of its monomer.

Proteins and peptides of a therapeutic or diagnostic value should be compared with corresponding CRMs of polypeptide materials with established traceability to the International System of Units. This ensures the CRM traceability to SI units. The first and necessary step in the development of such materials is to verify their purity with established traceability to SI units.

Conclusion

The described approach to the characterization of CRMs for virus-like particles is expected to facilitate their future development and harmonization of approaches to their characterization and testing procedures.

The development of CRMs for protein-based VLPs will ensure the metrological traceability of measurement results of their characteristics, calibration of measuring instruments used in bioanalysis [14], and conditions for creating vaccines devoid of side effects. The study of VLPs and CRMs based thereon will improve the system of metrological support for laboratory medicine [15].

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Reference Materials for the Composition of Antimicrobial Substances

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Abstract The present study aims to generalize the experience of creating state reference materials (GSOs) of active substances and determine their main characteristics, standardized both in the State Pharmacopoeia of the Russian Federation and in the regulatory documents of the measurement uniformity assurance system. In connection with the violation of supply chains, the acquisition and use of foreign reference active substances became quite problematic or even impossible. As a consequence of the current difficult situation with the insufficient nomenclature of GSOs, the domestic manufacturers and developers faced urgent problems in creating them. The development of antimicrobial reference active substances will solve urgent issues of strengthening the technological sovereignty of Russia, minimize the import dependence of the Russian economy, as well as ensure the targets of the Strategy for Scientific and Technological Development of the Russian Federation to be achieved. The conclusions of the study can be applied in creating GSOs to substitute imported reference materials or surpass their level.

Keywords Reference material · Active substances · Antimicrobials · Metrological traceability · RF scientific and technological development strategy

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Introduction

Antimicrobials are one of the most widely used groups of medicines. They apply to various areas of medicine both in terms of a treatment and precautions. The quality control of medicines in accordance with the requirements of the State Pharmacopoeia of the Russian Federation typically provides for the use of reference materials (RMs), including chemical reference substances (CRSs) [1–4]. The need to use the RM of not only the active substance, but also its impurities, is enshrined in regulatory legal acts and in the State Pharmacopoeia of the Russian Federation.

To date, the disrupted supply chain of foreign RMs[1] has led to problems in the production of medicines, since RMs of active substances and their impurities are used by manufacturers as a daily basis for comparing the characteristics when producing each batch of medicines. The logistical difficulties of supplying the RMs of active substances result in the need to solve urgent issues of their import substitution and strengthening of the Russian technological sovereignty.

A comparative analysis of RMs, necessary for the production of medicines, included in the list of Vital and Essential Medicinal Products of the Russian Federation,[2] as well as RMs, registered in Federal Information Fund for Ensuring the Uniformity of Measurements (FIF OEI)[3] as of May 2022 showed the absence of approved type RMs of active substances, antimicrobials [5–7], including aminoglycosides, oxalzolidines, glycopeptides, macrolides, and lincomycins. Taking into account the requirements of the Decree of the Government of the Russian Federation dated April 14, 2022 No. 653 [8], as well as the general requirements of the Federal Law dated June 26, 2008, No. 102-FZ [9], the Russian scientific community faces the task of developing a unified approach that summarizes the best practices of complete studies on the RMs of active substances and their impurities. The implementation of this work will contribute to the strength of the Russian technological sovereignty and minimize the import dependence of the Russian economy on foreign supplies. It is worth noting that the idea and content of the study is among the most significant areas of scientific development that affect world science as a whole and refers to biotechnology, physical chemistry, and cellular technologies [10].

The present study aims to summarize the information on the existing domestic approved type RMs of active substances, as well as to establish generalized approaches to the development and characterization of antimicrobial reference substances.

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[1] Reference materials of active substances, manufactured in accordance with the requirements of United States Pharmacopoeia (USP), European Pharmacopoeia (EP), and British Pharmacopoeia (BP) by the National Institute of Biological Standards and Control (NIBSC) and national metrological institutes of other countries.

[2] Scroll vital and essential medicines for medical applications. Available via https://www.consultant.ru/document/cons_doc_LAW_335635/a80818c7d9593b31dbdbd0d3418aec02298bd57d6d/. Accessed 4 August 2022 (In Russ.).

[3] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology. Accessed 10 August 2022 (In Russ.).

Terminology

Table 1 shows the RM definitions, provided in the International Vocabulary of Metrology—VIM 3 [11] and the XIV State Pharmacopoeia of the Russian Federation [12].

Active substances—medicinal products in the form of one or more pharmacologically active substances, both synthetic and natural, intended for the production, manufacture of medicinal products and determining their effectiveness [13].

Table 1 Reference material terminology

International vocabulary of metrology—VIM 3	XIV RF state pharmacopoeia
5.13 (6.13) *Reference material (RM)*—a material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties	*Reference materials* (RMs)—substances, used to perform the quality control of the investigated medicinal products using physico-chemical and biological methods in order to confirm the compliance of medicinal products with the requirements of regulatory documentation established during the implementation of the state registration, as well as to calibrate the reference materials of the manufacturer of medicinal products used for the quality control and other purposes in the circulation of medicinal products *Chemical Reference Substance (CRS)* is a reference material manufactured in accordance with the pharmacopoeial monograph (PM). [OFS.1.1.0007.18. Reference materials]
5.14 (6.14) *Certified reference material (CRM)*—a reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures	*Certification of reference materials*—a study aimed at establishing the values of the certified characteristics in accordance with the test program and/or methodology, followed by the execution of appropriate documents (passport, RM application data sheet, layouts of primary and secondary packaging labels). The RM certification involves testing for homogeneity, stability, determining optimal storage and transportation conditions *Certified value of reference materials* is the value of the RM certified characteristic, established during its certification and provided in the passport and other RM documents with its error or uncertainty. The certified value is represented by a number expressing the value of the reproduced RM value in the established units. [OFS.1.1.0007.18. Reference materials]

Antimicrobials—substances of natural, semi-synthetic or synthetic origin that causes death or delays the growth of bacteria, mycoses, viral diseases, and protozoal infections (caused by protozoa) [14].

Antibiotics—natural compounds, as well as semi-synthetic or synthetic analogues, that suppress the development of microorganisms at low concentrations (up to 1 μg/mL). First of all, their action is aimed at bacteria and fungi, at the same time as being used as cytostatic (antitumor medicines). A number of them are able to block the replication of viruses [14].

Materials and Methods

In most cases, in terms of materials for the manufacture of RMs, commercially available substances of known manufacturers, supplied with certificates of analysis indicating the mass fraction of the main component and, preferably, the methods of analysis, are used. Otherwise, RM manufactures themselves synthesize and purify the RM material. However, when the information on the mass fraction and methods is not available, it is necessary to conduct a mandatory procedure for identifying the main substance. NMR spectroscopy, mass spectrometry, UV spectrophotometry or IR spectrometry can serve as the main physical and chemical identification method [15, 16].

The general pharmacopoeial monograph "OFS.1.1.0007.18. Reference Materials" [17] establishes the following test methods: NMR spectrometry, mass spectrometry, infrared spectrometry, and element analysis for the structural description; establishing the content of organic and inorganic impurities, water, and residual solvents, loss on drying for the purity determination; method of mass balance for the quantitative determination. When testing the RMs of active substances for the type approval, it is mandatory to determine the content of related compounds.

As an example, let us consider antibiotic RMs developed jointly by the FSBI Gause Institute of New Antibiotics and All-Russian Research Institute of Metrological Service. As a result of the work,[4] GSO 11413-2019 (natamycin composition), GSO 11532-2020 (olivomycin A composition), GSO 11532-2020 (amphotericin B mass fraction) state reference materials were developed together with a reference measurement procedure (RMP) for the composition (purity) of the initial active

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[4] The work was performed within the framework of the "Bioanalitika" research and development work, an integral part of the "Komponent" research and development work, a state contract dated 06/16/2017, No. 120-93.

This work was carried out in order to ensure the comprehensive development of the metrological assurance in the field of bioanalytical measurements; creation of tools and methods for the metrological assurance of measurements in the field of bioanalytical measurements, including laboratory diagnostics; ensuring reliable measurement results obtained using measuring instruments by creating the reference materials of active substances; ensuring the reliability of measuring the composition (purity) of initial active substances used for the manufacture of medicinal products traceable in accordance with the legislation on the measurement uniformity assurance.

substances: Amphotericin B, Olivomycin A, Natamycin.[5] The main feature of these RMs is that they comply with both the norms of the RF Pharmacopoeia and GOSTs of the Measurement Uniformity Assurance System (OEI GOST). In the course of the study, the error of the metrological characteristics of the GSOs, presented in Table 2, was established as directly depended on the quality of the RM raw material, synthesis and purification processes.

The specified reference measurement procedure establishes the requirements related to the conditions of operations, whose implementation ensures the obtainment of the given measurement results with the established accuracy indicators. During the developmnet of the RMP, works were carried out on the selection of measurement methods and used equipment; the full uncertainty budget was calculated. In the procedure of measuring the composition (purity) of initial active substances, a measurement method of determining the mass fraction of the analyzed active substance as "100% minus impurities" was implemented.

Summarizing the results of the performed tests and the developed approaches to the methods of the antibiotic RM characterization, the following theses can be formulated.

If the RM manufacturer has to achieve a compliance with the requirements of the RF State Pharmacopoeia and OEI GOSTs, it is necessary to conduct tests for determining the following characteristics:

- appearance [13];
- solubility [17];
- identification (or "authenticity" in the terminology of the RF State Pharmacopoeia) and confirmation of the substance chemical structure is typically carried out by the primary method of NMR spectrometry, HPLC-MS/MS, ultraviolet-visible absorption spectrophotometry [18], infrared absorption spectroscopy [19];
- mass fraction of the main substance component is determined by the mass balance method, where the mass fractions of all impurities are subtracted from 100%;
- related substances are determined using gas chromatography (GC) or HPLC methods [20], for residual organic solvents, for example, GC method is used [21];
- the total amount of inorganic impurities is determined using the sulfate ash method [22], componentwise analysis if performed using atomic emission spectrometry [23] and atomic absorption spectroscopy [24];
- since, for specific substances, pharmacopoeial monographs normalize either the water content or the loss on drying, depending on the substance, either the water content by Karl Fischer titration method, or the loss on drying should be determined [25].

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[5] FR.R1.31.2019.00005 Reference method for measuring the composition (purity) of the original pharmaceutical substances: Amphotericin B, Olivomycin A, Natamycin. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/8/items/834985. Accessed 10 August 2022 (In Russ.).

Table 2 Certified metrological characteristics of active substance GSOs

Certified characteristics	Interval of accepted certified values, %	Accepted values of relative expanded uncertainty, at P = 0.95 and k = 2, %
GSO 11413-2019 reference materials of natamycin composition		
Mass fraction of natamycin	95.0–99.5	3
Mass fraction of related compounds	0.1–5	3
Total mass fraction of inorganic impurities	0.1–5	3
Potassium (K) mass fraction Calcium (Ca) mass fraction Sodium (Na) mass fraction	0.01–5	2
Total mass fraction of water and organic solvents	0.1–5	3
GSO 11532-2020 reference materials of olivomycin A composition		
Olivomycin A mass fraction	95.0–99.5	3
Mass fraction of related compounds	0.1–5	3
Total mass fraction of inorganic impurities	0.1–5	3
Chromium (Cr) mass fraction Calcium (Ca) mass fraction Iron (Fe) mass fraction	0.01–5	2
Mercury (Hg) mass fraction	0.01–5	2
Total mass fraction of water and organic solvents	0.1–5	3
GSO 11532-2020 reference materials of amphotericin B mass fraction		
Amphotericin B mass fraction	95.0–99.5	3
GSO 11732-2021 reference materials of gramicidin C hydrochloride (soviet) substance composition		
Gramicidin C hydrochloride (soviet) mass fraction	95.0–99.5	6
Mass fraction of related compounds	0.1–5	3
Total mass fraction of inorganic impurities	0.003–5	5
Chromium (Cr) mass fraction Calcium (Ca) mass fraction Iron (Fe) mass fraction	0.001–2 0.001–2 0.001–1	3 2 2
Total mass fraction of water and organic solvents	0.1–5	1

In order to comply with the test program for the type approval of RMs, it is necessary to conduct additional tests for RM uniformity, short-term and long-term stability, as well as to establish requirements to RM storage, transportation conditions, and their shelf life. Determining the metrological traceability of RM characteristics is also a mandatory test section. The characteristics of these RMs can be traced to State Primary Standards GET 196-2015.[6]

Verified measuring instruments of approved types are used to perform all described test methods. For example, the main measuring instruments can include: analytical scales,[7] dispensers,[8] spectrophotometer,[9] IR-Fourier spectrometer,[10] high-performance liquid chromatograph,[11] pH-meter,[12] as well as measuring glassware, necessary auxiliary equipment, reagents, and solvents.

Results and Discussion

Applying the approaches described above to the characterization of active substance RMs, the approved type RMs were manufactured with the following certified characteristics given in Table 2.

Considered in Table 2 RMs are used in the manufacture of different batches of medicines to analyze and compare various batches of each medicine. The same procedure is carried out for the case of changes in the technological process or replacement of excipients, which can be carried out using the RMs of the composition and properties for these medicines.

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[6] GET 196-2015 State primary standard of units of mass (molar) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on spectral methods: Custodian Institute All-Russian Research Institute for Optical and Physical Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397882. Accessed 10 August 2022 (In Russ.).

[7] FIF OEI No. 54391-13 Non-automatic scales, Sartorius Weighing Technology GmbH, Germany. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/366830. Accessed 10 August 2022 (In Russ.).

[8] FIF OEI No. 36152-07 Automatic and mechanical single-channel dispensers, BIOHIT OYJ, Finland. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/345455. Accessed 10 August 2022 (In Russ.).

[9] FIF OEI No. 67389-17 Spectrophotometers, JASCO International Co., Ltd, Japan. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/381335. Accessed 10 August 2022 (In Russ.).

[10] FIF OEI No. 66036-16 Fourier transform infrared spectrometers, Thermo Fisher Scientific, USA. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/379856. Accessed 10 August 2022 (In Russ.).

[11] FIF OEI No. 47938-17 High-performance liquid chromatographs, LLC IH EkoNova, Russia. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/359326. Accessed 10 August 2022 (In Russ.).

[12] FIF OEI No. 15495-02 pH meters, Metrohm Ltd., Switzerland. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/313568. Accessed 10 August 2022 (In Russ.).

Conclusion

In the course of the study, the authors analyzed the main factors affecting the metrological assurance of studies aimed at the RMs of antimicrobial active substances. The main approaches to the methods of characterizing antibiotic RMs are formulated. The developed approach was tested by creating the state reference materials of antimicrobial medicines GSO 11413-2019, GSO 11532-2020, GSO 11532-2020, and GSO 11732-2021.

The development of domestic antimicrobial RM production solves the urgent issues of strengthening the Russian technological sovereignty, minimizing the import dependence of the Russian economy, and ensuring the achievement of the targets set by the Scientific and Technological Development of the Russian Federation, its priorities and perspectives.

These GSOs will ensure reliable and metrologically traceable measurement results obtained using medical products related to measuring instruments.

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Author Contribution Kulyabina E. V.—development of research concept, obtaining funding, development of methodology, supervision of research; Shchekotikhin A. E.—management of research work, provision of research materials; Tevyashova A. N.—conducting research work, curating data; Kulyabina T. V.—text verification and editing, validation, control; Morozova V. V.—draft writing, preparation and creation of visual materials.

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Nominal Reference Materials of Biological Substances

Valeria V. Morozova, Elena V. Kulyabina, Vitaly Yu. Morozov, and Tatiana V. Kulyabina

> *I personally saw what looked like an animal, but I can't be absolutely positive that it wasn't a mineral. I think that what was involved was really energy rather than matter. Relatively speaking, it would be easiest to describe the whole thing as a phenomenon hovering somewhere on borderland of dimensions and designations, on the abutment of color, shape, odor, mass, length and breadth, contours, shadows, darkness and so on and so forth.*
> *Slawomir Mrożek, "Streap-Tease"*
> *Translation by Edward Rothert (Bettencourt da Silva R., Ellison S. L. R. (eds.) (2021) Eurachem/CITAC Guide: Assessment of performance and uncertainty in qualitative chemical analysis. First Edition, Eurachem. Available via Eurachem.* https://www.eurachem.org. *Accessed 8 November 2022).*

Abstract For biological substances, nominal examination traceability currently remains an unresolved issue. This is primarily associated with the lack of certified nominal reference materials (RMs), as well as requirements and practices for using tools in the field of metrology that could ensure nominal examination traceability for such properties as "nucleic acid sequence (RNA and DNA)," urine color; and letter codes. The study justifies the relevance of developing an application theory for such tools in Russian metrology, as well as the specified aspects. The differences

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between measurement and nominal examination are outlined. Also, a draft chain of nominal examination traceability to an international harmonization protocol is proposed. Methods for ascertaining certified RM characteristics of human mitochondrial DNA fragment and inactivated SARS-CoV-2 “GC2020/1” strain are presented, including additional characteristics attributed to the “nucleic acid sequence” property. It is established that the uncertainty of nominal properties can be expressed as probability or confidence level. The authors note the role of nominal RMs in the reliable identification of nominal properties in biological substances. This study provides the foundation for implementing the presented conclusions in a new branch of metrology concerned with developing methods and means for ensuring reliable nominal examination of substances and materials.

Keywords Nominal properties · Nominal reference materials · Nominal examination · Traceability · JCTLM · Quality criteria

Introduction

Since its establishment as a scientific discipline, metrology has been concerned with measurement, as well as the ways and means of achieving the required accuracy. However, new branches of human knowledge that have existed for over half a century now do not apply the term “measurement.” Instead, they use the terms “testing” and “examination,” which are usually associated with the methods of scientific knowledge rather than measurement methods. This refers to the studies of the human genome [1], comparison of study results against known color scales, as well as the analysis of shapes, surface smoothness, and border serrations in different cell types, etc. In all of the above examples, scientists face the need to compare study results in order to ensure the traceability of obtained values without measuring them, i.e., without any reference to the units of physical quantities of the International System of Units (SI). For example, when mapping the human genome, the most important property is the nucleotide sequence of human DNA, which cannot be measured via comparison against a unit of length, concentration, or any other unit of measurement. Traceability is ensured to an elementary nucleotide—adenine, guanine, cytosine, and thymine. That is, this traceability system is based on fundamental constants in the form of nitrogenous bases that make up any living thing—humans, animals, plants, and even viruses, which are not considered to be alive, yet are also made up of nucleotides (only in their case, uracil nucleotide replaces thymine).

The nominal examination of foodstuffs is dictated by the need to provide the population with quality products. For example, consumers need to be certain that they buy a product that is exactly as advertised on the label or that they have paid for and purchased expensive, healthy seafood rather than something that only looks similar. Consumption of counterfeit foods can lead to serious health consequences, including fatal outcomes.

In clinical and laboratory medicine, nominal examination is often of key importance when executing the orders of judicial and prosecutorial authorities. For example, when establishing paternity, it is crucial to determine the exact nucleotide sequence of a test subject's DNA. Also, reliable identification of the SARS-CoV-2 coronavirus is essential to containing the spread of the virus and reducing the socioeconomic burden [2, 3].

A theoretical literature analysis and an analysis of implemented practical works reveal that the nominal examination of nucleic acid sequences (RNA and DNA) has been considered extensively by the leading national metrology institutes: e.g., National Institute of Standards and Technology)[1] [4, 5], National Institute of Metrology of China (NIM, China),[2] and Korea Research Institute of Standards and Science (KRISS) [6]. However, a series of specific (procedural) issues pertaining to the nominal examination of "nucleic acid sequence (RNA and DNA)" remain underexplored. Russia is yet to address the issues associated with the wide use of terminology relating to nominal properties; development and certification of procedures for determining such properties; as well as creation, testing, and type approval of nominal reference materials (RMs).

According to the literature review, further examination is required to better understand tools for ensuring nominal examination traceability for such properties as "nucleic acid sequence (RNA and DNA)," urine color, and letter codes, as well as to formulate requirements and establish application practices.

The study aims to identify and analyze the main factors affecting the creation of means and methods for the metrological support of nominal examination, ensuring the traceability of nominal properties.

Theoretical Analysis

According to ISO/IEC Guide 99:2007, a nominal property constitutes "a property of a phenomenon, body, or substance, where the property has no magnitude." These include, for example, the shape of the leukocyte nucleus, urine color, cerebrospinal fluid color, taxon, nucleic acid sequence (RNA and DNA), i.e., such properties of an object to which the terms "magnitude" and "measurement" are not applicable. Nominal properties are inherent in any living organism—humans, animals, and plants, any creature that contains nucleic acids, ribonucleic acid (RNA) and

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[1] Certificate of Analysis, Standard Reference Material 2392 Mitochondrial DNA Sequencing (Human). Available via NIST. https://tsapps.nist.gov/srmext/certificates/2392.pdf. Accessed 8 November 2022.

Certificate of Analysis, Standard Reference Material 3246 Ginkgo biloba (Leaves). Available via NIST. https://tsapps.nist.gov/srmext/certificates/3246.pdf. Accessed 8 November 2022.

[2] Reference material certificate Chinese quartet family 1 (monozygotic twin family-mother). Human whole genomic DNA sequence reference material from B-lymphoblastoid cell lines. Available via NCRM. https://www.ncrm.org.cn/Repository/0950c4e0-e829-420c-b494-f88cba1fd576.pdf. Accessed 8 November 2022 (In Cn).

deoxyribonucleic acid (DNA), in its composition. Future scientific and technological advances (including measurement technologies) will probably permit the "measurement" of nominal properties; however, the values such properties are currently determined through their "examination" [7]. According to [7], a nominal property is a "property of a phenomenon, body, or substance, where the property has no size," whereas nominal property value is a "feature common to equivalent individual nominal properties."

Despite difficulties associated with ensuring the traceability of nominal properties, such work is necessary to ensure reliable identification of humans, animal source foods, fishery resources, and, finally, the SARS-CoV-2 coronavirus. In turn, reliable identification is impossible without using a measure of nominal properties, such as RMs.

As noted above, the terminology of nominal properties differs from that of physical quantities. Thus, a nominal examination standard realizes the definition of a given nominal property, with specified nominal property value and associated examination uncertainty, serving as a reference [8]. Essentially, the only difference from the classical concept of "standard" lies in the use of the term "examination" instead of the term "measurement."

When it comes to defining the primary reference procedure for nominal examination, the difference lies in the use of the term "examination result." So, how is the term "examination result" defined? An examination result constitutes nominal property values attributed to the examined property along with any other relevant and available data [8].

Since the conventional metrology terminology used for the units of physical quantities is not applicable to nominal properties, the hierarchical traceability scheme must be presented in a form corresponding to the traceability of nominal properties, for example, for the nucleotide sequence of DNA or RNA, or of any nucleic acids. Drawing on the interim guidelines approved by the Ministry of Health of the Russian Federation,[3] we propose a draft chain of nominal examination traceability (see Fig. 1).

The approach to ensuring the nominal examination traceability shown in the figure serves as a guideline for our projects that are underway.

To date, only RMs for quantitative properties have been approved and registered in the Federal Information Fund for Ensuring the Uniformity of Measurements, e.g.:

- mass fraction of various substance components, e.g., pharmaceutical substances: GSO 11413-2019, GSO 11732-2021, GSO 11360-2019, and GSO 11532-2020[4];

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[3] Interim guidelines dated April 16, 2020 "Drug therapy for acute respiratory viral infections (ARVI) in outpatient practice during the COVID-19 epidemic. Version 2" (developed by the scientific community: I. M. Sechenov First Moscow State Medical University, National Medical Research Center of Phthisiopulmonology and Infectious Diseases, National Medical Research Center for Therapy and Preventive Medicine). Available via Information and legal portal Garant.ru. https://www.garant.ru/products/ipo/prime/doc/73816786/. Accessed 8 November 2022 (In Russ.).

[4] GSO 11413-2019 Reference material type-approved for natamycin composition. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/889620. Accessed 8 November 2022 (In Russ.).

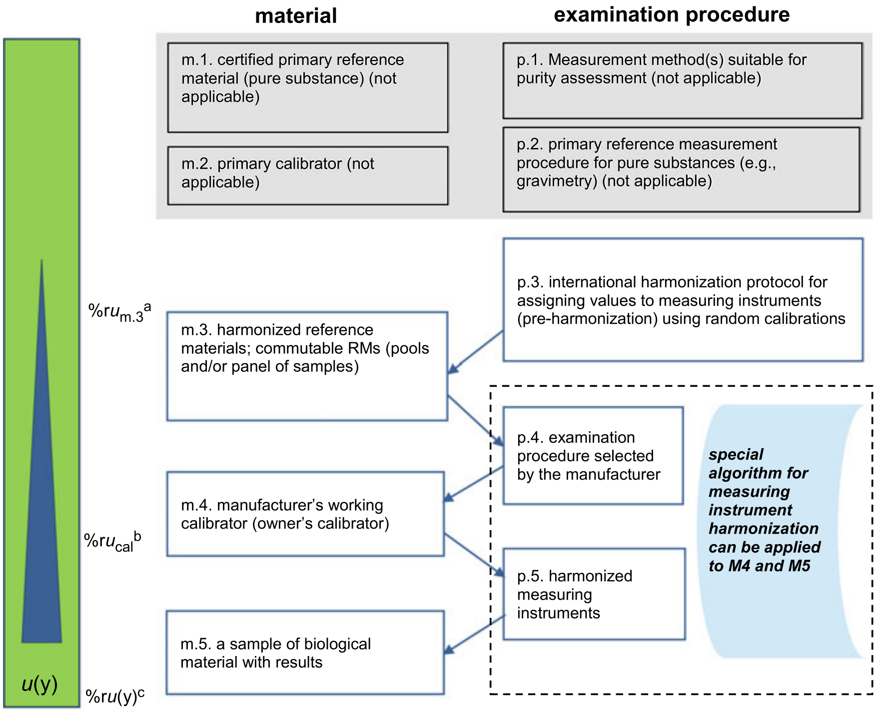

Fig. 1 Draft chain of nominal examination traceability (nucleotide sequence of DNA or RNA, or nucleic acids) established through calibration hierarchy

- mass concentration: e.g., protective antigen in a solution—GSO 10922-2017, recombinant virus protein in a solution—GSO 10921-2017[5];
- catalytic concentration RM, e.g. of enzymes: GSO 11387-2019 and GSO 11386-2019.

GSO 11732-2021 Reference material for the composition of gramicidin S hydrochloride (Soviet). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1392008. Accessed 8 November 2022 (In Russ.).

GSO 11360-2019 Reference material type-approved for the mass fraction of amphotericin B. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/719091. Accessed 8 November 2022 (In Russ.).

GSO 11532-2020 Reference material of type-approved for the composition of olivomycin A. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1236979. Accessed 8 November 2022 (In Russ.).

[5] GSO 10922-2017 Reference material type-approved for the mass concentration of a recombinant protective antigen against *Bacillus anthracis* in phosphate-saline solution. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/389279. Accessed 8 November 2022 (In Russ.).

GSO 10921-2017 Reference material type-approved for the mass concentration of Ebola virus recombinant glycoprotein in phosphate saline. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/389280. Accessed 8 November 2022 (In Russ.).

However, RMs for which nominal, as well as quantitative, properties of a substance can be certified are already available. For example, in addition to the certified characteristics of mass fraction/concentration, GSO 11607-2020[6] and GSO 11661-2020[7] must be certified for DNA and RNA nucleotide sequences, due to which these certified reference materials (CRMs) are actually used and without which it is impossible to control the operation of genome analyzers/sequencers.

Materials and Methods

The study presents methods and means of determining the certified characteristics of specified CRMs, including non-certified, nominal properties provided in the type description.

Type Approval Procedure in Case of Readily (Commercially) Available Source Material: On the Example of GSO 11607-2020

When the source material for CRM production, specifically a purified PCR product, was readily (commercially) available, the following procedure was used. A purified PCR product obtained via high-precision amplification of a commercial DNA preparation having a known nucleotide composition was used as the original sample for CRM preparation.

The purified and desalted PCR product was 50-fold diluted in 1000 mm^3 of deionized water, and this solution was used to fill a quartz cuvette having an optical path length of 10 mm. The optical density was determined at 260 nm (A_{260}) and 320 nm (A_{320}), thus determining DNA concentration in the test sample. The obtained DNA concentration was used to dilute the product to the final concentration.

The DNA nucleotide sequence of the CRM was determined using type-approved and verified measuring instruments (MIs)—genome analyzers and sequencers, e.g., a genome analyzer (Roche Diagnostics GmbH, Germany) or a capillary electrophoresis genetic analyzer (Institute for Analytical Instrumentation RAS, St. Petersburg).

The known nucleotide sequence was used to determine the mass fraction of each nucleotide. For each nucleotide, the relative fraction of the nucleotide in the DNA nucleotide sequence D_i was calculated using Eq. (1):

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[6] GSO 11607-2020 Type-approved reference material of human mitochondrial DNA fragment from HL-60 cell line culture (fragment 5999-7792). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1383975. Accessed 8 November 2022 (In Russ.).

[7] GSO 11661-2020 Type-approved reference material of the inactivated SARS-CoV-2 "GK2020/1" strain. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1384811. Accessed 8 November 2022 (In Russ.).

$$D_i = (K_i \cdot 100)/N, \tag{1}$$

where

D_i is the relative fraction for each nucleotide in the DNA nucleotide sequence, %;
K_i is the number of each nucleotide in the sequence, units;
i is the designation for a nucleotide, which is a "tetrit," a unit of nucleotide measurement that takes four values: A, G, C, and T;
N is the sequence size equal to the number of nucleotides in the sequence, units.

Taking the molecular weight of each nucleotide into account, nucleotide mass fraction was calculated as per Eq. (2):

$$W_i = (D_i \cdot M_i)/m, \tag{2}$$

where

W_i is the mass fraction of each nucleotide, %;
M_i is the molecular weight of each nucleotide, g;
m is the mass of the DNA nucleotide sequence comprising N nucleotides, g.

Following the type approval procedure, CRMs can be used for MI verification/ calibration, provided that they comply with the mandatory requirements set in the hierarchy schemes, procedures for certifying measurement standards, or procedures for MI verification, as well as for accuracy control and certification of measurement procedures and for providing traceability of measurement results.

Type Approval Procedure in the Absence of a Readily (Commercially) Available Source Material: On the Example of GSO 11661-2020

In the absence of a readily (commercially) available source material for CRM production, sufficient amounts of the culture of a pathogenic biological agent (e.g., SARS-CoV-2 coronavirus) were produced via PCR (polymerase chain reaction)[6] to determine the concentration of coronavirus RNA. The most available, highly sensitive, and specific method for the identification of SARS-CoV-2 nucleic acids in biological samples is the real-time PCR method with fluorescence-hybridization detection [9, 10]. Therefore, special attention was paid to studying the analytical sensitivity of reagent kits for the detection of SARS-CoV-2 nucleic acids.

RNA concentration in the CRM was determined using real-time PCR with fluorescence-hybridization detection. The calibration characteristic (calibration line) was plotted as a dependence between the number of threshold cycles and the logarithm of initial concentration. In order to plot the calibration line, we used calibration solutions comprising a diluted plasmid preparation of a known concentration (determined via a spectrophotometric method) and containing SARS-CoV-2 fragments. The concentration was measured via the spectrophotometric method using a V-730 spectrophotometer (Jasco International Co. LTD, Japan). An integral step in CRM creation consisted in the inactivation of the pathogenic biological agent at + 95 °C.

The certified value was determined in accordance with the certified procedure for determining the certified RM value (mass concentration), which was developed and certified by a national metrology institute. In the case of SARS-CoV-2, the All-Russian Scientific Research Institute of Metrological Service (VNIIMS) developed and approved a procedure for measuring the certified value of mass concentration for the CRM of the inactivated SARS-CoV-2 "GK2020/1" strain.[8]

The DNA nucleotide sequence of the CRM was determined via next-generation sequencing (NGS) [11] using type-approved and verified MIs—genome analyzers/sequencers, which were indicated above. In addition, the study used a mini centrifuge equipped with rotors for 0.2 cm^3/0.6 cm^3/1.5 cm^3 microtubes, having a speed of at least 2400 rpm; pipettes, such as Eppendorf Research Plus single-channel pipettes having a variable volume of (0.5–200) μL; an electric household refrigerator as per GOST 26678-85, having a refrigerating compartment to maintain temperatures from 2 to 8 °C and a freezing compartment to maintain a temperature not exceeding −18 °C; as well as auxiliary reagents and solutions.

Following the type-approval procedure, CRMs can be used first of all for the reliable identification of pathogenic biological agents (viruses or bacteria); certification of procedures and accuracy control intended to determine viral and bacterial RNA concentration, number of nucleotides in the coding RNA genomic region and the nucleotide sequence in a specific single-stranded RNA genomic region of viruses and bacteria; as well as in the assessment of the sensitivity and specificity of reagent kits intended for the detection of pathogenic biological agents. Also, the CRMs can be used for the verification and calibration of analyzers, real-time PCR thermocyclers, and MIs designed to identify/determine the concentration of viral and bacterial strains and to perform genetic analysis (genetic analyzers), provided that the metrological and technical characteristics of these CRMs meet the requirements of procedures for MI verification and calibration.

Results and Discussion

Revisiting the discussion about the relevance and possibility of creating nominal CRMs, we should consider the traceability assurance of characteristics attributed to such CRMs and note the opinion of our colleagues from CCQM Working Group on Nucleic Acid Analysis (CCQM-NAWG), specifically Dr. Alison Devonshire, the

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[8] FR.1.31.2020.38562 Procedure for measuring the certified value of mass concentration for the CRM of the inactivated SARS-CoV-2 "GK2020/1" strain. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16. Accessed 8 November 2022 (In Russ.).

leader of Analyte Group 2: Nucleic Acids Review Team,[9] regarding the traceability of DNA sequence CRMs. JCTML-EC-07 Annex II [12] cites molecular biology experts who call for full coordination in order to ensure a harmonized approach to nucleotide sequence traceability. One of the criteria for confirming the identification of a nucleic acid sequence CRM is estimated uncertainty, which is recommended to be expressed as the probability of sequencing the examined nucleic acid fragment correctly. If all the criteria presented in JCTML-EC-07 Annex II are met, then the certified nominal property values of the sequence CRM will be traceable to nucleotides that are not SI units but are internationally recognized referent units [12].

Conclusion

For biological substances, nominal examination traceability remains an unresolved issue at the moment. This is primarily associated with the lack of nominal CRMs, as well as requirements and practices for using tools in the field of metrology that could ensure nominal examination traceability for such properties as “nucleic acid sequence (RNA and DNA)”, urine color; and letter codes.

In the course of the study, we analyzed the main factors affecting the metrological support of nominal examination and the traceability of nominal properties. These factors include inadequate terminology; lack of internationally-accepted provisions in Russian GOSTs regarding the traceability of nominal properties; lack of legislative regulations ensuring traceability, etc.

On the example of GSO 11607-2020 and GSO 11661-2020, it is established that CRMs certified for the quantitative characteristics of mass fraction/concentration can also be certified for DNA and RNA nucleotide sequences. The presented conclusions enable the application of these CRMs for the metrological support of clinical and laboratory medicine, as well as ensuring the quality of food items and the identification reliability of the test object. Thus, it is shown that nominal CRMs of biological substances fit into the draft traceability chain presented in the figure, establishing the DNA or RNA nucleotide sequence through a hierarchy of calibrations traceable to nucleotides, which are not SI units but are internationally recognized referent units [12].

The specified example of traceability assurance illustrates how the global metrology community can successfully work together to solve an important problem. It seems reasonable to adopt a unified view on ensuring the traceability of nominal properties, as well as other characteristics, which will be discussed in detail by the present authors in future works. The presented conclusions can be applied to the creation of a new branch of metrology that will be concerned with developing methods and means for ensuring reliable nominal examination of substances and materials.

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[9] Analyte Group 2: Nucleic Acids Review Team. Available via BIPM. https://www.bipm.org/en/committees/jc/jctlm/wg/jctlm-rt-nucleic-acids. Accessed 8 November 2022 (In Russ.).

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Caffeine Quantification Via High-Precision Coulometric Titration

Alexandr Yu. Shimolin

Abstract Caffeine has traditionally been used to assess and control the metrological characteristics of liquid chromatographs. In recent years, with increasing requirements for the traceability of measurement results, the use of caffeine as a reagent for verification, calibration, and type approval tests has started to contradict the effective regulations governing measurement uniformity. This study aims to develop and test a procedure of caffeine quantification in caffeine via the primary method of coulometric titration for the metrological support of high-performance liquid chromatography (HPLC) with an accuracy of no more than 1%abs. In the course of work, a detailed analysis of methods for determining caffeine content in various matrices was carried out; in addition, a procedure for determining caffeine mass fraction in caffeine via coulometric titration was developed and tested. A comparison of the obtained results with caffeine mass fraction measurements performed using HPLC confirmed the commutativity of coulometric titration and HPLC. The developed procedure of caffeine quantification in caffeine via coulometric titration employing GET 176 can be used to develop a caffeine composition reference material and to provide metrological traceability of HPLC measurements on its basis to state primary standards reproducing the "mass fraction of components" and to SI units.

Keywords Standard · GET 176 · Coulometry · Iodometry · Caffeine · HPLC · High-performance liquid chromatography · Traceability · Procedure · Coulometric titration

Abbreviations

FIF EUM	Federal Information Fund for Ensuring the Uniformity of Measurements
HPLC	High-performance liquid chromatography

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GVET 208-1	State Secondary Standard of the units of mass fraction and mass (molar) concentration of organic components in liquid and solid substances and materials based on gas and liquid chromatography
GET 176	State Primary Standard of the units of mass (molar, atomic) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on coulometry
IR	Spectrum—infrared wavelength spectrum
MI	Measuring instrument
RM	Reference material
UV spectrum	Ultraviolet wavelength spectrum
SS CT	Standard system implementing coulometric titration as part of GET 176

Introduction

The method of high-performance liquid chromatography (HPLC) finds wide application in chemical, pharmaceutical, food, and oil refining, and other industries, as well as in environmental protection. The HPLC is used for the quality control of raw materials and finished products, monitoring of technological processes, in scientific research, as well as for the monitoring of environmental safety indicators and for other analytical tasks. As a chemical compound, caffeine has traditionally been used to control the metrological characteristics of liquid chromatographs equipped with spectrophotometric (including those with a diode matrix), refractive index, and evaporative light scattering detectors. However, as of January 01, 2022, Russia had no one type of certified reference materials (CRMs) required for the verification, calibration, and testing of measuring instruments (MIs), including type approval testing, as well as for the plotting of calibration characteristics for liquid chromatographs and accuracy control of measurement procedures in the process of their application. An analysis of the Federal Information Fund for Ensuring the Uniformity of Measurements (FIF EUM)[1] also revealed a lack of methods for measuring caffeine mass fraction within the range of 97–100% with an accuracy of at least 1%.

According to data from the Federal Information Fund as of May 2022, caffeine is used to verify 14 out of 62 types of liquid chromatographs. In 2021 alone, 1421 such chromatographs were verified using a caffeine reagent. Taking into account Rosstandart Order No. 2510 as of July 31, 2020 [1] and the general requirements of Federal Law No. 102-FZ as of June 26, 2008 [2], such practice is flawed as it violates the principle of measurement result traceability to State Primary Standards. What is more, the current level of science and technology imposes serious requirements for the metrological characteristics of CRMs used as calibrants. Thus, it is necessary

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology. Accessed 4 August 2022 (In Russ.).

to develop a highly accurate procedure for determining caffeine mass fraction in caffeine using a primary method of analysis [3] for the subsequent release of a CRM of caffeine.

In analytical practice, several methods for quantifying caffeine in caffeine-containing matrices are known. Thus, the determination of caffeine microquantities in drugs as per [4] is proposed to be carried out via voltammetry with the use of iodine photogenerated from a potassium iodide solution.

As the main method for analyzing caffeine in coffee, GOST 29148-97 [5] proposes a photometric method relying on the hydrolytic oxidation of caffeine to tetramethyl purpuric acid and subsequent photometric determination of the color intensity of its solution at a wavelength of 540 nm. However, the photometric method is designed for a relatively low content of caffeine (up to 2.3%), specifying a measurement repeatability of 0.15%abs (6.5%rel) and an interlaboratory precision of 0.3%abs (13%rel), which clearly does not satisfy the requirements for the procedure under development. In addition, according to [6], the method does not take into account the features of actual coffee samples, specifically changes in the spectral characteristics of the examined coffee extract due to the possible presence of impurities depending on the type of coffee. The shift of the solution absorption peak from 540 nm can result in significantly underestimated data. Also, an appendix to GOST 29148-97 provides a complicated reference spectrophotometric method, which requires the use of diatomaceous earth; it is designed for similarly low levels of caffeine and does not significantly increase the accuracy of analysis.

Prior to the introduction of GOST 29148-97 to determine caffeine in coffee and caffeine-containing products, the method of iodometric titration was widely used. This method is based on the quantitative precipitation of caffeine in the form of its periodide in the presence of sulfuric acid using iodine solution in potassium iodide; then this compound is broken down with ethyl alcohol. The released iodine is titrated with sodium thiosulfate (hypo) solution with the addition of starch as an indicator near the endpoint of titration. Determination is also possible with the potentiometric equivalence point fixation [7]. The method is still used for the qualitative identification of "caffeine" substance according to the eponymous article GF RF XIV FS.2.1.0116.18 of State Pharmacopoeia [8].

The anhydrous acid-base titration method considered in [9, 10] is based on caffeine exhibiting pronounced basic properties in glacial acetic acid, which allows it to be titrated with a standard perchloric acid solution. With a slight modification, the method was included in GF RF XIV FS.2.1.0116.18 as the most preferable caffeine quantification method. This method provides an accuracy of up to 0.3%rel. However, its use is associated with working with substances included in the list of narcotic drugs, psychotropic substances, and their precursors to be controlled approved by Government Decree No. 665 as of July 02, 2015 [11], which complicates the application of this procedure.

The procedure proposed in this article is based on a primary method of analysis [3]—coulometric titration—which establishes metrological traceability to SI units (kilogram, ampere, and second). The method is based on the use of electrogenerated iodine and a coulometrically standardized sodium thiosulfate solution, which is essentially a development of iodometry. While conditions for 100% current efficiency when electrogenerating iodine and the procedure of sodium thiosulfate standardization are described in [12–14], a procedure for determining caffeine via coulometric titration has not been described before. In Russia, coulometric titration is implemented in GET 176-2019 State Primary Standard of the units of mass (molar, atomic) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on coulometry (GET 176)[2] [15]. Its measurement capabilities in the application field of methods relying on electrogenerating iodine for CRM certification were confirmed within international key comparisons CCQM K152 *Assay of Potassium Iodate* [16] and published in the BIPM calibration and measurement capability database [17].

In [6, 7], the authors present data on the comparability of caffeine measurements in organic matrices via iodometry, HPLC, as well as photocolorimetry and capillary electrophoresis within the caffeine mass fraction measurement range of 0.02–6.0%. However, despite the availability of published data on good comparability of caffeine determination results via the specified methods, additional studies are required on the comparability (commutativity) of coulometric titration and HPLC when determining caffeine in a pure substance with an accuracy of not more than 1% abs.

Thus, this study aims to develop a procedure of caffeine quantification in caffeine via coulometric titration for metrological support of HPLC when determining caffeine in a pure substance with an accuracy of not more than 1% abs.

To this end the following objective has been set: development and testing of a procedure for determining caffeine mass fraction in pure caffeine via coulometric titration; establishment of metrological characteristics defining the method; comparison of results with those obtained using HPLC.

Materials and Methods

CRM Material

As test material, the study used a caffeine reagent, batch No. 0618, industrially produced for the pharmacopoeia (supplied by CHEMMIX, Russia). The material constitutes white crystals soluble in water. The molar mass is 194.1908 g/mol [18].

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[2] GET 176-2019 State primary standard of units of mass (molar, atomic) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on coulometry (includes 3 installations on the basis of constant current coulometry, controlled potential coulometry and inductively coupled plasma mass spectrometry): Custodian Institute UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1382712. Accessed 4 August 2022 (In Russ.).

Reagents

The study used the following substances and materials: GSO 11713-2021 Certified reference material of potassium iodate,[3] batch No. 1 [19]; 5-aqueous sodium thiosulphate, solution having a concentration of 0.1 mol/dm^3; acetate buffer solution (pH 4.4–4.6) having a concentration of 0.5 mol/dm^3 (anolyte); sodium nitrate, solution having a concentration of 0.5 mol/dm^3 (catholyte); chemically pure potassium iodide; chemically pure sulfuric acid, solution having a concentration of 2 mol/dm^3; chemically pure sodium carbonate.

Equipment

The studies were carried out using a standard system implementing coulometric titration (SS CT) that is part of GET 176 (Fig. 1). The SS CT was adjusted to conduct iodometric titration in accordance with the flowchart, used MIs, reagents, and materials described in [14].

Caffeine Identification

For the identification of analyzed substance (caffeine), UV and IR spectral data were collected (for results see Figs. 2, 3 and 4).

The test substance was identified on the basis of the UV spectrum by means of GVET 208-1-2016 State Secondary Standard of the units of mass fraction and mass (molar) concentration of organic components in liquid and solid substances and materials based on gas and liquid chromatography (GVET 208-1)[4] [20] under the following conditions:

Method	HPLC, GF RF XIV FS.2.1.0116.18
Column	ZORBAX eclipse XDB-C18 (analytical 4.6 × 250 mm 5-μ)
Mobile phase (MPA)	Tetrahydrofuran
Mobile phase B (MPB)	Acetonitrile

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[3] GSO 11713-2021 Reference material for the composition of potassium iodate (UNIIM KIO_3 CRM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1391862. Accessed 4 August 2022 (In Russ.).

[4] GVET 208-1-2016 State Secondary Standard of the units of mass fraction and mass (molar) concentration of organic components in liquid and solid substances and materials based on gas and liquid chromatography: Custodian Institute UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/11/items/399340. Accessed 4 August 2022 (In Russ.).

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Mobile phase C (MPC)	1.36 g of sodium acetate trihydrate dissolved in 1000 cm^3 with glacial acetic acid at pH = (4.5 ± 0.1)
Elution mode	Isocratic, MPA:MPB:MPC (2.0:2.5:95.5)
Solvent	2.0 cm^3 MPA; 2.5 cm^3 MPB; 95.5 cm^3 MPC
Mass concentration of the solution	0.21 mg/cm^3
Input volume	10 mm^3
Flow rate	1 cm^3/min
Temperature of the column oven	25 °C
Detection conditions	275 nm
Retention time	13.1 min

Substance identification on the basis of the IR spectrum was performed by means of a Nicolet iS5 FT-IR spectrometer (Thermo, USA).

Measurement conditions are as follows:

Detector	DTGS KBr
Beamsplitter	KBr
Wavenumber range, cm^{-1}	3495.26–499.96
Number of sample scans	16
Number of reference scans	16
Resolution	4.000
Mirror speed	0.4747
Aperture	100.00

The UV spectrum of the test substance was 275 nm, which corresponds to the “caffeine” substance (273 nm according to GF RF XIV FS.2.1.0116.18).

The chromatograms 2 and 3 revealed impurity peaks corresponding to the following substances: theobromine, theophylline, and benzoic acid.

The results of substance identification according to the IR spectrum are shown in Table 1.

The correspondence between the examined substance and the “caffeine” compound was estimated at 97.70%.

Coulometric Titration Procedure

A caffeine sample sufficient for ten measurements (1 g) was dried for 12 h in a drying chamber (Binder, Germany) at (70 ± 1) °C; then it was cooled to room temperature in a desiccator over phosphorus pentoxide.

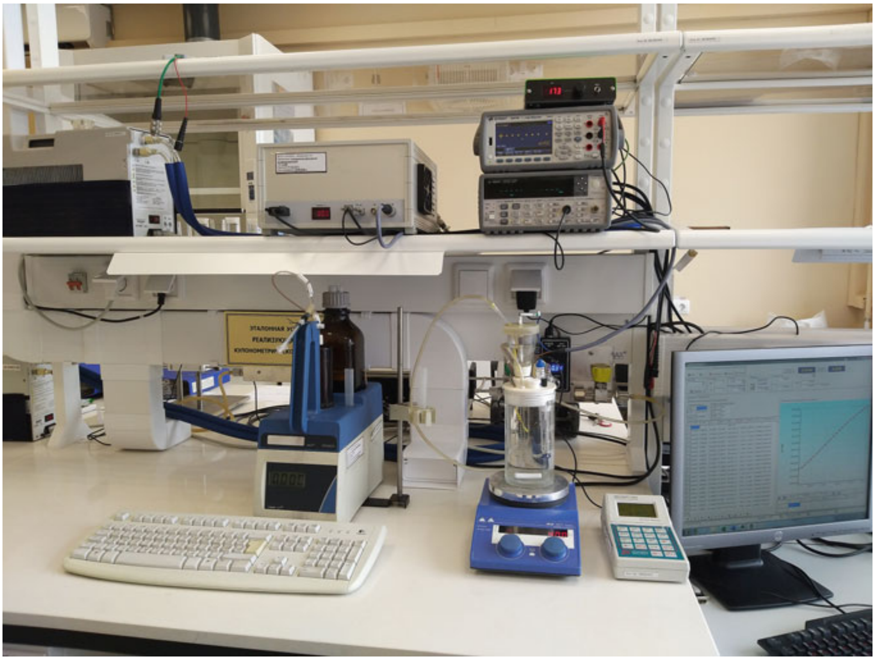

Fig. 1 Standard system implementing the coulometric titration as a part of GET 176-2019 state primary standard of the units of mass (molar, atomic) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on coulometry

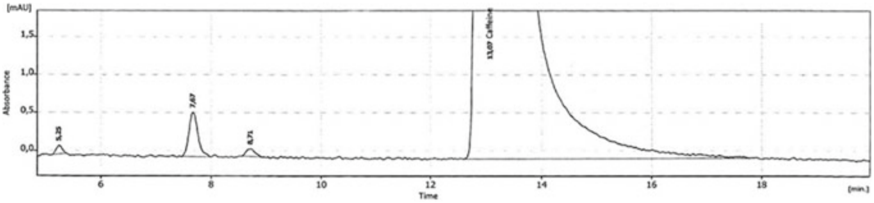

Fig. 2 Chromatogram of the test substance (caffeine)

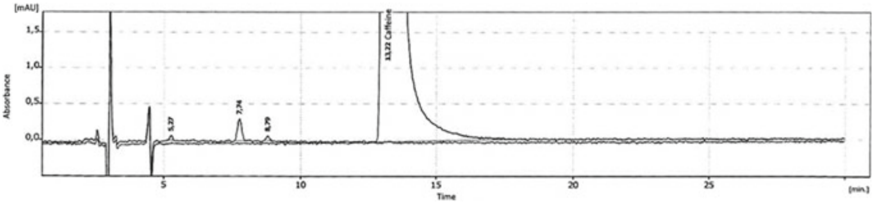

Fig. 3 Chromatograms of the test substance (caffeine) and solvent

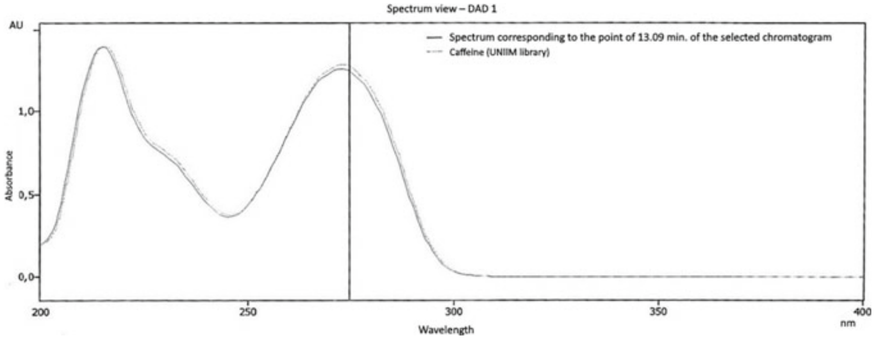

Fig. 4 UV spectrum of the substance with a retention time of 13.1 min at a wavelength of 275 nm

Table 1 Results of caffeine identification on the basis of the IR spectrum using the Nicolet iS5 FT-IR spectrometer

Index	Degree of correspondence, %	Reference compound	Spectral library
10	98.08	Caffeine	HR spectra IR demo
184	97.70	Caffeine	Georgia state forensic drugs
184	97.70	Caffeine in KBR	Georgia state crime lab sample library

A potassium iodate sample sufficient for ten measurements (3 g) was dried for two hours in the drying chamber at (110 ± 5) °C; then it was cooled to room temperature in a desiccator over phosphorus pentoxide.

All electrolytes were prepared using deionized water, which was additionally stripped of oxygen, and stored under a constant flow of high-purity argon (henceforth referred to as 'degassed water').

A (0.050 ± 0.002) g caffeine probe was placed in a pre-weighed 100 cm^3 flask and weighed by means of a CP225D analytical balance (Sartorius, Germany) with an accuracy of $1{\cdot}10^{-5}$ g via substitution weighing, taking the air buoyancy correction into account [21]. The probe mass was calculated as the difference of two weighings.

A (0.070 ± 0.002) g potassium iodate probe was placed in a flask containing the caffeine test portion and weighed using substitution weighing with an accuracy of 1×10^{-5} g, factoring in the air buoyancy correction. The probe mass was calculated as the difference of two weighings.

A probe of sodium thiosulfate solution (about 10 cm^3) pre-standardized according to [14] under stability conditions [22] was collected using a disposable syringe and weighed by means of a balance with an accuracy of up to 1×10^{-5} g, factoring in the air buoyancy correction. The probe mass was calculated as the difference of two weighings. Degassed water was added to the mixture of caffeine and potassium iodate, which increased the volume of the solution to 30 cm^3. After that, the flask was sealed tightly with a cork and mixed thoroughly until crystals were completely dissolved.

About 2 g of potassium iodide was added to a conical flask containing 50 cm^3 of dilute sulfuric acid and mixed intensively until crystals dissolved; then about 20 cm^3 of the mixture was immediately collected and added to the flask containing the solution of caffeine and potassium iodate. The sample solution turned a deep purple, and a brown precipitate was formed. The flask containing the solution was tightly sealed with a cork and weighed using a balance with an accuracy of 1×10^{-4} g, factoring in the air buoyancy correction. Following the weighing operation, the flask was kept in a dark for 15 min. A preliminary titration procedure was carried out using a SS CT as per [14]. Then, an aliquot of about 5 cm^3 was collected from the flask using a syringe fitted with an end filter whose membrane size did not exceed 0.45 μm; the filter was removed; the syringe with the aliquot was weighed by means of a balance having an accuracy of 1×10^{-5} g via substitution weighing, taking the air buoyancy correction into account, to be transferred into the coulometric cell. Then the sodium thiosulfate probe was added drop by drop to the working chamber of the cell until the indicator signal dropped. The excess sodium thiosulfate was additionally titrated using iodine electrogenerated at a current of 1 mA with 5-s pulses.

The result of a single measurement was determined using a summary formula including each measurement step:

$$A_{\mathrm{Cfn}} = \frac{\frac{1}{4} \cdot M_{\mathrm{Cfn}} \cdot \left(\frac{6 \cdot A_{\mathrm{KIO_3}} \cdot m_{\mathrm{KIO_3}} \cdot m_{\mathrm{aliq}}}{M_{\mathrm{KIO_3}} \cdot m_{\mathrm{sol}}} - C_{\mathrm{hypo}} \cdot m_{\mathrm{hypo}} + \frac{(U_1 \cdot (t_1' - t_1) + U_2 \cdot t_2)}{z \cdot F \cdot R} \right) \cdot m_{\mathrm{aliq}}}{m_{\mathrm{Cfn}} \cdot m_{\mathrm{sol}}} \times 100\%,$$

where

A_{Cfn}	caffeine mass fraction, %;
$A_{\mathrm{KIO_3}}$	mass fraction of potassium iodate determined via coulometric titration with an absolute expanded uncertainty ($k = 2$; $P = 0.95$) of no more than 0.05%;
$M_{\mathrm{KIO_3}}$, M_{Cfn}	molar mass of potassium iodate and caffeine, respectively, g/mol;
$m_{\mathrm{KIO_3}}$, m_{Cfn}, m_{aliq}, m_{sol}	masses of the potassium iodate, caffeine, aliquot, and solution following the reaction, respectively, g;
C_{hypo}	quantity of sodium thiosulfate in the probe, mol/kg of the solution;
U_1	voltage of the generator current during preliminary titration, V;
U_2	voltage of the generator current during the main titration, V;
U_3	voltage of the generator current during the final titration, V;
t_1'	total duration of preliminary titration, s;
t_1	time corresponding to the preliminary titration endpoint, s;
t_2	time of main titration, s;

Table 2 Results of measuring caffeine mass fraction in five dissolved samples of caffeine

Sample number, *n*	Results of parallel determinations, %			Average caffeine mass fraction in the sample, %	SD of the average value for the sample, %
	1	2	3		
1	99.750	99.770	100.283	99.934	0.17
2	99.986	99.867	99.608	99.820	0.11
3	99.435	99.977	100.410	99.941	0.28
4	99.989	100.492	99.841	100.107	0.20
5	100.222	100.020	99.866	100.036	0.10
Average caffeine mass fraction in the material, %					99.968
Standard deviation of the average measurement result (n = 15), %					0.075

t_3	time corresponding to the final titration endpoint, s;
z	number of electrons involved in the reaction ($z = 2$);
F	Faraday constant, C/mol ($F = 96{,}485.33212$ C/mol);
m_{hypo}	probe mass, taking into account the air buoyancy correction, g;
R	resistance of the standard resistor, Ω.

In order to determine the comparability of caffeine mass fraction measurements via coulometric titration and HPLC, caffeine was quantified using GVET 208-1 under the conditions specified in GF RF XIV FS.2.1.0116.18.

Results and Discussion

In the course of the study, measurements were performed for five dissolved caffeine samples, i.e., three parallel determinations for each sample (for measurement results see Table 2).

Uncertainty Budget

The uncertainty budget was estimated using algorithms described in GOST 34100.3-2017/ISO/IEC Guide 98-3:2008 [23]. The calculation results are shown in Table 3.

In order to confirm the commutativity, caffeine mass fraction in caffeine was measured five times via HPLC using GVET 208-1. The measurement results are presented in Table 4.

The results of measuring caffeine mass fraction in caffeine showed good agreement between the applied methods of coulometric titration and HPLC.

Table 3 Uncertainty budget of caffeine mass fraction measurement in caffeine via coulometric titration by means of GET 176

Type	Characteristic	Value		Uncertainty		Distribution	Sensitivity coefficient		Product		$\nu\iota$
		x_i	Unit	u_i	Unit		c_i	Unit	$c_i u_i$	Unit	
A	Repeatability	99.968	%	0.075	%	N	1	1	7.48×10^{-2}	%	15
B	U_1	0.01007	V	6.89×10^{-5}	V	R	6.00×10^{-1}	%/V	4.13×10^{-5}	%	∞
B	t_1'	67.6056	s	1.03×10^{-4}	s	R	2.10×10^{-4}	%/s	2.16×10^{-8}	%	∞
B	t_1	38.84140	s	5.59×10^{-5}	s	R	2.10×10^{-4}	%/s	1.17×10^{-8}	%	∞
B	U_3	0.01006	V	7.83×10^{-5}	V	R	3.36	%/ V	2.63×10^{-4}	%	∞
B	t_3	160.8926	s	1.03×10^{-4}	s	R	2.10×10^{-4}	%/s	2.16×10^{-8}	%	∞
B	m_{test}	5.288701	g	1.14×10^{-5}	g	R	18.9	%/g	2.16×10^{-4}	%	∞
B	m_{hypo}	0.51711	g	7.78×10^{-5}	g	R	2.03×10^{-2}	%/g	1.58×10^{-6}	%	∞
B	R	10.001477	Ω	5.00×10^{-6}	Ω	R	3.98×10^{-1}	%/Ω	1.99×10^{-6}	%	∞
B	M	194.1908	g/mol	8.32×10^{-3}	g/mol	R	5.15×10^{-1}	%/(g/mol)	4.28×10^{-3}	%	∞
B	F	96,485.332120	C/mol	5.00×10^{-6}	C/mol	R	4.13×10^{-7}	%/(C/mol)	2.06×10^{-12}	%	∞
B	z	1	–	0	–	N	1	%/–	0	%	∞

(continued)

Table 3 (continued)

Type	Characteristic	Value		Uncertainty		Distribution	Sensitivity coefficient		Product		ν_ι
		x_i	Unit	u_i	Unit		c_i	Unit	$c_i u_i$	Unit	
B	C_{hypo}	0.19583	mol/kg	3.77×10^{-5}	mol/kg	R	10.4	%/(mol/kg)	3.92×10^{-4}	%	∞
B	Diffusion	–	mol/kg	9.94×10^{-7}	mol/kg	R	1	%/(mol/kg)	9.94×10^{-7}	%	∞
B	Impact of O_2	–	mol/kg	1.00×10^{-2}	mol/kg	R	1	%/(mol/kg)	1.00×10^{-2}	%	∞
B	Illumination	–	mol/kg	1.00×10^{-3}	mol/kg	R	1	%/(mol/kg)	1.00×10^{-3}	%	∞
B	Ar purity	–	mol/kg	1.86×10^{-7}	mol/kg	R	1	%/(mol/kg)	1.86×10^{-7}	%	∞
B	Impurities	–	mol/kg	1.65×10^{-8}	mol/kg	R	1	%/(mol/kg)	1.65×10^{-8}	%	∞
B	m_{KIO_3}	0.157204	g	7.78×10^{-5}	g	R	25.0	%/g	1.95×10^{-3}	%	∞
B	M_{KIO_3}	219.9998	g/mol	2.20×10^{-3}	g/mol	R	3.72×10^{-5}	%/(g/mol)	8.18×10^{-8}	%	∞
B	m_{sol}	115.95859	g	7.78×10^{-5}	g	R	2.93×10^{-4}	%/g	2.28×10^{-8}	%	∞
B	m_{aliq}	5.28870	g	7.78×10^{-5}	g	R	1.21×10^{-3}	%/g	9.43×10^{-8}	%	∞
B	Completeness of precipitation	–	mol/kg	5.00×10^{-2}	mol/kg	R	1	%/(mol/kg)	5.00×10^{-2}	%	∞

(continued)

Table 3 (continued)

Type	Characteristic	Value		Uncertainty		Distribution	Sensitivity coefficient		Product		$\nu\iota$
		x_i	Unit	u_i	Unit		c_i	Unit	$c_i u_i$	Unit	
B	A_{KIO_3}	0.99964	g/g	3.00×10^{-4}	g/g	R	3.94	%/(g/g)	1.18×10^{-3}	%	∞
Certified value									**99.968**	%	
Type A absolute standard uncertainty, u_A, %									0.075	%	
Type B absolute standard uncertainty, u_B, %									0.051	%	
Absolute combined standard uncertainty, u_c, %									0.091	%	
Absolute expanded uncertainty ($k = 2$; $P = 0.95$), U, %									0.18	%	

Table 4 Results of measuring caffeine mass fraction in caffeine via HPLC by means of GVET 208-1

Number of the measurement	Caffeine mass fraction in caffeine, %
1	99.970
2	99.971
3	99.972
4	99.972
5	99.969
Average mass fraction of caffeine, A, %	**99.97**
Type A absolute standard uncertainty, u_A, %	0.0005
Type B absolute standard uncertainty, u_B, %	0.199
Absolute combined standard uncertainty, u_c, %	0.4
Absolute expanded uncertainty ($k = 2$; $P = 0.95$), U, %	0.8

The standard deviation of measurement results obtained for samples at the level of 0.05% indicates sufficient completeness of iodine extraction, completeness of precipitation, degree of filtration, as well as minimization of errors associated with aliquot sampling and the influence of atmospheric oxygen.

The accuracy indices of the developed procedure exceed those of widely used anhydrous titration procedures (0.3%) [9], as well as significantly exceeding those of the described iodometric procedures (0.9%) [9]. Thus, the developed procedure for determining caffeine mass fraction in caffeine via coulometric titration can be applied in the development of a caffeine composition RM of for metrological support of HPLC measurements.

Conclusion

Due to the wide application of high-performance liquid chromatographs in various industries, it is essential to establish and control their metrological characteristics. For verification, calibration, type approval tests, as well as for the construction of calibration characteristics for liquid chromatographs and accuracy control of measurement procedures during their use, the most convenient means of metrological support are reference materials for the composition of pure substances and their solutions with SI-traceability.

In the study, a procedure for measuring caffeine mass fraction in caffeine via coulometric titration was developed and tested. The following metrological characteristics were determined for the developed procedure: caffeine mass fraction measurement range—97.00–99.99%; expanded uncertainty ($P = 0.95$; $k = 2$) – 0.18%, which in terms of accuracy is 2–6 times better than the used titration-based procedures. Another advantage of the described procedure consists in avoiding the need to use reagents that have to be taken into special account (precursors). A comparison of the obtained results with caffeine mass fraction measurements performed using HPLC confirmed the commutativity of coulometric titration and HPLC, which allows coulometric titration to be used for the development of caffeine composition RMs for the metrological support of HPLC.

The article proves the theoretical possibility of using coulometric iodometric titration for high-precision quantification of pure purines.

The developed procedure of caffeine quantification in caffeine via primary coulometric titration employing GET 176 can be used to develop a caffeine composition RM and to provide metrological traceability of HPLC measurements on its basis to state primary standards reproducing the "mass fraction of components" and SI units.

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Contribution of the Authors Morozova V. V.—writing a draft of the article, conducting research, preparing and creating visual materials; Kulyabina E. V.—management of research work, development of the research concept, development of methodology; Morozov V. Yu.—formal analysis, data curation; Kulyabina T. V.—revision of the text, validation and control of measurements.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Primary Reference Procedure for Measuring the Mass Fraction and Molar Concentration of Copper and Zinc in Biological Materials by Isotope Dilution Mass Spectrometry

Elena V. Vostroknutova, Tatiana N. Tabatchikova, Pavel V. Migal, Elena L. Lebedeva, Egor P. Sobina, Alena V. Sobina, and Marina F. Kuznetsova

Abstract This study describes the process of developing a primary reference procedure for measuring the mass fraction and molar concentration of copper and zinc in biological materials by isotope dilution mass spectrometry (ID-ICP-MS). In order to improve the measurement accuracy, optimal conditions for sample preparation and measurement were determined. Uncertainty sources were examined, with each source assessed in terms of its contribution to the uncertainty budget. The measurement range of copper and zinc mass fraction and molar concentration comprised from $1{\cdot}10^{-6}$ to $1.5{\cdot}10^{-3}$% and from 2 to 20 μmol/dm^3, respectively. The relative expanded measurement uncertainties of copper mass fraction, zinc mass fraction, copper molar concentration, and zinc molar concentration were found to be equal to 7.1–7.5%, 8.9–9.2%, 8.8%, and 8.6%, respectively. The developed procedure can be used to establish metrological characteristics of reference materials (RMs) for the

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composition of lyophilized blood serum and reconstituted lyophilized blood serum, to control the accuracy of measurement results obtained using other measurement techniques of similar quantities, and to carry out high-precision measurements of copper and zinc mass fraction and molar concentration for arbitration purposes in lyophilized blood serum and reconstituted lyophilized blood serum.

Keywords Primary reference procedure · Lyophilized blood serum · Inductively coupled plasma mass spectrometry method · Isotope dilution method

Abbreviations

DRC	Dynamic reaction cell
KED	Kinetic energy discrimination
SPRP	State primary reference procedure
ID-ICP-MS	Isotope dilution inductively coupled plasma mass spectrometry
FIF AMU	Federal Information Fund for Assuring Measurement Uniformity
AS	Assessment samples
RM	Reference material

Introduction

One important field of medical research deals with the determination of those trace elements in the human body that ensure the processes of growth, hematopoiesis, respiration, metabolism, etc. Among the variety of such trace elements, only nine can be considered essential. This means that any deficiency in these elements or their imbalance may lead to clinical symptoms or significant changes in the state of human organs and systems. Copper and zinc are both essential elements. Copper is involved in iron metabolism, connective tissue formation, energy production at the cellular level, melanin production, nervous system maintenance. Zinc is a trace element necessary for normal cell growth and cell division, having a stabilizing effect on cell membranes and participating in antioxidant defense systems. Deficiency or excess of copper and zinc in the human body may cause or indicate pathological conditions. The deficiency of copper causes disorders associated with stunted growth, skin depigmentation, anemia, etc. However, its excess levels may harm the nervous and excretory systems, trigger allergic reactions, etc. Zinc deficiency results in stunting, dermatitis, infertility, immunodeficiency, and lymphoid tissue reduction. Measurements of copper and zinc contents are used to support the diagnosis of intoxications or occupational diseases, to assess copper and zinc balance, to identify their deficiency or excess states [1–21]. Accurately measured copper and zinc contents are informative markers at early clinical diagnostic stages.

Trace element contents can be measured using atomic absorption spectrometry, inductively coupled plasma atomic emission spectrometry, inductively coupled plasma mass spectrometry, and inverse voltammetry [22–26]. As of 01.02.2022, the Federal Information Fund for Assuring Measurement Uniformity (FIF AMU),[1] provides a list of approved (certified) measurement procedures. This list includes 10 reference measurement procedures for potassium, calcium, sodium, magnesium, and lithium in blood serum and blood plasma, as well as 18 certified reference procedures for measuring mercury, niobium, tantalum, rare-earth elements, aluminum, isotopes uranium-238, uranium-235, total uranium, thallium, lead, nickel, cadmium, beryllium, arsenic, selenium, bismuth, manganese, chromium, titanium, copper, zinc in various biological objects, including blood plasma, blood, urine, serum, red blood cell mass, etc. The relative error limits of the measurement procedures presented in both FIF AMU and available literature [23–26] range from 15 to 45% depending on the measurand.

Foreign publications describe methods for measuring the content of mercury, lead, and cadmium in biological materials by neutron activation analysis, inductively coupled plasma mass spectrometry, including isotope dilution [27–29]. Leading metrological institutes use isotope dilution inductively coupled plasma mass spectrometry (ID-ICP-MS) for accurate determination of the content of such toxic metals, as lead, mercury, and cadmium [30]. The researchers in [29] found the characteristic relative expanded uncertainty of lead mass fraction measured by isotope dilution at 47.76 ng/g to be $< 1\%$ at $P = 0.95$ and $k = 2$. The work [27] described the method of cadmium determination in blood and serum samples and determined the relative expanded uncertainty of cadmium measurements to be $< 1\%$ at $P = 0.95$ and $k = 2$ for the mass fraction of 90.85 μg/kg, and $< 10\%$ at $P = 0.95$ and $k = 2$ for the mass fraction of 0.0468 μg/kg. It should be noted that isotope dilution, as one of the most high-precision methods, has been recognized as having the potential to be the primary method by the Consultative Committee for Amount of Substance (CCQM) [31]. This allows primary reference measurement procedures to be developed on its basis.

An analysis of the FIF AMU list shows a lack of state primary reference procedures (SPRPs) for measuring copper and zinc in biological materials, which could provide a metrological basis for the creation of reference materials (RMs). The FIF AMU also lacks RMs for the composition of biological materials with certified characteristics of copper and zinc content. Therefore, it appears relevant to develop an SPRP for the content of copper and zinc that could provide the basis not only for characterization of RMs for the composition of biological materials, but also for ensuring reliability, objectivity, and comparability of measurement results in medical laboratory diagnostics. The ID-ICP-MS methods seems promising for the development of an SPRP on its basis.

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry. Accessed 17 October 2022 (In Russ.).

In this work, we aim to develop an SPRP for the content of copper and zinc based on the ID-ICP-MS method. To achieve this aim, the following objectives were formulated: to determine optimal sample preparation conditions, to evaluate the effect of matrix elements, to determine and optimize the conditions of mass spectrometry analysis, to correct the mass discrimination effect, to evaluate the metrological characteristics of the developed SPRP, and to confirm its accuracy.

Materials and Methods

Measurement Method

The ID-ICP-MS method is based on changing the natural isotope composition of the element under determination (measurand) in sample (*x*) by adding a known quantity of an RM with the isotope composition different from that found in nature (*y*), followed by measuring the isotope ratio in sample (*x*) and in the sample mixture with the additive (referred to as a spike) (*b*). Given the natural isotope composition of the element in the sample, the mass of the added nonnatural RM, and the mass of the sample, the mass fraction (molar concentration) of the measurand can be found using the following equation:

$$X_x = X_y \cdot \frac{m_y}{m_x} \cdot \frac{M_x}{M_y} \cdot \frac{\left(K_{y1} \cdot R_{y1} - K_{b1} \cdot R_{b1}\right)}{\left(K_{b1} \cdot R_{b1} - K_{x1} \cdot R_{x1}\right)} \cdot \frac{\sum_i K_{xi} \cdot R_{xi}}{\sum_i K_{yi} \cdot R_{yi}} \cdot K - X_{bl}, \qquad (1)$$

where X_y is the mass fraction (molar concentration) of the measurand in the RM solution with a modified isotope composition (hereinafter referred to as the spike), % (μmol/dm^3);

M_x	is the molar mass of the measurand in the sample, g/mol;
M_y	is the molar mass of the measurand in the spike, g/mol;
R_{y1}	is the measured isotope ratio in the spike;
R_{b1}	is the measured isotope ratio in the mixture of sample and spike;
R_{x1}	is the measured isotope ratio in the sample;
K_{x1}, K_{y1}, K_{b1}	are correction coefficients of the isotope ratio in the sample, spike, and the sample and spike mixture, related to the mass discrimination effect;
m_y	is the spike mass, mg;
m_x	is the sample mass, mg;
$\sum_i R_{xi}$	is the sum of the measurand isotope ratio to the reference isotope in the sample;
$\sum_i R_{yi}$	is the sum of the measurand isotope ratio to the reference isotope in the spike;

i is the number of isotopes of the measurand;
K is the sample dilution factor;
X_{bl} is the mass fraction (molar concentration) of the measurand in a blank sample, % (μmol/dm^3).

An isotope free from interferences is taken as the reference isotope of the element under determination. The index 1 refers to the variable isotope ratio when a spike is introduced in the sample.

The main idea of the ID-ICP-MS method consists in measuring the isotope ratios R_{yi}, R_{bi}, R_{xi}, which are shifted relative to the true value due to the mass discrimination effect. This effect is corrected by introducing the coefficients K_{xi}, K_{yi}, K_{bi} for each isotope ratio. These coefficients are estimated on the basis of experimental data and data from isotope composition standards, as well as reference data on the natural isotope composition of elements [32].

Equipment and Materials

The measurement procedure was developed using a Nexion 2000 inductively coupled plasma mass spectrometer (Perkin Elmer, USA) included in the GET 176-2019 state primary standard[2] [33]. A distillacid BSB-939-IR acid purification system (Berghof, Germany) and a DVS-M/1H-2 combined membrane unit (NPK Mediana-Filter, Russia) for nitric acid and water preparation were used as auxiliary equipment.

In order to implement the procedure based on the isotope dilution method, RMs with the following metrological characteristics were developed:

- GSO 11931-2022 RM for the isotope composition of copper solution (Cu65-10): the range of permissible certified values of copper mass fraction from $0.9 \cdot 10^{-3}$% to $1.1 \cdot 10^{-3}$%, the permissible relative expanded uncertainty of the certified value of mass fraction (at $k = 2$, $P = 0.95$) not exceeding 0.5%; the range of permissible certified values of ^{63}Cu isotope fraction from 0.3% to 0.5%, the permissible expanded relative uncertainty of the certified value of ^{63}Cu isotope fraction (at $k = 2$, $P = 0.95$) not exceeding 10%; the range of permissible certified values of ^{65}Cu isotope atomic fraction from 99.5 to 99.7%, the permissible relative expanded uncertainty of the certified value of ^{65}Cu isotope atomic fraction (at $k = 2$, $P = 0.95$) not exceeding 0.1%;
- GSO 11933-2022 RM for the isotope composition of zinc (Zn68-10): the range of permissible certified values of zinc mass fraction from $0.9 \cdot 10^{-3}$% to $1.1 \cdot 10^{-3}$%, the permissible relative expanded uncertainty of the certified value (at $k = 2$, P

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[2] GET 176-2019 State primary standard of units of mass (molar, atomic) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on coulometry (includes 3 installations on the basis of constant current coulometry, controlled potential coulometry and inductively coupled plasma mass spectrometry): Custodian Institute UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1382712. Accessed 17 October 2022 (In Russ.).

= 0.95%) not exceeding 0.5%; the range of permissible certified values of ^{64}Zn isotope fraction from 0.1% to 0.6%, the permissible relative expanded uncertainty of the certified value of ^{64}Zn isotope fraction (at k = 2, P = 0.95%) not exceeding 10%; the range of permissible certified values of ^{66}Zn atomic fraction from 0.1% to 0.6%, the permissible expanded relative uncertainty of the certified value of ^{66}Zn atomic fraction (at k = 2, P = 0.95) not exceeding 10%; the range of permissible certified values of ^{67}Zn isotope fraction from 0.1% to 0.6%, the permissible relative expanded uncertainty of the certified ^{67}Zn isotope fraction (at k = 2, P = 0.95) not exceeding 10%; the range of permissible certified values of ^{68}Zn isotope atomic fraction from 98.0% to 99.0%, the permissible expanded relative uncertainty of the certified value of ^{68}Zn isotope atomic fraction (at k = 2, P = 0.95) not exceeding 0.1%; the range of permissible certified values of ^{70}Zn isotope fraction from 0.01% to 0.05%, the permissible relative expanded uncertainty of the certified value of ^{70}Zn isotope fraction (at k = 2, P = 0.95) not exceeding 20%.

RMs for the composition of pure metal solutions in mineral acids were used as RMs of natural isotope composition:

- GSO 10942-2017 RM for the composition of copper (Cu-1000) solution: the range of permissible certified values of Cu mass fraction from 800 to 1200 mg/kg, the range of permissible relative error of the certified mass fraction value (P = 0.95) within ± 0.5%, the permissible relative expanded uncertainty of the certified mass fraction (at k = 2, P = 0.95) not exceeding 0.7%;
- GSO 11243-2018 RM for the composition of zinc (Zn-1000) solution: the range of permissible certified values of Zn mass fraction from 800 to 1200 mg/kg, the range of permissible relative error of the certified mass fraction value (P = 0.95) within ± 0.5%, the permissible expanded relative uncertainty of the certified mass fraction value (at k = 2, P = 0.95) not exceeding 0.7%.

Research Object

The research object comprised lyophilized human serum produced by Vector-Best JSC, lyophilized fetal bovine blood serum produced by Biolot LLC, and reconstituted lyophilized human and fetal bovine blood serum.

Results and Discussion

Selection of Optimal Methodological Decisions

Sample Preparation Conditions

Lyophilized human and animal serum, as a rule, is readily soluble in water. However, its dissolution in water leads to a risk of incomplete extraction of the elements under determination. In order to select an optimal solvent, preliminary measurements of the mass fraction of copper and zinc in lyophilized human serum dissolved by two methods were carried out. Within the framework of the first method (acid decomposition), lyophilized blood serum weighing 0.3 g was placed in a polyethylene test tube followed by addition of 5 cm^3 pre-purified concentrated nitric acid and incubation for 1 h. Subsequently, the test tube was exposed to 80 °C in a desiccator for 1 h followed by cooling and bringing the solution mass to 50 g by 1% nitric acid solution. For measurements, the obtained solution was five-fold diluted with 1% nitric acid solution. Within the framework of the second method (dissolution in water), lyophilized blood serum weighing 0.3 g was placed in a polyethylene test tube followed by addition of 5 cm^3 deionized water and dissolution of the sample by small circular movements during 30 min. Upon visual inspection of the end of dissolution, the solution mass in the test tube was brought to 50 g by 1% nitric acid solution. For measurements, the obtained solution was five-fold diluted with 1% nitric acid solution.

In the as-obtained solutions, the mass fraction of copper and zinc was measured by inductively coupled plasma mass spectrometry in the dynamic reaction cell (DRC) mode. The standard addition method was used for measurements. RMs for the composition of copper (GSO 10942-2017) and zinc (GSO 11243-2018) solutions were introduced as additives.

Table 1 presents the measurement results of the mass fraction of copper and zinc by the most common naturally occurring isotopes for lyophilized blood serum samples prepared by the described two methods.

Table 1 shows that acid decomposition is the most optimal method for preparing samples of lyophilized blood serum. In comparison with acid decomposition, the method of dissolution in water leads to underestimation of the measurement results of the mass fraction of copper and zinc.

Table 1 Measurement results of the mass fraction of copper and zinc in lyophilized blood serum samples prepared by two different methods, i.e., acid decomposition and dissolution in water

Element, mass of the isotope for which the mass fraction is measured	Measurand mass fraction, mg/kg	
	Acid decomposition	Dissolution in water
^{63}Cu	9.44	8.32
^{64}Zn	9.64	8.15

A preliminary mass spectrometry analysis by the Total Quant method showed that the main matrix elements in blood serum are sodium, magnesium, phosphorus, sulfur, and potassium. Table 2 shows the obtained results of measuring the mass fractions of matrix elements in lyophilized human and fetal bovine blood serum by the Total Quant method.

The matrix elements of lyophilized serum can potentially form polyatomic ions that match the isotopes of the elements under study in terms of the m/z ratio. Table 3 summarizes the possible polyatomic ions formed from the matrix elements of lyophilized serum.

In order to assess the effect of possible interference and matrix effects on the measurement results of copper and zinc contents, model solutions were prepared similar in composition to those of lyophilized blood serum samples under study. The mass fraction of copper and zinc comprised 9 μg/kg. The mass fraction of matrix elements varied across the range: sodium from 0 to 150 mg/kg, magnesium from 0 to 1 mg/kg, sulfur from 0 to 50 mg/kg, phosphorus from 0 to 2 mg/kg. A 1% nitric acid solution was used as a background. The mass fraction of elements in the prepared model solutions is given in Table 4.

Measurements of the mass fraction of copper and zinc in model solutions were performed by linear graduation in different measurement modes of the inductively coupled plasma mass spectrometer, including the standard mode, dynamic reaction cell (DRC) mode, and kinetic energy discrimination (KED) mode.

Table 2 Measurement results of the mass fraction of elements in lyophilized human and fetal bovine blood serum obtained by the Total Quant method

Element	Element mass fraction, mg/kg		Optimal dilution factor of lyophilized serum
	Lyophilized human blood serum	Lyophilized fetal bovine blood serum	
Na	24,246	43,839	800
Mg	162	426	700
P	672	1063	700
S	350	421	40
K	1050	6324	-
Ca	806	2244	-

Table 3 Possible polyatomic ions formed from the matrix elements of lyophilized serum

Polyatomic ion formation	Isotope under study
$^{23}Na + {}^{40}Ar \rightarrow {}^{23}Na^{40}Ar^{+}$ $^{31}P + {}^{16}O^{16}O \rightarrow {}^{31}P^{16}O^{16}O^{+}$	^{63}Cu
$^{25}Mg + {}^{40}Ar \rightarrow {}^{25}Mg^{40}Ar^{+}$	^{65}Cu
$^{24}Mg + {}^{40}Ar \rightarrow {}^{24}Mg^{40}Ar^{+}$ $^{32}S + {}^{16}O^{16}O \rightarrow {}^{32}S^{6}O^{16}O^{+}$	^{64}Zn
$^{26}Mg + {}^{40}Ar \rightarrow {}^{26}Mg^{40}Ar^{+}$	^{66}Zn

Table 4 Mass fraction of elements in the prepared model solutions

Model solution No	Mass fraction of the element, µg/kg					
	Cu	Zn	Na	Mg	S	P
Solution 1	9	9	0	0	0	0
Solution 2	9	9	30,000	200	10,000	400
Solution 3	9	9	60,000	400	20,000	800
Solution 4	9	9	120,000	800	40,000	1600
Solution 5	9	9	150,000	1000	50,000	2000

Figures 1 and 2 present the dependences of the measured value of the mass fraction of copper by ^{63}Cu isotope on the mass fraction of the sodium and phosphorus matrix elements, obtained in different measurement modes.

It can be seen from Figs. 1 and 2 that an increase in the mass fraction of the sodium and phosphorus matrix elements leads to an increase in the mass fraction of copper by ^{63}Cu isotope in the standard mode of measurements. This is associated with the formation of polyatomic ions given in Table 4. The results obtained in the DRC and KED measurement modes are not affected by the increase in the mass fraction of sodium and phosphorus.

Figure 3 shows the obtained correlation between the increase in the mass fraction of the magnesium matrix element and the copper mass fraction measured by ^{65}Cu isotope.

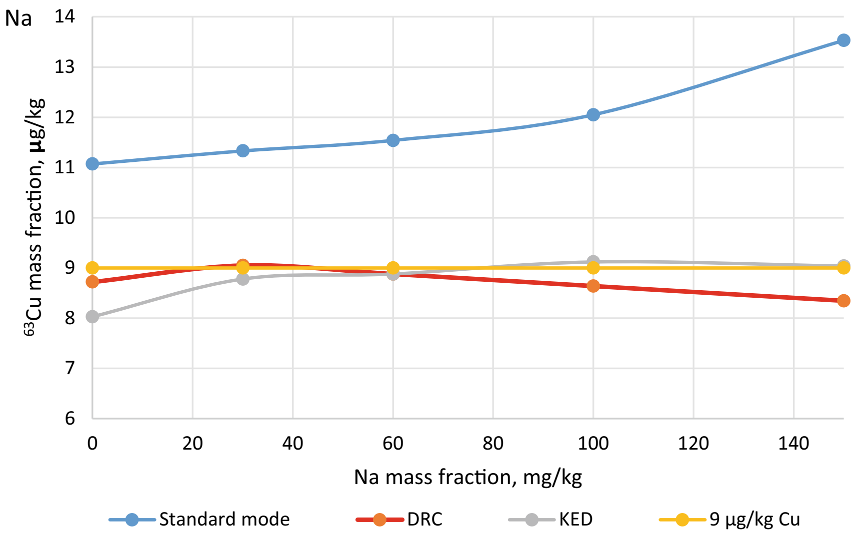

Fig. 1 Measurement results of copper mass fraction by ^{63}Cu isotope as a function of the mass fraction of the sodium matrix element

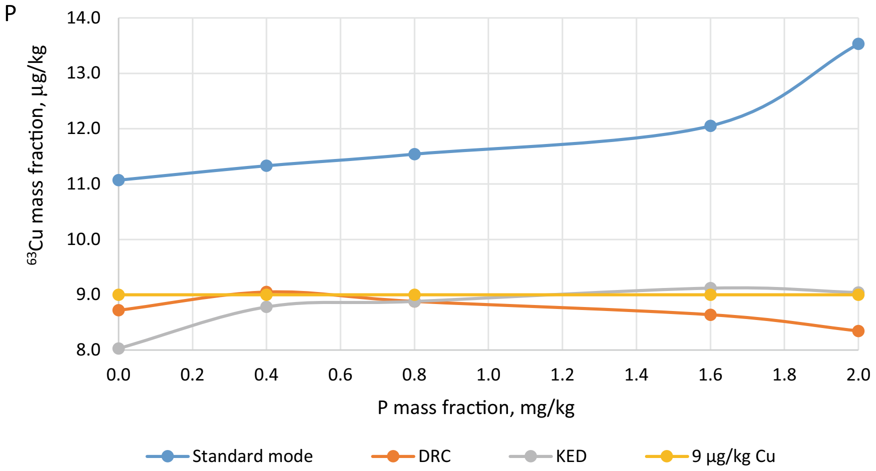

Fig. 2 Measurement results of copper mass fraction by ^{63}Cu isotope as a function of the mass fraction of the phosphorus matrix element

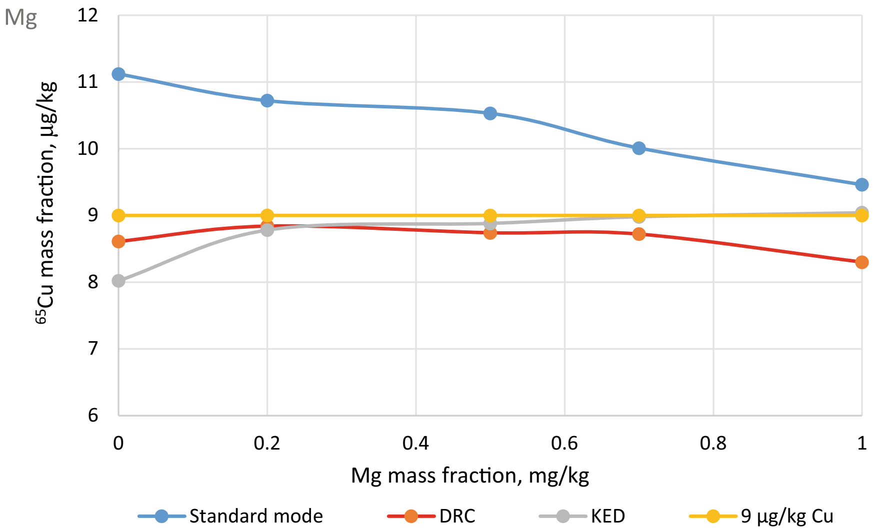

Fig. 3 Measurement results of copper mass fraction by ^{65}Cu isotope as a function of the mass fraction of the magnesium matrix element

It was found that, in the standard mode, an increase in the mass fraction of magnesium in a model solution from 0 to 1 mg/kg leads to a decrease in the mass fraction of copper measured by ^{65}Cu isotope. However, in the DRC and KED modes, the amount of magnesium has no significant effect on the mass fraction of copper by ^{65}Cu isotope. Therefore, a matrix effect manifested in the suppression of a signal

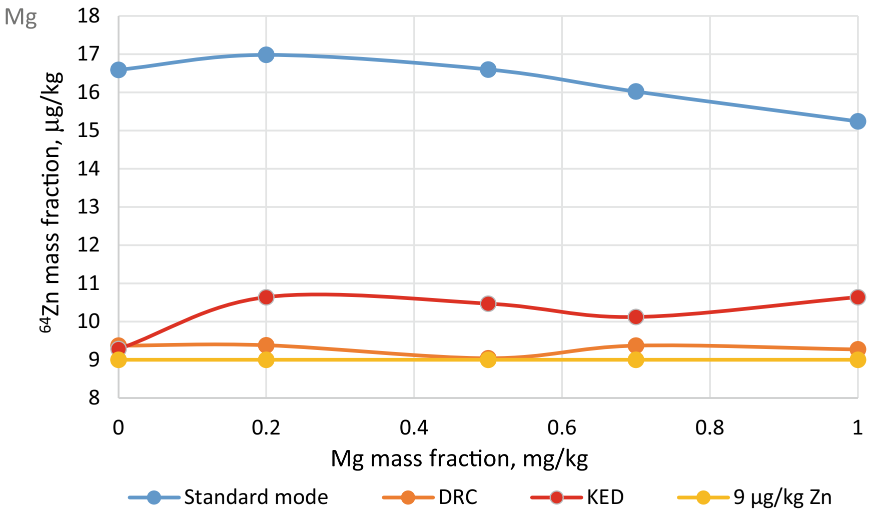

Fig. 4 Measurement results of zinc mass fraction by ^{64}Zn isotope as a function of the mass fraction of the magnesium matrix element

of the measurand isotope, ^{65}Cu in this case, is observed in the standard mode. As a consequence, the copper mass fraction measured by ^{65}Cu isotope decreases under an increase in the magnesium mass fraction.

Figures 4 and 5 shows the correlation between an increase in the mass fraction of the magnesium matrix element and the mass fraction of zinc measured by ^{64}Zn and ^{66}Zn isotopes in different measurement modes.

The DRC mode eliminates spectral overlaps on the ^{64}Zn and ^{66}Zn masses, thus reducing the matrix effect. In the DRC mode, an increase in the magnesium mass fraction in a model solution from 0 to 1 mg/kg is not significant when measuring the zinc mass fraction by ^{64}Zn and ^{66}Zn isotopes.

Figure 6 shows the dependence of the zinc mass fraction by ^{64}Zn isotope on the mass fraction of the sulfur matrix element in different measurement modes.

The DRC mode eliminates spectral overlaps on ^{64}Zn isotope under the sulfur mass fraction of < 20 mg/kg. When the sulfur mass fraction exceeds 20 mg/kg, the zinc mass fraction measured by ^{64}Zn isotope increases and the DRC mode ceases to be effective in eliminating spectral overlaps. Therefore, the mass fraction of the sulfur matrix element in the studied solution should not exceed 20 mg/kg. The dilution factor of the original lyophilized serum should be selected such that possible spectral overlaps and matrix effects be avoided. Otherwise, the results of isotope ratio measurements by the ID-ICP-MS method will be distorted.

According to the conducted studies into spectral overlaps and matrix effects, the optimal dilution factors of the original lyophilized blood serum are presented in Table 2.

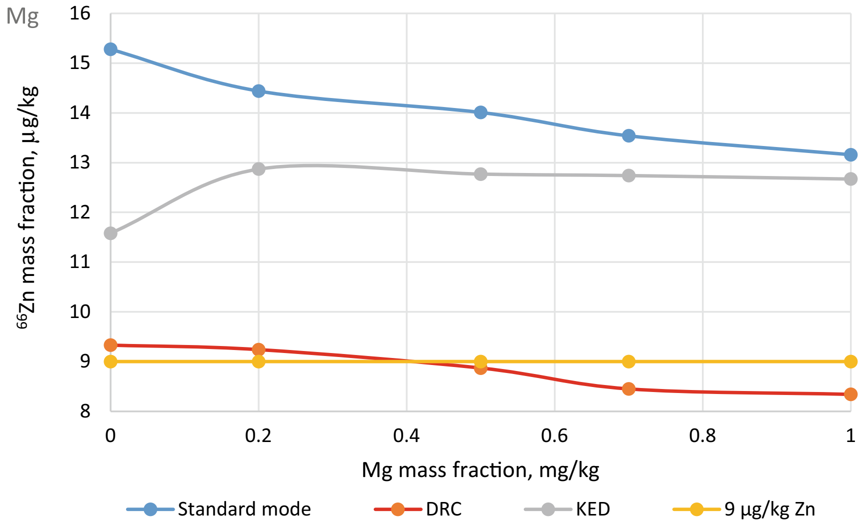

Fig. 5 Measurement results of zinc mass fraction by ^{66}Zn isotope as a function of the mass fraction of the magnesium matrix element

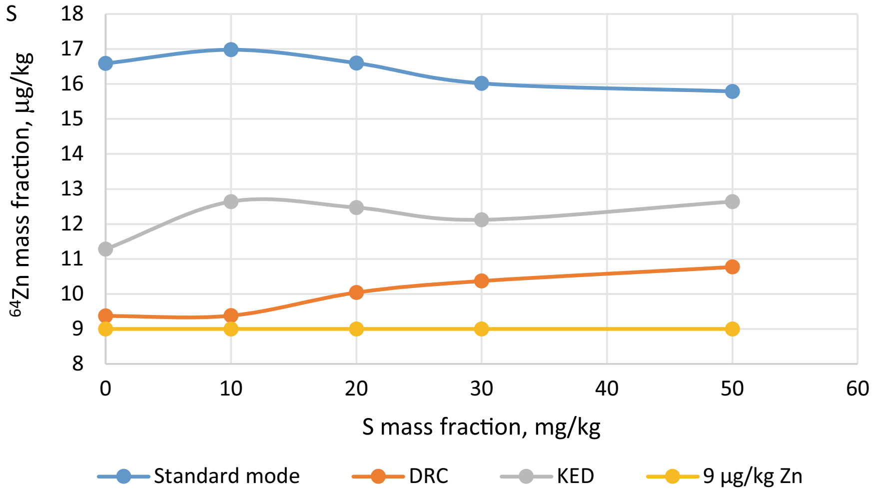

Fig. 6 Measurement results of zinc mass fraction by ^{64}Zn isotope as a function of the mass fraction of the sulfur matrix element

As a result of the conducted studies, the following optimal sample preparation conditions were determined:

(1) A weighted portion of lyophilized human serum is dissolved in pre-purified concentrated nitric acid followed by incubation for at least 1 h in a desiccator

at 80 °C until termination of nitric oxide vapor release. Subsequently, working solutions for measurements in 1% nitric acid solution are prepared.

(2) Reconstituted lyophilized blood serum is prepared by dissolving 0.6 g of lyophilized blood serum in 5.0 cm^3 of deionized water followed by stirring until a homogeneous suspension is formed. Afterwards, aliquots are taken and working solutions in 1% nitric acid solution are prepared.

The conducted investigation into the effect of matrix elements on analytes in different mass spectrometer modes found DRC to be the most optimal mode for measuring blood serum solutions. The DRC mode not only neutralizes possible overlaps, but also demonstrates sufficient sensitivity at low analyte concentrations. Matrix effects are reduced by diluting the sample by at least 800 times.

The following solutions were prepared for subsequent measurements:

(1) A sample solution in 1% nitric acid solution under the dilution factor of not < 800;
(2) A sample solution with a spike: the mass fraction of the analyte from $6.0 \cdot 10^{-7}$ to $3.0 \cdot 10^{-5}$% and that of the introduced spike were selected depending on the mass fraction (molar concentration) of the analyte in the sample solution; the amount of the spike was calculated from the basic equation of isotope dilution method (1) such that the isotope ratios $^{65}Cu/^{63}Cu$ and $^{68}Zn/^{64}Zn$ were close to unity.
(3) A blank sample solution consisting of a solution of reagents in the same quantities as those used for preparing the sample solutions;
(4) A blank sample solution with addition of RMs for the composition of copper and zinc (GSO 11931-2022 and GSO 11933-2022).

All solutions were prepared using the gravimetric method.

Selection and Optimization of Mass Spectrometry Conditions in the DRC Mode

When selecting optimal conditions for measurements using a mass spectrometer in the DRC mode, the main parameters included the detection limit of copper and zinc, the relative standard deviation (RSD) in the measurements of the $^{65}Cu/^{63}Cu$, $^{66}Zn/^{64}Zn$, $^{67}Zn/^{64}Zn$, $^{68}Zn/^{64}Zn$, and $^{70}Zn/^{64}Zn$ isotope ratios, as well as the maximum intensity of the studied ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes in model solutions and the studied solutions of lyophilized blood serum. The following parameters affecting the process of measurements in the DRC mode were investigated: reaction gas consumption, high-frequency voltage applied to the cell quadrupole (RPq), and the rate of argon flow through the atomizer.

Ammonia was used as a reaction gas for operation of the mass spectrometer in the DRC mode. In order to establish the optimal flow rate of ammonia, the detection limits of copper and zinc were determined by measuring the mass fraction of copper and zinc by ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes in 1% nitric acid solution by linear graduation. During the experiment, the ammonia flow rate was varied across the range of 0.1 to 1 cm^3/min. The detection limits of copper and zinc were determined for all isotopes at different ammonia flow rates by the 3σ criterion. The obtained results are presented in Figs. 7 and 8.

In addition, the isotope ratios of ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn in the working solution of lyophilized blood serum at different ammonia

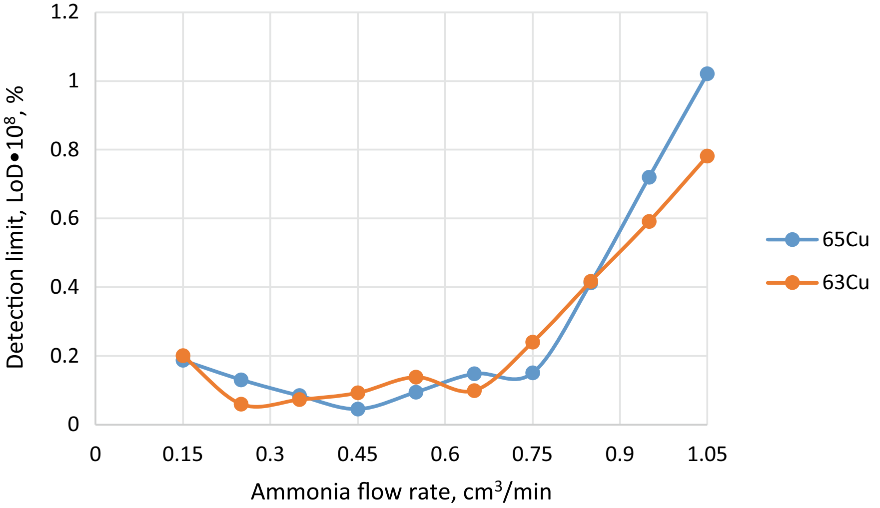

Fig. 7 Dependence of copper detection limits by ^{63}Cu and ^{65}Cu isotopes as a function of ammonia flow rate in the reaction cell

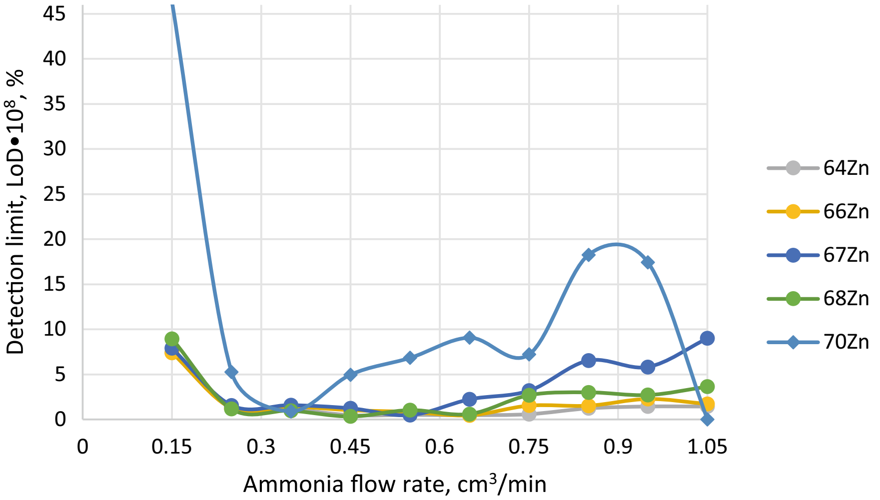

Fig. 8 Dependence of zinc detection limits by ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes as a function of ammonia flow rate in the reaction cell

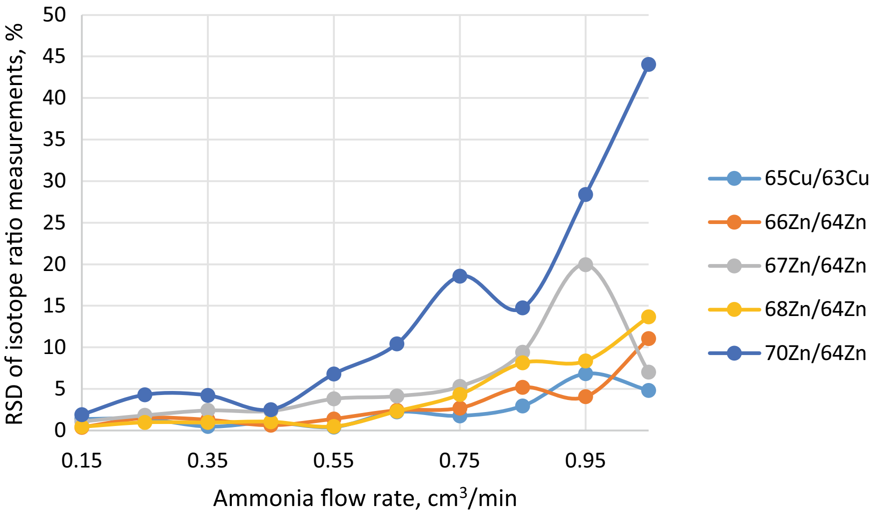

Fig. 9 Dependence of the RSD of isotope ratio measurements on the ammonia flow rate in the reaction cell

flow rates were measured. Figure 9 shows the dependence of the RSD of measurements of isotope ratios on the ammonia flow rate in the reaction cell.

The optimum value of ammonia flow rate was determined to be 0.45 cm^3/min. Under this value, the lowest detection limits of copper and zinc for all isotopes and the lowest relative RSD of measurements of the ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn isotope ratios are observed.

In order to establish optimal values of high-frequency voltage applied to the cell quadrupole (RPq), the signal intensities of ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes were measured in the working solution of lyophilized blood serum. The RPq value was varied from 0.1 to 0.9 V. Dependences of the signal intensity of ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes on RPq values are shown in Fig. 10.

Measurements of the isotope ratios of ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn in the working solution of lyophilized blood serum at different RPq values were conducted. The dependence of the RSD of measurements of isotope ratios on the RPq value is shown in Fig. 11.

The maximum signal intensity of ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes is observed at RPq = 0.2 V. However, the minimum RSD of measurements of ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn isotope ratios is observed at RPq = 0.3 V. Under this value, the output signal intensity decreases insignificantly compared to RPq = 0.2 V. For the isotope dilution method, the determining factor in selecting the optimum RPq value consists in the minimum value of the RSD of isotope ratio measurements. Thus, the optimum RPq value was determined to be 0.3 V.

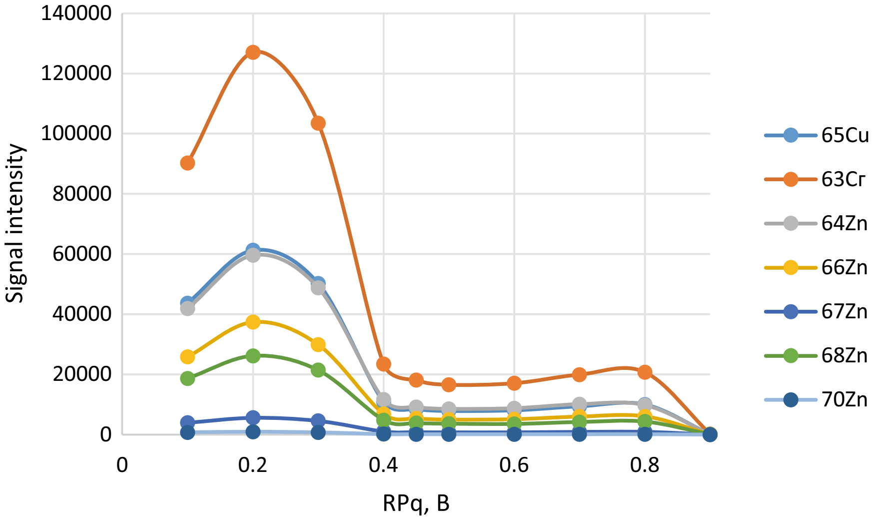

Fig. 10 Dependences of the signal intensity of ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes on RPq values

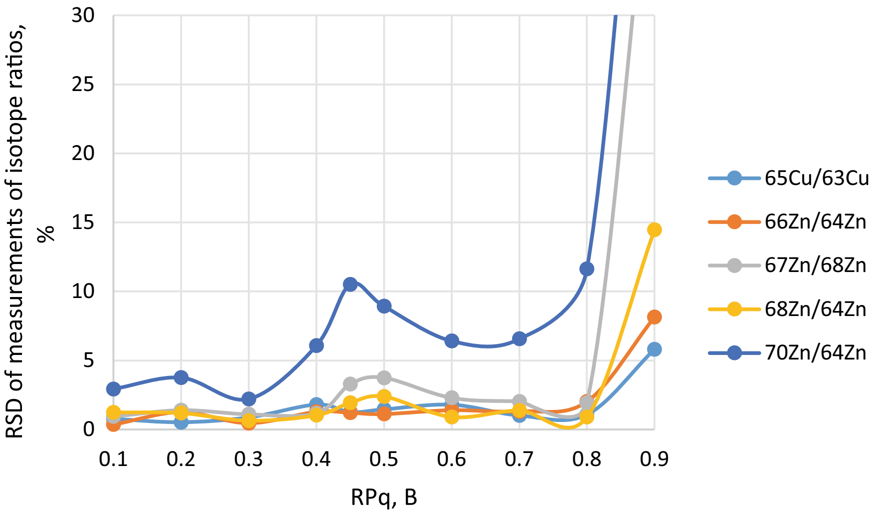

Fig. 11 Dependences of RSD of measurements of isotope ratios ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn on RPq values

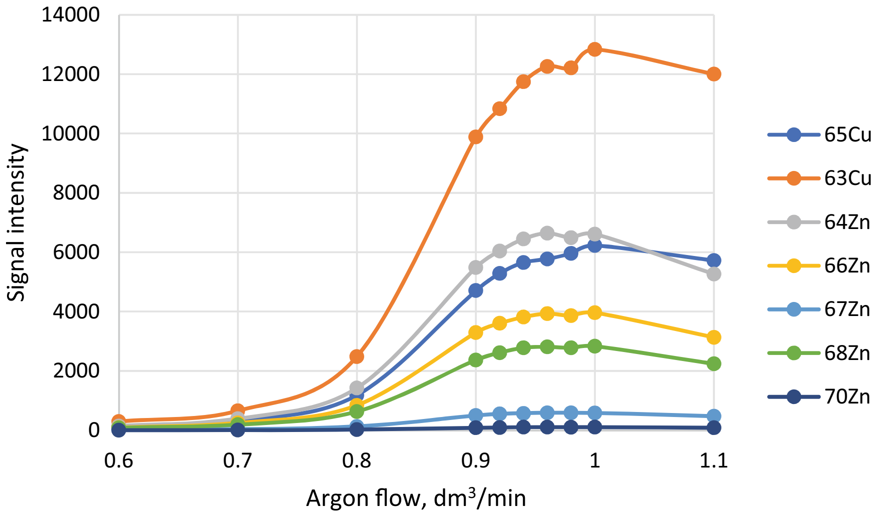

Fig. 12 Dependence of the signal intensity of ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes on the rate of argon flow through the atomizer

In order to estimate the effect of argon flow rate through the atomizer, the signal intensity of ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn isotopes in the working solution of lyophilized blood serum was studied under the argon flow rate varied across the range from 0.6 to 1.0 dm^3/min. The results obtained are shown in Fig. 12.

The isotope ratios of ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, and ^{70}Zn/^{64}Zn were also studied in the working solution of lyophilized blood serum at different rates of argon flow. The results are shown in Fig. 13.

As can be seen from Figs. 12 and 13, the optimum argon flow rate ranges between 0.96–0.98 dm^3/min. At this value, the maximum signal intensity from ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn isotopes and the minimum value of the RSD of the ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn isotope ratios are observed.

Table 5 summarizes the established optimal values for determination of copper and zinc mass fractions in lyophilized blood serum by mass spectrometry in the DRC mode.

The parameters of plasma power and argon flow rate through the outer burner tube were taken from the manufacturer' specification.

Correction of Mass Discrimination Effect

Mass discrimination is manifested in a decrease in the measurement sensitivity when moving from heavier to lighter isotopes, expressed as a bias in the measured isotope ratio relative to its true value. In addition to the mass discrimination effect, biases in measured isotope ratios also depend on the mass spectrometer settings [34, 35].

The following solutions were used to correct the mass discrimination effect:

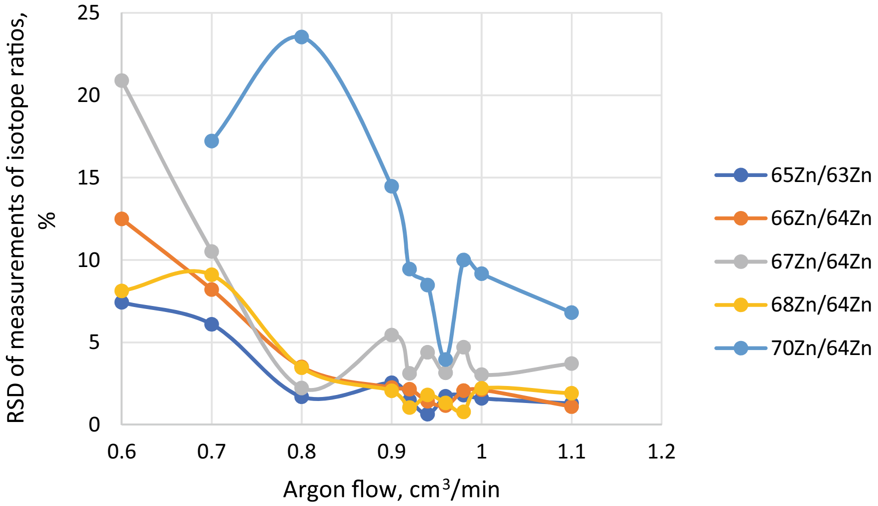

Fig. 13 Dependence of the RSD of measurements of ^{65}Cu/^{63}Cu, ^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn isotope ratios on the rate of argon flow through the atomizer

Table 5 Mass spectrometry measurement parameters

Parameter	Value
Plasma power, W	1350
Argon flow rate through the outer burner tube, dm^3/min	16
Argon flow rate through the atomizer, dm^3/min	From 0.96 to 0.98
High-frequency voltage applied to the cell quadrupole (RPq), B	0.3
Resolution, u	From 0.7 to 0.8
Atomizing chamber	Cyclone chamber
Ammonia flow rate into the reaction cell, cm^3/min	0.45

(1) A solution of RMs for copper (GSO 10942-2017) and zinc (GSO 11243-2018) with natural isotopic composition and the mass fraction of copper and zinc from $6.0 \cdot 10^{-7}$ to $3.0 \cdot 10^{-5}$%. The isotope ratios of copper and zinc were calculated according to the natural isotopic composition of elements by IUPAC [32] using the expression

$$R_{istand} = \frac{A_i}{A_{ref}}, \tag{2}$$

where A_i is the atomic fraction of the i-th isotope according to IUPAC data [32];

A_{ref} is the atomic fraction of the reference isotope.

(2) Solutions of copper and zinc RMs with equimolar isotopic composition, which comprise a mixture of copper and zinc RMs with natural isotopic composition (GSO 10942-2017, GSO 11243-2018) and RMs for the isotopic composition of copper and zinc (GSO 11931-2022, GSO 11933-2022) (hereinafter the RM mixture). The amount of RM mixed solutions was calculated from the basic equation of the isotope dilution method such that the obtained isotope ratios for ^{65}Cu/^{63}Cu and ^{68}Zn/^{64}Zn isotope pairs in the resulting mixture were close to unity.

(3) A solution of RMs for the isotopic composition of copper and zinc (GSO 11931-2022 and 11933-2022, respectively) with the mass fraction of copper and zinc from $6.0 \cdot 10^{-7}$ to $3.0 \cdot 10^{-5}$%. The isotope ratios of copper and zinc were calculated according to the CRM data sheets and an expression similar to (2).

Correction coefficients were established during measurements of the as-prepared solutions and calculated by the formula

$$K_i = \frac{R_{istand}}{R_{imsr}} + K_{bias}, \tag{3}$$

where R_{istand} is the actual value of i-th isotope ratio calculated by (2); for the RM mixture, R_{istad} was calculated by the basic equation of ID-ICP-MS (1);

R_{imsr} is the i-th measured isotope ratio;

K_{bias} are the coefficients associated with the correction for the optimization of mass spectrometer operation parameters; in our calculations, $K_{bias} = 0$ due to the conducted optimization of mass spectrometer operation parameters.

The values of correction coefficients obtained from the measurement results of the RMs of natural isotopic composition were further applied to the measured values of isotope ratios in the samples. The values of the correction coefficient obtained from the measurement results of the RM mixture were applied to correct the bias of isotope ratios measured in the samples with a spike. The values of the correction coefficient obtained from the measurement results of the RM solution for isotopic composition were applied to correct the bias of isotope ratios in the blank sample with a spike.

Measurement Procedure

Figure 14 outlines the procedure of measurements using the isotope dilution method.

In order to correct the effect of mass discrimination, the sequence of measurements of prepared solutions was designed as follows:

(1) before and after measurements of isotope ratios in the sample and blank sample, isotope ratios were measured in a solution of RMs of natural composition;

(2) before and after measurements of isotope ratios in the sample with a spike, isotope ratios were measured in the RM mixture solution;

(3) before and after isotope ratios were measured in the blank sample with a spike, isotope ratios were measured in the spike solution.

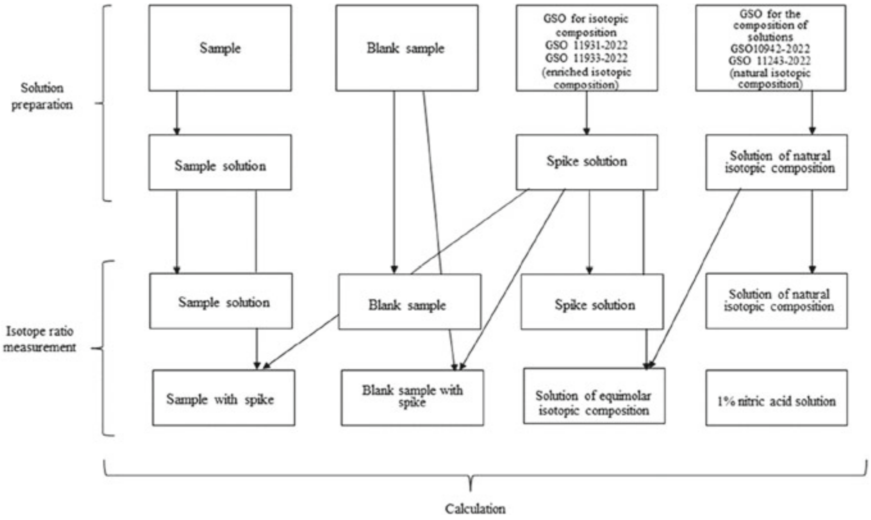

Fig. 14 Measurement procedure for the mass fraction and molar concentration of copper and zinc in biological materials (matrices)

Between the introduction of samples and solutions, the input system was flushed with a 1% nitric acid solution.

The sequence of measurements started with measurements of isotope ratios in solutions of natural isotopic composition (sample solution, blank sample, solution of natural isotopic composition). Afterwards, isotope ratios were measured in equimolar mixtures (sample with spike, solution of equimolar isotope composition) and then in solutions with modified (enriched) isotopic composition (blank sample with spike, spike solution).

The mass fraction of copper and zinc in lyophilized serum and the molar concentration of copper and zinc in reconstituted serum were calculated using expression (1).

Accuracy Estimation

The metrological characteristics of the developed measurement procedure were established following algorithms described in RMG 61–2010, GOST 34100.3–2017/ISO/IEC Guide 98–3:2008 [36, 37]. Statistical data obtained by measuring the mass fraction and molar concentration of copper and zinc in assessment samples (AS) were used to estimate the random uncertainly component (type A measurement uncertainty).

The relative total standard measurement uncertainty (u_c) is the sum of the type A (u_A) and type B (u_B) relative standard uncertainties:

$$u_c = \sqrt{u_A^2 + u_B^2}, \tag{4}$$

The type A relative standard uncertainty is estimated based on measurements of the mass fraction and molar concentration of copper and zinc in AS obtained under conditions of inter-laboratory testing precision, i.e., at different times and by different operators.

The type B relative standard uncertainty is estimated based on the equation of measurements (1) by the formula

$$u_B = \frac{100}{\underline{X_i}} \cdot \sqrt{\sum_{\varepsilon=1}^{m} c_\varepsilon^2 \cdot u_\varepsilon^2}, \tag{5}$$

where c_ε is the sensitivity coefficient of Σ-th uncertainty component determined from the equation of measuring the mass fraction (molar concentration) of the measurand in a sample as a partial derivative of the function by Σ-th uncertainty component;

u_ε is the standard uncertainly of measuring the Σ-th equation component;

$\underline{X_i}$ is the arithmetic mean value of the mass fraction (molar concentration) of the measurand, % (μmol/dm^3).

Standard uncertainties of the mass fraction $[u(X_{yi})]$ of the measurand in the spike are calculated from the relative expanded uncertainties of certified mass fraction given in the RMs data sheets for isotopic composition (GSO 11931-2022 and GSO 11933-2022)

$$u(X_{yi}) = \frac{U_0 \cdot X_{yi}}{2 \cdot 100}, \tag{6}$$

where U_0 is the relative expanded uncertainty of the certified value of copper or zinc mass fraction at k = 2 and P = 0.95, %;

X_{yi} is the certified value of copper or zinc mass fraction in RMs for the isotopic composition of solutions, %.

Standard weighing uncertainties ($u(m)$) are calculated from the data provided in the calibration certificates of the balances used.

The molar weight of copper and zinc in the sample and in the spike are calculated as follows

$$M = \sum M_{ai} \cdot A_i, \tag{7}$$

where M_{ai} is the molar weight of the *i*-th isotope given in [32], g/mol;

A_i is the atomic fraction of the *i*-th isotope given in [32] for natural isotopic composition or in the data sheets of RMs for the isotopic composition of copper and zinc solutions (GSO 11931-2022 and GSO 11933-2022).

The standard uncertainty of the copper and zinc molar weight is estimated as

$$u(M) = \sqrt{\sum_{i=1}^{n}(A_i \cdot u(M_{ai}))^2 + \sum_{i=1}^{n}(M_{ai} \cdot u(A_i))^2}, \quad (8)$$

where $u(M_{ai})$ is the standard uncertainty of the molar weight of the i-th isotope given in [32], g/mol;

$u(A_i)$ is the standard uncertainty of the atomic fraction the i-th isotope taken from the data sheets of RMs for the isotopic composition of copper and zinc solutions, or from [32].

Data on the atomic weights of the isotopes of various elements can be found in [32].

The standard uncertainties of the mass discrimination correction coefficients are calculated as follows

$$u(K_i) = \sqrt{\left(\frac{1}{R_{imsr}} \cdot u(R_{istand})\right)^2 + \left(-\frac{R_{istand}}{R_{imsr}^2} \cdot u(R_{imsr})\right)^2 + u(K_{bias})^2}, \quad (9)$$

where $u(R_{istand})$ are the standard uncertainties of isotope ratios in the RM for the natural isotopic composition of copper solution and the RM for the enriched isotopic composition of copper solution, calculated as follows

$$u(R_{istand}) = \sqrt{\left(\frac{1}{A_{ref}} \cdot u(A_i)\right)^2 + \left(\left(-\frac{A_i}{A_{ref}^2}\right) \cdot u(A_{ref})\right)^2}, \quad (10)$$

where $u(A_i)$ is the standard uncertainty of the atomic fraction of the i-th isotope;

$u(A_{ref})$ is the standard uncertainty of the atomic fraction of the reference isotope (^{63}Cu for copper and ^{64}Zn for zinc);

$u(R_{imsr})$ are the standard uncertainties of the measured isotope ratios, which are the standard deviation of the arithmetic mean of isotope ratio measurements.

$$u(R_{irms}) = \sqrt{\sum_{j=1}^{2} \frac{\left(R_{irms} - \underline{R_{irms}}\right)^2}{n \cdot (n-1)}}. \quad (11)$$

where $u(K_{bias}) = 0.001$ is the standard uncertainty of correction for optimization of mass spectrometer operation parameters; $j = 2$ is parallel determinations of the i-th isotope ratio.

The dilution factor of a lyophilized serum sample is calculated according to the sample preparation procedure

$$K = \frac{m_{r.0}}{m_0} \cdot \frac{m_{r.1}}{m_1}, \quad (12)$$

where m_0 is the weight of the sample portion taken for the preparation of the initial sample solution, g;

$m_{r.0}$ is the weight of the initial sample solution, g;

m_1 is the weight of an aliquot of the sample solution taken for dilution, g;

$m_{r.1}$ is the weight of the sample solution after dilution, g.

The standard uncertainty of the dilution factor of a lyophilized blood serum sample was calculated as follows

$$u(K) = \sqrt{\begin{array}{l} \left(\frac{1}{m_0} \cdot \frac{m_{r.1}}{m_1} \cdot u(m_{r.0})\right)^2 + \left(-\frac{m_{r.0}}{m_0^2} \cdot \frac{m_{r.1}}{m_1} \cdot u(m_0)\right)^2 \\ +\left(\frac{m_{r.0}}{m_0} \cdot \frac{1}{m_1} \cdot u(m_{r.1})\right)^2 + \left(-\frac{m_{r.0}}{m_1^2} \cdot \frac{m_{r.1}}{m_0} \cdot u(m_1)\right)^2 \end{array}}, \tag{13}$$

where $u(m_{r.0})$, $u(m_0)$, $u(m_{r.1})$, $u(m_1)$ are standard uncertainties of weighing of sample portions and sample solutions, g.

The sample dilution factor of a reconstituted lyophilized serum sample was calculated according to the sample preparation procedure

$$K' = \frac{m_0}{V_0 \cdot M_{sti}} \cdot \frac{m_{r.1}}{m_1} \cdot 10^6, \tag{14}$$

where m_0 is the weight of an aliquot of lyophilized serum solution taken for measurements, g;

V_0 is volume of an aliquot of reconstituted serum taken for measurements, cm^3;

m_1 is the weight of an aliquot of the solution taken for dilution, g;

$m_{r.1}$ is the weight of the diluted solution, g;

M_{sti} is the molar weight of copper and zinc in reconstituted serum, g/mol.

The standard uncertainty of the dilution factor of the reconstituted lyophilized serum sample was calculated as follows

$$u\left(K'\right) = 10^6 \cdot \sqrt{\begin{array}{l} \left(\frac{1}{V_0 \cdot M_{sti}} \cdot \frac{m_{r.1}}{m_1} \cdot u(m_0)\right)^2 + \left(-\frac{m_0}{V_0^2 \cdot M_{sti}} \cdot \frac{m_{r.1}}{m_1} \cdot u(V_0)\right)^2 \\ +\left(\frac{m_0}{V_0 \cdot M_{sti}} \cdot \frac{1}{m_1} \cdot u(m_{r.1})\right)^2 + \left(-\frac{m_{r.1}}{m_1^2} \cdot \frac{m_0}{V_0 \cdot M_{sti}} \cdot u(m_1)\right)^2, \\ +\left(-\frac{m_{r \cdot 1}}{m_1} \cdot \frac{m_0}{V_0 \cdot M_{sti}^2} \cdot u(M_{sti})\right) \end{array}} \tag{15}$$

where $u(V_0)$ are the standard uncertainties of measuring the selected aliquot volume, cm^3;

$u(M_{sti})$ is the standard uncertainty of the molar weight of the i-th element in the sample, calculated according to [32], g/mol.

The mass fraction (molar concentration) of measurands in the blank sample was calculated based on the measured isotopic ratios in the blank sample and the blank sample with a spike, using the basic measurement equation of the isotopic dilution method. The evaluation of the uncertainty of measuring the mass fraction (molar concentration) in a blank sample was carried by analyzing the measurement equation in accordance with GOST 34100.3-2017/ISO/IEC Guide 98-3:2008 [37]. The relative standard uncertainty of measuring the mass fraction (molar concentration) of measurands in a blank sample comprised 45% (at P = 0.95).

The expanded standard uncertainty of measurements of the mass fraction (molar concentration) of copper and zinc was calculated by the formula

$$U = 2 \cdot u_c. \tag{16}$$

The relative expanded uncertainty of measurements of copper and zinc mass fraction in blood serum is calculated according to the formula

$$U_o = \frac{U}{\underline{X_i}} \cdot 100. \tag{17}$$

Tables 6 and 7 present typical uncertainty budgets for measurements of copper and zinc mass fraction in blood serum.

The maximum values from those presented in Table 8 are taken as the metrological characteristics of the developed state primary reference procedure (SPRP).

Confirmation of Highest Accuracy

The highest accuracy of the developed SPRP was confirmed by its comparative analysis with similar SPRP produced in other countries, information about which is available in the database of the Joint Committee for Traceability in Laboratory Medicine (JCTLM). Foreign procedures for measuring the concentration of copper and zinc in biological objects included in the JCTLM database [38] were developed on the basis of neutron activation analysis, with the relative expanded uncertainty of measurements ranging from 2 to 10%. Table 9 presents information on such procedures registered in the JCTLM database.

The highest accuracy of the developed SPRP was confirmed experimentally by the COOMET 849/RU/2021 comparisons "Pilot comparison to determine the content of metals in blood serum." The comparison results were processed in accordance with the COOMET document R/GM/14:2006 "COOMET Recommendation. Guidelines for the evaluation of COOMET key comparisons data" [39] according to the COOMET protocol 849/RU/21. The conducted comparisons showed that the results obtained by the developed SPRP agree well with those obtained by other procedures at the same time as exhibiting the lowest uncertainty levels.

The highest accuracy of measurements obtained using the SPRP for the mass fraction and molar concentration of copper and zinc in biological materials (matrices) is achieved and confirmed by:

- the use of inductively coupled plasma mass spectrometry with isotope dilution as a measurement method. Isotope dilution has been recognized by the Consultative

Table 6 Uncertainly budget of measuring copper mass fraction in blood serum

Uncertainty source		Value	u_ε	Unit of measurement	c_ε	$u_\varepsilon \cdot c_\varepsilon$	Contribution (%)
Description	Designation						
Repetition	$\underline{X}_{Cu}$	0.000975	$1.8{\cdot}10^{-5}$	%	1.000	$1.76{\cdot}10^{-5}$	27.0
Component mass fraction in the spike	X_{yCu}	$2.12{\cdot}10^{-5}$	$4.1{\cdot}10^{-8}$	%	46.101	$1.90{\cdot}10^{-5}$	2.9
Spike weight	m_{yCu}	1.01105	$1.0{\cdot}10^{-4}$	g	$2.51{\cdot}10^{-6}$	$2.51{\cdot}10^{-10}$	0.0
Sample weight	m_x	49.773	0.0001	g	$1.83{\cdot}10^{-9}$	$1.83{\cdot}10^{-13}$	0.0
Mass discrimination correction for $R_{y65/63}$	$K_{y65/63}$	2.2492	0.0701	–	$-4.04{\cdot}10^{-8}$	$-2.84{\cdot}10^{-9}$	0.004
Measured 65/63 ratio in the spike	$R_{y65/63}$	135.104	0.6755	–	$7.20{\cdot}10^{-6}$	$4.86{\cdot}10^{-6}$	7.5
Mass discrimination correction for $R_{b65/63}$	$K_{b65/63}$	0.8855	0.0063	–	– 0.00202	$-1.27{\cdot}10^{-5}$	19.5
Measured 65/63 ratio in the sample with the spike	$R_{b65/63}$	1.1082	0.0055	–	– 0.00161	$-8.93{\cdot}10^{-6}$	13.7
Mass discrimination correction for $R_{x65/63}$	$K_{x65/63}$	0.9193	0.0053	–	0.00088	$4.69{\cdot}10^{-6}$	7.2
Measured 65/63 ratio in the sample	$R_{x65/63}$	0.4844	0.0078	–	0.00167	$1.30{\cdot}10^{-5}$	20.0
Mass discrimination correction for $R_{y63/63}$ in the spike	$K_{y63/63}$	1.0000	0	–	– 0.00098	0	0.0
Measured 63/63 ratio in the spike	$R_{y63/63}$	1.0000	0	–	– 0.00098	0	0.0
Mass discrimination correction for $R_{x63/63}$ in the sample	$K_{x63/63}$	1.0000	0	–	0.00098	0	0.0
Measured 63/63 ratio in the sample	$R_{x63/63}$	1.0000	0	–	0.00098	0	0.0
Molar weight of the measurand in the sample	M_{stCu}	63.5460	0.068	g/mol	0.00002	$1.04{\cdot}10^{-6}$	1.6

(continued)

Table 6 (continued)

Uncertainty source		Value	u_ε	Unit of measurement	c_ε	$u_\varepsilon \cdot c_\varepsilon$	Contribution (%)
Description	Designation						
Molar weight of the measurand in the spike	M_{yCu}	64.9212	0.011	g/mol	$-$ 0.00002	$-$ 1.69·10^{-7}	0.3
Copper mass fraction in the blank sample	X_{blCu}	5.96·10^{-10}	2.7·10^{-10}	%	$-$ 865.44	$-$ 2.36·10^{-7}	0.4
Dilution factor	K	865.437	0.287	–	$-$ 3.79	$-$ 1.04·10^{-9}	0.0
Type A standard uncertainly, u_A					1.76·10^{-5}		
Type B standard uncertainly, u_B					2.14·10^{-5}		
Total standard uncertainty, u_C					2.77·10^{-5}		
Expanded uncertainly (k = 2 P = 0.95), U, %					5.54·10^{-5}		
Relative expanded uncertainty (k = 2 P = 0.95), U_o, %					5.68		

Table 7 Uncertainly budget of measuring zinc mass fraction in blood serum

Uncertainty source		Value	u_ε	Unit of measurement	c_ε	$u_\varepsilon \cdot c_\varepsilon$	Contribution (%)
Description	Designation						
Repetition	$\underline{X}_{Zn}$	0.000998	$1.8{\cdot}10^{-5}$	%	1.000	$1.80{\cdot}10^{-5}$	33.67
Component mass fraction in the spike	X_{yZn}	$1.89{\cdot}10^{-5}$	$4.3{\cdot}10^{-8}$	%	27.061	$1.15{\cdot}10^{-6}$	2.16
Spike weight	m_{yZn}	1.0117	$6.0{\cdot}10^{-5}$	g	$5.05{\cdot}10^{-4}$	$3.03{\cdot}10^{-8}$	0.06
Sample weight	m_x	49.773	0.000433	g	$-1.03{\cdot}10^{-5}$	$-4.45{\cdot}10^{-9}$	0.01
Mass discrimination correction for $R_{y68/64}$	$K_{y68/64}$	2.1022	0.0403		$2.69{\cdot}10^{-6}$	$1.08{\cdot}10^{-7}$	0.20
Measured 68/64 ratio in the spike	$R_{y68/64}$	126.118	0.6306		$4.49{\cdot}10^{-8}$	$2.83{\cdot}10^{-8}$	0.05
Mass discrimination correction for $R_{b68/64}$	$K_{b68/64}$	0.80821	0.00521		$-9.91{\cdot}10^{-4}$	$-5.17{\cdot}10^{-6}$	9.68
Measured 68/64 ratio in the sample with the spike	$R_{b68/64}$	1.2474	0.0062		$-6.42{\cdot}10^{-4}$	$-4.01{\cdot}10^{-6}$	7.50
Mass discrimination correction for $R_{x68/64}$	$K_{x68/64}$	0.8272	0.0160		$3.48{\cdot}10^{-4}$	$5.57{\cdot}10^{-6}$	10.44
Measured 68/64 ratio in the sample	$R_{x68/64}$	0.4391	0.0022		$6.56{\cdot}10^{-4}$	$1.44{\cdot}10^{-6}$	2.70
Mass discrimination correction for $R_{y64/64}$ in the spike	$K_{y64/64}$	1.0000	0			0	0.00
Measured 64/64 ratio in the spike	$R_{y64/64}$	1.0000	0		$2.97{\cdot}10^{-6}$	0	0.00

(continued)

Table 7 (continued)

Uncertainty source		Value	u_ε	Unit of measurement	c_ε	$u_\varepsilon \cdot c_\varepsilon$	Contribution (%)
Description	Designation						
Mass discrimination correction for $R_{y66/64}$ in the spike	$K_{y66/64}$	1.1427	0.0384		$2.26{\cdot}10^{-6}$	$8.68{\cdot}10^{-8}$	0.16
Measured 66/64 ratio in the spike	$R_{y66/64}$	0.7625	0.0038		$3.39 \cdot 10^{-6}$	$1.29{\cdot}10^{-8}$	0.02
Mass discrimination correction for $R_{y67/64}$ in the spike	$K_{y67/64}$	0.0093	0.0938		$1.24{\cdot}10^{-6}$	$1.16{\cdot}10^{-7}$	0.22
Measured 67/64 ratio in the spike	$R_{y67/64}$	0.4171	0.0021		$2.75 \cdot 10^{-8}$	$5.74{\cdot}10^{-11}$	0.00
Mass discrimination correction for $R_{y70/64}$. in the spike	$K_{y70/64}$	0.5585	0.0426		$3.28 \cdot 10^{-7}$	$1.40{\cdot}10^{-8}$	0.03
Measured 70/64 ratio in the spike	$R_{y70/64}$	0.1104	0.0006		$1.66{\cdot}10^{-6}$	$9.15{\cdot}10^{-10}$	0.00
Mass discrimination correction for $R_{x64/64}$ in the sample	$K_{x64/64}$	1.0000	0		$4.91{\cdot}10^{-4}$	0	0.00
Measured 64/64 ratio in the sample	$R_{x64/64}$	1.0000	0		$4.91{\cdot}10^{-4}$	0	0.00
Mass discrimination correction for $R_{x66/64}$ in the sample	$K_{x66/64}$	0.9263	0.0304		$2.98{\cdot}10^{-4}$	$9.04{\cdot}10^{-6}$	16.93

(continued)

Table 7 (continued)

Uncertainty source		Value	u_ε	Unit of measurement	c_ε	$u_\varepsilon \cdot c_\varepsilon$	Contribution (%)
Description	Designation						
Measured 66/64 ratio in the sample	$R_{x66/64}$	0.6066	0.0030			$1.38{\cdot}10^{-6}$	2.58
Mass discrimination correction for $R_{x67/64}$ in the sample	$K_{x67/64}$	1.0517	0.0195			$8.62{\cdot}10^{-7}$	1.62
Measured 67/64 ratio in the sample	$R_{x67/64}$	0.0900	0.0004			$2.32{\cdot}10^{-7}$	0.43
Mass discrimination correction for $R_{x70/64}$ in the sample	$K_{x70/64}$	2.2506	0.1035		$4.85{\cdot}10^{-6}$	$5.02{\cdot}10^{-7}$	0.94
Measured 70/64 ratio in the sample	$R_{x70/64}$	0.0099	0.000049		$4.85{\cdot}10^{-6}$	$2.40{\cdot}10^{-10}$	0.00
Molar weight of the component in the sample	M_{stZn}	65.3778	0.460	g/mol	$7.82{\cdot}10^{-6}$	$3.60{\cdot}10^{-6}$	6.74
Molar weight of the component in the spike	M_{yZn}	67.9000	0.021	gol	$-\ 7.53{\cdot}10^{-6}$	$-\ 1.61{\cdot}10^{-7}$	0.30
Zinc mass fraction in the blank sample	X_{blZn}	$4.11{\cdot}10^{-9}$	$1.81{\cdot}10^{-9}$	%	$-\ 8.65{\cdot}10^{2}$	$-\ 1.57{\cdot}10^{-6}$	2.94
Dilution factor	K	865.4370	0.2873		$1.15{\cdot}10^{-6}$	$3.31{\cdot}10^{-7}$	0.62
Type A standard uncertainly, u_A					$1.80{\cdot}10^{-5}$		
Type B standard uncertainly B, u_B					$33{\cdot}10^{-5}$		
Total standard uncertainty, u_C					$2.24{\cdot}10^{-5}$		

(continued)

Table 7 (continued)

Uncertainty source		Value	u_ε	Unit of measurement	c_ε	$u_\varepsilon \cdot c_\varepsilon$	Contribution (%)
Description	Designation						
Expanded uncertainly (k = 2 P = 0.95), U (%)					$4.47 \cdot 10^{-5}$		
Relative expanded uncertainty (k = 2 P = 0.95), U_o (%)					4.48		

Table 8 Measurement range, accuracy parameters

Research object	Measurand, unit of measurement	Measurement range	Relative total standard uncertainty, $u_{,l,0}$, %	Relative expanded uncertainty at k = 2 and P = 0.95, $U_{L,0}$, %
Lyophilized human and fetal bovine blood serum	Copper mass fraction, %	From $1{\cdot}10^{-6}$ to $5{\cdot}10^{-5}$ inclusive	3.7	7.5
		Above $5{\cdot}10^{-5}$ up to $1.5{\cdot}10^{-3}$ inclusive	3.4	6.9
	Zinc mass fraction, %	From $1{\cdot}10^{-6}$ to $5{\cdot}10^{-5}$ inclusive	4.3	8.7
		Above $5{\cdot}10^{-5}$ up to $1.5{\cdot}10^{-3}$ inclusive	4.4	8.8
Reconstituted humn and fetal bovine blood serum	Copper molar concentration, μmol/dm^3	From 2 to 20 inclusive	4.1	8.3
	Zinc molar concentration, μmol/dm^3	From 2 to 20 inclusive	4.1	8.1

Table 9 Measurement procedures included in the database of the Joint Committee for Traceability in Laboratory Medicine (JCTLM)

Procedure, country	Measurement method	Measurement range	Relative expanded measurement uncertainty, %	Registration in JCTLM
Radiochemical neutron activation analysis method for copper in blood serum (USA)	Radiochemical neutron activation analysis	From 100 ng/g to unlimited with dilution	From 3 to 10%	+
Radiochemical neutron activation analysis method for copper in whole blood (USA)	Radiochemical neutron activation analysis	From 100 ng/g to unlimited with dilution	From 3 to 10%	+
Instrumental neutron activation analysis method for zinc in blood serum (USA)	Radiochemical neutron activation analysis	From 100 ng/g to unlimited with dilution	From 2 to 10%	+

Committee for Amount of Substance (CCQM) as the primary and highly accurate method in comparison with other measurement methods;
- the use of state-of-the-art equipment that implements the method of inductively coupled plasma mass spectrometry with isotope dilution, i.e., a NexION 2000B mass spectrometer with inductively coupled plasma included in GET 176–2019;
- the use of RMs for isotopic composition, as well as IUPAC reference data to account for the output signal drift and to calculate the correction coefficients of isotope ratios associated with the mass discrimination effect;
- the involvement of highly-qualified personnel with large experience in the field of analytical chemistry and metrology, as well as in international key comparisons.

Conclusion

The establishment and control of metrological characteristics of procedures used to determine trace elements in biological materials represents a relevant research task due to wide application of such measurements in medical laboratory diagnostics. The metrological assurance of comparability and traceability of measurement results in clinical medicine is carried out using RMs of biological materials characterized using high-precision measurement techniques.

There is a lack of primary reference methods for measuring copper and zinc in biological materials, as well as RMs for the composition of biological materials with certified characteristics of copper and zinc content. In view of this, the authors aimed to develop and test a procedure for measuring copper and zinc contents based on the ID-ICP-MS method.

The study included determination of optimal sample preparation conditions, evaluation of the effect of matrix elements, selection of optimal mass spectrometry measurement conditions, and accounting for the mass discrimination effect. Uncertainty sources were studied, with the contribution of each to the uncertainty budget being estimated. During characterization of the proposed procedure, the following metrological characteristics were determined: the measurement range of copper and zinc mass fraction from $1 \cdot 10^{-6}$ to $1.5 \cdot 10^{-3}\%$, the measurement range of copper and zinc molar concentration from 2 to 20 μmol/dm^3, the relative expanded uncertainty of copper mass fraction measurements from 7.1 to 7.5%, the relative expanded uncertainty of zinc mass fraction measurements from 8.9 to 9.2%, the relative expanded uncertainty of copper molar concentration measurements 8.8%, the relative expanded uncertainty of zinc molar concentration measurements 8.6%.

The developed measurement procedure was approved as the state primary reference measurement procedure of copper and zinc mass fraction and molar concentration in biological materials (matrices) by the order of the Federal Agency on Technical Regulation and Metrology (Rosstandart) No. 2693 from 26.10.2022 and was further included in the FIF EUM.

The practical significance of the obtained results consists in the possibility of applying the developed procedure for establishing the metrological characteristics of

RMs for the composition of blood, controlling the correctness of measurement results obtained using other measurement techniques of similar quantities, performing high-precision measurements of copper and zinc mass fraction in blood serum in disputable situations.

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Molecular Diagnostics of Oncological Disease: Prospects for the Development of a Reference Material for the *HER2* Gene Content

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Abstract The development of oncological pathologies is closely related to various genomic rearrangements. Studies of DNA isolated from a patient's biological material, as well as identification and determination of the content of nucleotide sequences acting as cancer biomarkers, can be used to determine genetic factors in cancer development, provide early diagnosis, determine a treatment strategy, monitor it, and confirm the patient's recovery. The study aims to develop the main approaches to the design of DNA reference materials (RMs) on the example of an *HER2* RM to provide metrological support for the molecular diagnostics of oncological pathologies. To that end, a procedure was developed for determining *HER2* gene copy number via the digital PCR method. The study demonstrated a repeatability of measurements for the results obtained according to the developed procedure and those obtained using an MLPA commercial kit with human biological material samples. Five permanent tissue cultures were characterized in terms of the copy number ratios of *HER2* to *CEP17* and *RPPH1* sequences. A tissue culture with *HER2* gene amplification was identified. The obtained results will be used to create an RM for the copy number ratio of *HER2* to *RPPH1* and *CEP17*.

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Keywords Reference materials · Cancer diagnostics · Laboratory medicine · Nucleotide sequence · Sequence copy number

Abbreviations

EGFR	Epidermal growth factor receptor
ISH	In situ hybridization
IS WHO	WHO international standards
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MLPA	Multiplex ligation-dependent probe amplification
SI	International System of Units
NGS	Next-generation sequencing
WHO	World Health Organization
DNA	Deoxyribonucleic acid
BIPM	International Bureau of Weights and Measures
NMI	National metrological metrology institute
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
dPCR	Digital polymerase chain reaction
ddPCR	Droplet digital polymerase chain reaction
CCQM-NAWG	Working Group on Nucleic Acid Analysis of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
RNA	Ribonucleic acid
RM	Reference material
CUC	Common Use Center

Introduction

According to the World Health Organization (WHO),[1] cancer is the leading cause of death in the world, responsible for 16% of all deaths [1]. The National Cancer Institute (USA) defines cancer as a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Cancer always associates with genetic alterations, complex changes at different levels of the genetic organization in malignantly transformed cells [2]. Such changes comprise various mutations that disrupt the primary DNA structure, gene copy number variation per genome and gene

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[1] World Health Organization. Available via WHO. https://www.who.int/news-room/fact-sheets/detail/cancer. Accessed 14 October 2022 (In Russ.).

activity alterations. It is true that malignant cell transformation manifests itself in the activation/hyperexpression of cancer-promoting oncogenes and the inactivation/suppressed expression of anti-oncogenes, tumor suppressor genes, whose quantitative parameters can be fully described using bioanalytical measurements.

The specified changes involve nominal properties—nucleotide sequences and quantities, i.e., the copy number of certain nucleotide sequences or the copy number ratio of nucleotide sequences.

A group of gene mutations is linked to inherited cancer predisposition. The risk of neoplasia in carriers of such mutations is significantly increased. For example, breast cancer is detected in approximately 13% of women throughout their lives. In women carrying mutant *BRCA1* or *BRCA2* (*BRCA1/2*) alleles, breast cancer is diagnosed in 55–72 and 45–69% of cases, respectively, by the age of 70–80 [3–5]. In addition, mutant *BRCA1/2* allele carriers have an increased risk of developing prostate (up to 22.9% in men), stomach (up to 3.5% in the population), and pancreatic cancers (up to 3% in the population) [6].

A better understanding of mutations and their role in the etiology and pathogenesis of tumors led to the increased use of molecular genetic studies in diagnosing, preventing, and treating malignant neoplasms [7]. It is essential to identity genetic abnormalities when using new antitumor drugs that target specific molecular mechanisms underlying tumor onset and development. Such genetic abnormalities detected via the nucleotide sequence analysis act as biomarkers that provide a molecular portrait of the tumor, enabling personalized clinical treatment decisions. For example, the detection of certain *EGFR* (epidermal growth factor receptor) mutations in patients with non-small cell lung cancer helps to identify patients indicated for therapy with tyrosine kinase inhibitors [8]; the identification of *KRAS* mutations is required to individualize antibody treatment in colorectal cancer [9].

In molecular diagnostics there are various methods for genetic biomarker analysis, including polymerase chain reaction with end-point detection (PCR), real-time PCR, quantitative PCR (qPCR), digital PCR (dPCR), in situ hybridization (ISH), multiplex ligation-dependent probe amplification (MLPA), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), targeted DNA sequencing (Sanger sequencing), and next-generation sequencing (NGS). Thus, it is crucial to ensure the reliability and comparability of measurement results obtained at different laboratories through different methods of molecular diagnostics at different times.

In laboratory medicine, metrological traceability is key in ensuring the comparability and reliability of measurement results. Noteworthy is that in molecular diagnostics, traceability is primarily achieved by means of reference materials (RMs) of nucleic acids, DNA and RNA, certified for such characteristics as nucleotide sequence, copy number concentration, or copy number ratio.

Presently, a limited number of reference materials are produced worldwide that can be used to transfer units to calibrators included in reagent kits for the molecular diagnostics of genetic tumor markers. For example, two RMs, including one certified reference material, are produced by the National Institute of Standards and Technology (NIST, USA), and five WHO international standards (IS WHO) are produced by the National Institute for Biological Standards and Control (NIBSC, UK). All the specified RMs are used to transfer the units of DNA sequence copy number ratio, i.e., NIST SRM 2373 [10] and RM 8366 [11]; IS WHO NIBSC 09/138, 16/120, 16/250, 18/118, and 18/130. Given that about 300 genetic biomarkers are involved in the diagnostics of cancer, it is imperative to ensure the metrological traceability of DNA sequence copy number measurements.

Noteworthy is that high priority is given to measurement uniformity assurance in the molecular diagnostics of cancer by the Working Group on Nucleic Acid Analysis of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM-NAWG) [12]. For example, the CCQM-P184[2] pilot study examined measurement capabilities for identifying mutations in *BRAF* and *EGFR* gene sequences and determining the ratio between the copy number of sequences carrying a mutation and the total copy number of these gene sequences. The study confirmed that the use of the dPCR method provides reliable, SI-traceable results of determining the content of genetic tumor markers.

Breast cancer is one of the most common malignant pathologies in women worldwide [13]. About 20 to 30% of invasive breast cancers are characterized by an increase in the gene copy number (or gene amplification) of *ERBB2* (*HER2*), i.e., human epidermal growth factor receptor 2. These types of tumors are the most aggressive, requiring special approaches to treatment [14, 15]. The *HER2* gene is a proto-oncogene, whose amplification results in an increased expression level, which, in turn, triggers a signaling pathway promoting cell growth, proliferation and an aggressive tumor metastasis [16]. *HER2* copy number measurements are essential for choosing the right treatment and drugs, as well as predicting the disease outcome.

The crucial role of measuring *HER2* gene copy number was noted by the International Bureau of Weights and Measures (BIPM): in 2021, the CCQM approved for the NAWG to conduct CCQM-K176 key comparisons aimed at confirming the capability of national metrology institutes (NMIs) to measure copy number ratios of *HER2* to *RPPH1* and *CEP17* considered to be single-copy.

This study is relevant since routine diagnostics often uses ISH methods to detect *HER2* gene amplification, which enable the detection of chromosomal rearrangements with high sensitivity and specificity; however, these are essentially subjective expert evaluation methods having uncharacterized accuracy indices. As a reference single-copy gene, test systems applied in laboratory diagnostics often use genes whose copy number per genome may vary [17]. In order to improve the measurement accuracy of gene copy number ratio, routine laboratory diagnostics employs the qPCR method, a relative method that requires the use of calibrators. Thus, it is necessary to ensure the comparability of measurements performed at different laboratories using various methods of molecular diagnostics.

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[2] CCQM-P184 Copy number concentration and fractional abundance of a mutation (SNV: EGFR or INDEL: BRAF) mixed with wild-type DNA. Comparison Coordinator: National Measurement Institute and LGC Standards, UK. Available via BIPM. https://www.bipm.org/documents/20126/61272029/CCQM-P184.pdf/46956277-38bc-0cd8-5ffd-ea4fa67c421b?version=1.1&t=1643100797100. Accessed 14 October 2022 (In Russ.).

RMs intended for traceability assurance should be provided not only as pure DNA preparations, but also as matrix samples. In this case, it is essential to ensure the commutability of the RM matrix and the matrix of human biological material under study, taking into account the preparation specifics, such as fixation, dehydration, etc. The creation of such RMs requires the development of corresponding measurement capabilities, which is prioritized by the CCQM-NAWG. Pilot and key comparisons explored and confirmed the measurement capabilities for identifying mutations and measuring the copy number proportion of corresponding sequences, demonstrating that the dPCR method provides reliable, SI-traceable results of measuring the content of genetic tumor markers.

The study aims to develop the main approaches to the creation of DNA RMs with SI-traceable assigned quantity value (DNA sequence copy number) for the metrological support of molecular cancer diagnostics on the example of the *HER2* gene sequence as a biomarker of breast cancer aggressiveness.

The study objectives are as follows: calculation of primers and probes to amplify the specific sequences of *HER2*, *RPPH1*, and *CEP17*; DNA isolation from biological material; optimization of the droplet digital PCR (ddPCR) performance; measurements of gene copy number concentrations; analysis of the comparability of measurement results obtained using the ddPCR and routine MLPA methods; determination of the *HER2* gene sequence copy number in breast tumor tissue cultures, as well as the feasibility of creating RMs certified for the gene copy number ratios of *HER2* to *RPPH1* and *CEP17*.

Materials and Methods

The study used cell cultures acquired from the Common Use Center (CUC) "Collection of Vertebrate Cell Cultures"[3] of the Institute of Cytology RAS: HeLa S3 cervical cancer tissue culture [18] and five cultures obtained from breast tumors, which could contain an increased genome-wide *HER2* gene copy number. The tissue culture was performed in accordance with the culture certificate and took no more than 4–6 passages. Prior to DNA isolation, the cellular biological material was tested for the presence of mycoplasmas via cytochemical methods as per the Pharmacopoeia of the Russian Federation (OFS.1.7.2.0031.15).[4] The study also used formalin-fixed and paraffin-embedded samples of biological material from 12 patients diagnosed with breast cancer who were treated at the clinic of the Pavlov First Saint Petersburg State Medical University after giving their voluntary informed consent.

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[3] CUC "Collection of Vertebrate Cell Cultures" Federal State Budgetary Institution of Science Institute of Cytology of the Russian Academy of Sciences. Available via Community Center Collection of Vertebrate Cell Cultures. https://incras-ckp.ru/. Accessed 12 October 2022 (In Russ.).

[4] OFS.1.7.2.0031.15 Test for the presence of mycoplasmas. Available via Pharmacopoeia.ru. https://pharmacopoeia.ru/ofs-1-7-2-0031-15-ispytanie-na-prisutstvie-mikoplazm/. Accessed 16 October 2022 (In Russ.).

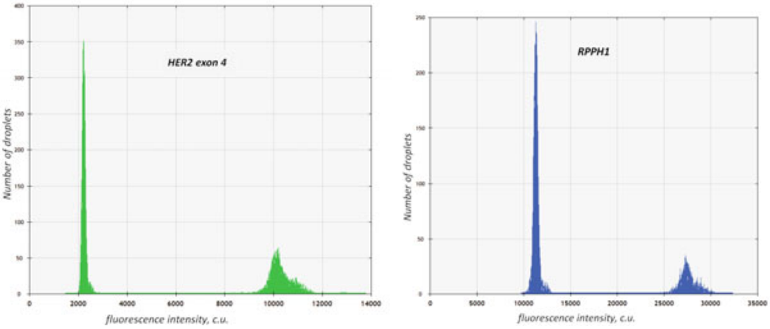
Prior to DNA isolation, all human biological materials were assessed for the malignant tumor volume: tumor tissue comprised at least 70% of the total tissue volume. DNA was isolated from the tissue using the ExtractDNA FFPE kit (Evrogen JSC, Russia) according to the manufacturer's instructions. The *HER2* gene copy number was examined via the MLPA method using SALSA MLPA Probemix P078 Breast tumor (MRC-Holland, Holland) according to the manufacturer's instructions.

For dPCR studies, DNA was isolated from tissue cultures using a commercial DNeasy Blood and Tissue Kit (QIAGEN, Germany) in strict compliance with the manufacturer's instructions. The purity and concentration of the isolated DNA were assessed spectrophotometrically with a BioSpec-nano instrument (Shimadzu, Japan). Following isolation and purification, the DNA was stored at − 70 °C, while avoiding repeated freeze-thawing. The copy number ratios of *HER2* to the gene considered to be a single-copy gene in the human genome was calculated using dPCR measurement results. The measurements were performed with a prototype DNA measuring system that we developed to implement the method of ddPCR; this prototype was calibrated with the use of a human DNA quantitation standard (SRM 2372a, NIST, USA) [19, 20].

Primers and probes for the dPCR amplification were designed using the PrimerQuest© Tool (Integrated DNA Technology, USA). Table 1 presents primer and probe sequences, specifying the size of the amplified sequences (amplicons).

Table 1 Primers and probes for the amplification of *HER2*, *RPPH1*, and *CEP17* gene sequences

Gene/sequence	Nucleotide sequence of primers/probe	Amplicon size (in base pairs)
***HER2*-EXON4** NC_000017.11: 39,694,639–39,695,571	5′-GGTGGCAAAGCAAAGCTATATTC-3′	102
	5′-CGTTTGTCCTGGCCATTCTA-3′	
	5′-HEX-ACATGCAAAGCTACTCCCTGAGCA-BHQ1-3′	
***HER2*-EXON16** NC_000017.11: 39,715,445–39,716,387	5′-CCTCTTGGCATGGCTTCTC-3′	96
	5′-TGTAGGGAGAGGAAGAGTTCTG-3′	
	5′-HEX-AAGGATGCCAAGGCAGGTAGGAC-BHQ1-3′	
***HER2*-EXON24** NC_000017.11: 39,724,733–39,725,827	5′-ATGAGCTACCTGGAGGATGT-3′	103
	5′-CCAGCCCGAAGTCTGTAATTT-3′	
	5′-HEX-TTGGGACTCTTGACCAGCACGTTC-BHQ1-3′	
RPPH1 NC_000014.9: 20,343,050–20,343,764	5′-CGGAGCTTGGAACAGACTCA-3′	97
	5′-GGAGAGTAGTCTGAATTGGGTTATG-3′	
	5′-FAM-CCTCACCTCAGCCATTGAACTCAC-BHQ1-3′	
CEP17 NC_000017.11: 26,981,721–26,983,000	5′-AAAGCCACAGGTAAGAAGTAGG-3′	97
	5′-CTAGATCACGGCAGCAAGAG-3′	
	5′-FAM-CTATTGCAGCACGTGGCACATGG-BHQ1-3′	



a b

Fig. 1 Distribution of ddPCR products by fluorescence intensity for: **a** *HER2* gene exon 4 sequences; **b** *RPPH1* gene sequences

The synthesis of primers and probes was performed by Syntol company (Moscow, Russia). The ddPCR was carried out using a ddPCR mastermix for probes (Bio-Rad Laboratories, USA). The ddPCR reactions were performed under the following conditions: pre-denaturation/enzyme activation at 95 °C for 10 min, followed by 60 cycles (94 °C for 30 s, 57 °C for 60 s) and the final stabilization at 98 °C for 10 min. The ddPCR results were analyzed using the QuantaSoft 1.7.4 software (Bio-Rad Laboratories, USA) in automatic and manual modes.

The amplification conditions were optimized using purified DNA isolated from HeLa S3 cervical cancer tissue culture. This culture is one of the most common and well-studied model objects in cell biology; it was chosen for the presence of a gene set of interest in the genome. The distribution of ddPCR products by fluorescence intensity for *HER2* exon 4 and *RPPH1* genes sequences is shown in Fig. 1 (*HER2* exon 4—Fig. 1a; *RPPH*—Fig. 1b).

Results and Discussion

The developed procedure for measuring the copy number (the number of the gene sequence copies per genome) of the *HER2* gene using the dPCR method was tested as part of the CCQM-K176 key comparisons, the report on which is under preparation. The correct choice of the primer/probe system was confirmed by the agreement between the copy number measurements of sequences belonging to different exons in the *HER2* gene. An analysis of the comparison results confirmed the applicability of the developed procedure for measuring the *HER2* copy number variation using the copy number ratios of *HER2* to *CEP17* and *RPPH1*. The relative standard uncertainty of measurements performed using the developed procedure did not exceed 6%, while the maximum expanded uncertainty amounted to 12% ($k = 2$).

Table 2 Copy number ratios of *HER2* to *RPPH1* and *CEP17* for samples of human biological material obtained via the MLPA and ddPCR methods

		Patient No.											
		1	2	3	4	5	6	7	8	9	10	11	12
Copy number ratio	*HER2/CEP17* (MLPA)	> 14	3	> 14	12	5.7	2.8	4	1.5	1	1	1	1
	*HER2/CEP17** (ddPCR)	18.4	2.99	44.4	13.2	5.3	2.5	4.2	1.7	1.1	1.0	1.1	1.0
	*HER2/RPPH1** (ddPCR)	24.7	2.44	43.6	22.5	5.6	4.3	3.4	1.6	1.1	1.0	2.2	1.0

* Expanded uncertainty for the results obtained using the ddPCR method amounts to 12% ($k = 2$)

This study compares the results of measuring the *HER2* copy number in the biological material of patients following the procedure that we tested as part of international key comparisons and the MLPA-based method used in clinical practice [21]. The measurement results obtained using the ddPCR and MLPA methods are shown in Table 2. Given that the sequence copy number is a counting quantity whose value, according to the *SI Brochure: The International System of Units* (BIPM),[5] is expressed using the unit one, which is a neutral element of any system of units, the ratio of such quantities is expressed as a dimensionless quantity or simply as a number. In all cases, *HER2*-to-*CEP17* copy number ratios obtained using the two techniques are in good agreement (i.e., MLPA values lie within the expanded uncertainty of 12% of the ddPCR values). It was shown that in four of twelve cases, the copy number ratio of *HER2* to *RPPH1* found on another chromosome differs from that to the *CEP17* gene found on the same chromosome 17 (patients 1, 4, 6, and 11). This result confirms the literature data on genomic rearrangements, including on the increased copy number of extensive chromosome regions during a malignant transformation in breast cancer [22]. The demonstrated repeatability of results obtained via the ddPCR and MLPA methods, as well as a wider range of copy number ratios of *HER2* to single-copy *RPPH1* and *CEP17*, show the potential of the ddPCR method in the certification of RMs for the copy number ratios of gene sequences. Such RMs will be designed primarily to transfer the gene copy number ratio unit to calibrators included in medical devices for in vitro molecular genetic diagnostics, as well as to be used in clinical practice to calibrate routine procedures and for intralaboratory quality control.

The development of DNA RMs certified for genetic abnormalities often requires solving the problem of obtaining renewable biological material. A possible solution to this problem lies in the use of in vitro cultured cell lines whose genetic material contains the required mutations. To examine the feasibility of creating an RM for the copy number ratio of the *HER2* gene to the single-copy *RPPH1* and *CEP17* genes, five breast tumor cell tissue cultures were analyzed. Following in vitro tissue

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[5] International System of Units (SI). BIPM. Available VNIIM. https://www.vniim.ru/files/SI-2019.pdf. Accessed 10 October 2022 (In Russ.).

culture growth, genomic DNA was isolated and purified as described above. The copy number ratios of the *HER2* gene to the *RPPH1* and *CEP17* genes were measured using the ddPCR method. The measurement results indicate that the examined tissue cultures differ in the copy number content of *HER2* gene sequences per genome: for three cultures, the copy number of the *HER2* gene sequence remained within the norm, while one culture exhibited a twofold decrease in the *HER2* gene copy number and another culture showed a significant increase in this parameter. In the latter case, the copy number ratios of *HER2* to *RPPH1* and *CEP17* showed a significant difference (12.5 ± 1.5 and 8.5 ± 1.0, respectively), indicating that this cell culture underwent multiplication of the chromosome 14 containing the *RPPH1* gene, as well as the chromosome 17 fragment where the *HER2* gene is localized.

The results we obtained on the example of measuring the *HER2* gene copy number clearly demonstrate the possibility of using established tissue cultures as a source of renewable biological material for the creation of RMs designed for use in the molecular DNA diagnostics of oncological disease.

Conclusion

The study has proposed and tested an approach to the creation of RMs designed to provide metrological support in molecular cancer diagnostics, which relies on the use of established cell cultures. A dPCR-based procedure was developed for measuring the *HER2* gene copy number variation. For the developed procedure and the SALSA MLPA Probemix P078 Breast tumor commercial kit (MRC-Holland, Holland), a study examining 12 samples of biological material from breast cancer patients yielded repeatable results. The use of dPCR produced a wider *HER2* copy number range as compared to MLPA results, which may inform clinical decision-making. Additional measurements of the *HER2*-to-*RPPH1* copy number ratio were shown to reveal cases of genomic rearrangements associated with the increased copy number of the extensive chromosome 17 fragment containing the *HER2* and *CEP17* genes, at which the *HER2*-to-*RPPH1* copy number ratio remains the same.

Five established cell cultures from the CUC "Collection of Vertebrate Cell Cultures" were characterized in terms of the copy number ratios of *HER2* to *CEP17* and *RPPH1*. A cell line with *HER2* gene amplification was identified. The obtained results will be used in the creation of an RM for the *HER2* gene content. The certified values of the copy number ratios of *HER2* to *RPPH1* and *CEP17* will be metrologically traceable to the state primary standard of the unit of DNA sequence copy number (under development). In the new state standard, the storage and transfer of the DNA sequence copy number unit will be realized using transfer standards—DNA reference materials, whose primary structure (nucleotide sequence) will be confirmed via target Sanger sequencing using a NANOFOR 05 genetic analyzer (Syntol, Russia).

The theoretical significance of obtained results lies in the justification of applying human cell cultures derived from cancerous tumors as a renewable source of biological material for the creation of matrix RMs of DNA tumor biomarkers.

The results of measuring the *HER2* gene content in human cell cultures enable a potential unit transfer of the DNA sequence copy number ratio to calibrators included in medical devices for in vitro molecular genetic cancer diagnostics and intralaboratory quality control.

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Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference materials in measurements and technologies" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized and the review procedure was carried out.

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Application of Reference Materials in the Analysis of Medicinal Plant Raw Materials and Herbal Medicinal Products

Tatyana K. Ryazanova and Vladimir A. Kurkin

Abstract In order to improve the standardization of medicinal plant raw materials and herbal medicinal products, new approaches to the analysis of plant objects are required. We substantiate the use of reference materials (RMs) in the development of assay procedures using a number of plant raw materials as an example. Biologically active compounds contained in these materials are studied in terms of their chemical composition, stability, and physicochemical properties. Active substances were determined by HPLC-UV. The results were processed using WinASPECT and Microsoft Excel 2016. Assay procedures were developed and validated for determining syringin in *Syringa vulgaris* bark and *Eleutherococcus senticosus* rhizomes and roots; rosavin and salidroside in *Rhodiola rosea* rhizomes and roots; arbutin in *Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea* leaves; isosalipurposide in *Helichrysum arenarium* flowers; anthracene derivatives in *Aloe arborescens* leaves. Spectrophotometric methods for determining the amount of biologically active phenylpropanoids in terms of eleutheroside B (syringin) in medicinal plant raw materials and herbal medicinal products of *Eleutherococcus senticosus* and the amount of aralosides in *Aralia mandschurica* roots were proposed. The feasibility of using the RMs of syringin, rosavin and salidroside, araloside ammonium salts, arbutin, aloins A and B mixtures in assay procedures was substantiated.

Keywords Medicinal plant raw materials · Herbal medicinal products · Biologically active compounds · Reference materials · Standardization · High performance liquid chromatography · Spectrophotometry

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Abbreviations

BACs	Biologically active compounds
HPLC	High performance liquid chromatography
MPRM	Medicinal plant raw material
HMP	Herbal medicinal product
RM	Reference material

Introduction

Herbal medicinal products (HMPs) occupy a large share of the pharmaceutical market. The development of novel HMPs is associated with the problem of identification of medicinal plant raw materials (MPRMs), which task requires a combination of physical, physical–chemical, chemical and biological analytical methods [1]. Among the related issues that should be solved are the determination of sample preparation conditions and selection of analyzed substances [individual compound or a group of biologically active compounds (BAC)], analytical methods, and reference materials (RMs) for calculating the BAC content [2–7]. A comparison of the methods used for analyzing MPRMs and HMPs reveals the problem of non-absolute application of the principle of end-to-end standardization in the "MPRM—substance—HMP" series [5–7].

The need to revise the existing pharmacopoeial requirements can be illustrated by the lack of effective methods for the analysis of MPRM containing various BAC, such as simple phenols (*Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea* leaves), phenylpropanoids (*Eleutherococcus senticosus* and *Rhodiola rosea* rhizomes and roots, *Syringa vulgaris* bark), flavonoids (*Helichrysum arenarium* flowers), saponins (*Aralia mandschurica* roots), anthracene derivatives (*Aloe arborescens* leaves) [8, 9]. Thus, according to the State Pharmacopoeia of the Russian Federation 14th edition, arbutin in *Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea* leaves should be determined by a spectrophotometric technique involving a preliminary purification of the water–ethanol extract by filtering through an alumina layer [10]. However, according to the European and American herbal pharmacopoeias, arbutin should be determined by high-performance liquid chromatography (HPLC) [11–13]. HPLC techniques involve the use of methanol, whose handling requires specific conditions. In addition, arbutin can be measured using the methods of gas chromatography–mass spectrometry and photocolorimetry. The latter is based on the reaction of azo coupling and azo compound [13–16].

The pharmacopoeial technique for quantitative determination of BAC in *Aralia manchurica* is a multistage and labor-intensive process. This technique involves methanol extraction for 7 h in a Soxhlet extractor combined with an acidic hydrolysis stage, sapogenin precipitation (oleanolic acid) by adding an equal volume of water to the methanol extract, sapogenin precipitate purification, dissolution in a mixture of methanol and isobutyl alcohol followed by potentiometric titration with 0.1 N sodium hydroxide solution in a mixture of methanol and benzene [10]. Ultraviolet spectrophotometry can also be used for determining the content of saponins [17].

According to the State Pharmacopoeia of the Russian Federation 14th edition, standardization of raw materials and medicinal preparations of *Rhodiola rosea* (FS.2.5.0036.15 "*Rhodiolae roseae* L. rhizomes and roots" and FS.3.4.0008.18 "*Rhodiolae roseae* L. rhizomes and roots extract liquid") assumes a quantitative determination of salidroside and the total amount of cinnamic alcohol glycosides expressed in terms of rosavin [10]. The analysis is carried out by the HPLC-UV method (salidroside determined at 219 nm; the total amount of cinnamic alcohol glycosides expressed as rosavin determined at 250 nm). However, the feasibility of determining the total amount of cinnamyl alcohol glycosides expressed in terms of rosavin remains to be controversial. Rosavin is known to be a highly unstable compound. In comparison with other cinnamyl alcohol glycosides, rosavin is more sensitive to the conditions of harvesting and storage of raw materials, largely due to the possibility of enzymatic destruction under the action of vicanosidase. Moreover, the 219 nm wavelength used in the above pharmacopoeial technique for salidroside detection is less specific with respect to related components compared to another absorption maximum of this compound of 278 nm (Fig. 1) [18, 19].

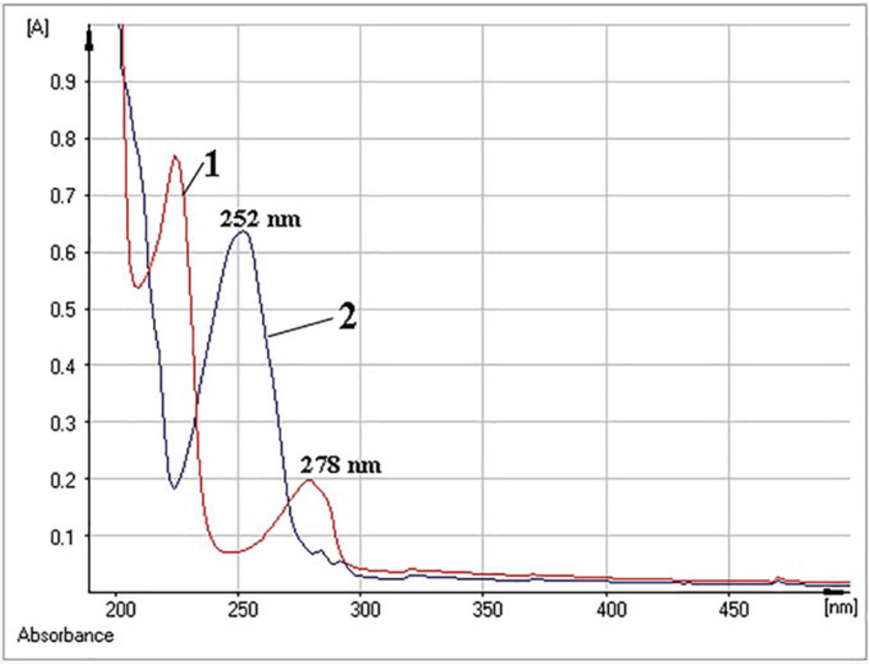

Fig. 1 UV absorption spectra of salidroside (1) and rosavin (2) (solvent—95% ethanol)

Various approaches are used for standardization of *Eleutherococcus senticosus* raw materials and medicinal preparations. Thus, the European Pharmacopoeia 10th edition suggests a qualitative assay of *Eleutherococcus* raw materials by thin-layer chromatography (TLC) using aesculin and catalpol RMs, a quantitative determination of the amount of B and E eleutherosides using ferulic acid as a standard by HPLC in the gradient elution mode with detection at 220 nm. However, all the three mentioned standards are not contained in the raw material [11]. In Russia, the "*Eleutherococcus senticosus* rhizomes and roots" pharmacopoeial article suggests a qualitative analysis of the main BAC groups by TLC using an eleutheroside B RM (synonym: syringin), qualitative reactions, and HPLC [10]. Identification of *Eleutherococcus* in a liquid extract from *Eleutherococcus* rhizomes and roots is carried out by HPLC, HPLC-UV, and qualitative reactions [10].[1] The content of eleutheroside B and the total amount of eleutherosides are determined by HPLC and the spectrophotometric method, respectively [10]. At the same time, eleutherosides represent different BAC classes, which raises the question about the feasibility of determining their total amount. Moreover, an optimal extractant for extracting eleutheroside B (syringin) and the total amount of biologically active eleutherosides is yet to be found. This is related to the fact that the pharmacopoeial method uses 70% ethanol, while 40% ethanol is used for obtaining a liquid extract [10]. Therefore, the existing regulatory approaches and requirements for the quality of *Eleutherococcus senticosus* rhizomes and roots, adopted in the State Pharmacopoeia of the Russian Federation 14th edition, require revision.

It also seems timely to develop a unified method for determining eleutheroside B in *Syringa vulgaris* bark. This material was suggested as a source of pharmacopoeial syringin RM by VFS 42 2088-92 "Syringin—reference material." Syringin in *Syringa vulgaris* bark is identified using spectrophotometric, chromatic and spectrophotometric, and HPLC methods [20]. Despite the biological activity and significance of this raw material for obtaining a pharmacopoeial RM of syringin, *Syringa vulgaris* bark is not included in the State Pharmacopoeia of the Russian Federation 14th edition [10]. The quality of *Syringa vulgaris* raw materials is assessed in accordance with VFS 42-2106-92 "*Syringa vulgaris* bark," which implies a chromatography mass–spectrophotometry determination of syringin.

According to FS.2.5.0007.1 "*Helichrysum arenarium* flowers,"[2] standardization of this MPRM is carried out by the total amount of flavonoids expressed in terms of isosalipurposide [10]. Since *Helichrysum arenarium* flowers serve as a source for obtaining an isosalipurposide RM, a methodology for quantitative determination of isosalipurposide in *Helichrysum arenarium* by HPLC is required [21–23].

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[1] FS.3.4.0009.18 Eleutherococcus prickly rhizomes and roots liquid extract. Available via Pharmacopoeia RF. https://pharmacopoeia.ru/fs-2-5-0053-15-eleuterokokka-kolyuchego-kornevishha-i-korni/. Accessed 26 August 2022 (In Russ.).

[2] FS.2.5.0007.1 Sand immortelle flowers. Available via Pharmacopoeia RF. https://pharmacopoeia.ru/fs-2-5-0007-15-bessmertnika-peschanogo-tsvetki/. Accessed 26 August 2022 (In Russ.).

Standardization of aloe species in the British, Japanese, European, and US Pharmacopoeias is performed according to the content of barbaloin (aloin A) by the spectrophotometric method [11, 12, 24–27]. In Russia, the draft versions of the "*Aloe arborescens* Mill. fresh leaves" and "*Aloe arborescens* Mill. leaves" pharmacopoeial articles (instead of FS 42 2191 84 and FS 42-2800-91, respectively) presented at the website of the Ministry of Health of the Russian Federation, the quantitative determination is also to be performed by the spectrophotometric method with the expression of the total amount of anthracene derivatives in terms of aloe emodin. All the methods described in literature involve many stages and imply a preliminary acidic hydrolysis combined with oxidation, a liquid–liquid extraction of the resulting aglycones, and subsequent complexation with magnesium acetate [24–28].

In the view of the abovementioned, we aim to substantiate both theoretically and experimentally the application of RMs when developing assay procedures. To this end, we use a number of plant raw materials and medicinal preparations on their basis as an example and determine the chemical composition, stability, and physicochemical properties of BAC contained in these products.

Materials and Methods

In this research, we used the following MPRM and HMP samples: commercial samples of *Aralia manchurica* roots (Ecogreen LLC, 2014); commercial samples of Aralia tincture (JSC Dalhimpharm and JSC Tverskaya Pharmaceutical Factory); *Vaccinium vitis-idaea* leaves harvested in the Republic of Mari El, Volzhsky District, Volzhsk, 2018; *Arctostaphylos uva-ursi* leaves harvested in the Republic of Mari El, Volzhskiy district, Volzhsk, 2018; *Rhodiolae roseae* rhizomes and roots harvested in Altai Krai in 2016–2018; experimental and commercial samples of liquid *Rhodiolae roseae* rhizome and root extract (extractant—40% ethanol); *Syringa vulgaris* bark harvested in 2018–2020 in the Botanical Garden of Samara University, in the Samara (Verkhny Suskan village, Ermakovo village) and Saratov (Natalino village) regions; *Eleutherococcus senticosus* rhizomes and roots harvested in Khabarovsk Krai in 2015–2018; *Aloe arborescens* Mill. fresh leaves cultivated at the Department of Pharmacognosy with Botany and Phytotherapy Basics and collected in the summer and autumn period of 2020; juice obtained *ex tempore* from *Aloe arborescens* Mill. fresh leaves; medicinal preparations "Aloe vera juice" (JSC Vifitech), "Aloe extract liquid," a solution for subcutaneous administration, by two different manufacturers (JSC Vifitech and JSC Dalkhimpharm).

Instrumental methods of analysis rely on the use of RMs. In Russia, however, there are certain difficulties concerning the provision of RMs for medicinal products (MPs), including the lack of national pharmacopoeial certified RMs and their long delivery time, as well as the high cost of foreign pharmacopoeial RMs (European, British Pharmacopoeia, US Pharmacopoeia) [1–3]. In this connection, in cases where RMs were needed, we obtained substances (a mixture of araloside ammonium salts, barbaloin) or individual compounds (arbutin, syringin, rosavin, salidroside, and isosalipurposide). All the obtained materials met the requirements for RMs according to the data of qualitative and quantitative assays, mass spectrometry, ^{1}H and ^{13}C–NMR spectroscopy [10, 11]. Individual compounds were isolated using the liquid column chromatography of laboratory water–ethanol extracts prepared from plant raw materials (extractant 70% ethanol at the ratio of raw material to extractant 1:5) using a silica gel sorbent L 40/100 μm (Czech Republic) [29–37]. Elution was performed using solvent mixtures of chloroform–ethyl alcohol 95% in different ratios (100:0 → 0:100). The chromatographic separation was controlled by TLC on Sorbfil PTC-AF-A-UF plates in the system "chloroform:95% ethanol:water 26:16:3 (vol%)" with UV-detection of fluorescence quenching areas (at 254 nm) and fluorescing areas (at 366 nm) using a chromatographic irradiator (UV complex UFK-254/365, Petrolaser Ltd.). In addition, the areas stained by treating the plates with a solution of diazobenzene sulfonic acid in a saturated solution of sodium carbonate were identified under natural light. For subsequent purification, chromatographic separation was repeated using other sorbents (polyamide, Sephadex LH-20); crystallization conditions were selected. The yield of the target substances was at least 60.0% of their content in the raw material calculated by quantitative assays. ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker AM 300 (300 MHz) and Bruker DRX 500 (126.76 MHz) NMR spectrometers. Electron-impact mass spectra were recorded using a Kratos MS-30 mass spectrometer at an ionizing electron energy of 70 eV and an ion source temperature of 250 °C.

In general cases, sample preparation for the quantitative determination of BAC was performed as follows: about 1 g of raw materials crushed to a particle size to pass through a 2-mm sieve (precisely weighted amount) was placed in a 100 cm^3 ground joint flask followed by addition of 30–50 cm^3 of water–ethanol mixture (the amount of solvent and the composition of mixture depended on the analyzed MPRM and BAC). The flask was capped and weighed with an accuracy of ± 0.01 g, and then attached to a backflow condenser and heated in a boiling water bath (moderate boiling) for 60 min. Subsequently, the flask was cooled for 30 min, closed with the same stopper, and weighed again. The missing extractant was replenished to the original mass. The extract was filtered through a paper filter (red stripe). For chromatographic studies, the solution was additionally filtered through an MF-Millipore membrane filter (0.45 μm).

RM solutions were prepared by solving 0.025–0.030 g (precisely weighted amount) of the particular substances in a suitable solvent in a 50-ml volumetric flask and bringing the solution volume to the mark with the same solvent.

For each object under study, the conditions of sample preparation for subsequent analysis were determined. To that end, the extraction ability of different extractants (purified water, 40, 70, and 95% ethanol) was compared, the effect of extraction time (30, 60, or 90 min) and the ratio of "raw material:extractant" (1:30; 1:50, and 1:100) on the yield of active substances was studied [29–37].

UV spectra were recorded using a Specord 40 (Analytik Jena) spectrophotometer. HPLC was performed using a Milichrom-6 (JSC Nauchpribor) chromatograph under the following conditions of reverse-phase chromatography under isocratic elution:

KAH-6-80-4 steel column (2 mm × 80 mm; Separon-C18), mobile phase—acetonitrile, 1% acetic acid solution in water in different ratios, elution rate—100 μl/min, eluent volume—1500–2500 μl. The optimum ratio of the mobile phase components was determined by peak resolution, the peak asymmetry coefficient. The BAC content was calculated using the method of external standard, with the latter being the individual compounds isolated from plant raw materials and identified with the degree of purity established earlier by the material-balance method. The suitability of the chromatographic system was evaluated in accordance with OFS.1.2.1.2.0001.15 "Chromatography."[3] Statistical processing of the results of quantitative assays was carried out in accordance with OFS 1.1.1.0013.15 "Statistical processing of the results of chemical experiments."[4] The methods were validated according to OFS.1.1.0012.15 "Validation of analytical methods."[5] The correctness of the methods applied was determined by adding a water–ethanol solution, the earlier isolated individual compound under study with the established degree of purity (RM candidate) to the test solution (80, 100, 120% in relation to the compound content in the extract).

In addition, experimental syrup samples of *Eleutherococcus senticosus*, *Arctostaphylos uva-ursi*, and *Vaccinium vitis-idaea* were obtained. Considering the recommended dosage of the registered preparation, an *Eleutherococcus senticosus* syrup was obtained from a liquid extract with a content of 5% of the total weight of the syrup. Syrups from *Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea* leaves were prepared according to a standard syrup technology using a decoction of herbal raw materials instead of purified water. Sucrose or sorbitol were used as corrigents. Prior to estimating the content of active components, sample preparation was carried out by diluting an aliquot of tested samples with tenfold of 95% ethanol. The as-obtained solutions were kept for some time until precipitation and filtered through an alumina layer (reagent grade, neutral, Brockmann grade II) with a height of 0.5 cm in a POR 100 glass filter 2 cm in diameter into a 25-cm^3 volumetric flask. BAC quantification in experimental and commercial preparations was performed using the approaches described for the respective MPRM.

Results and Discussion

Using the methodology described above and HPLC, we developed and validated assay procedures for quantitative determination of arbutin in *Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea* leaves, syringin in the MPRMs and HMPs of *Syringa*

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[3] OFS.1.2.1.2.0001.15 Chromatography. Available via Pharmacopoeia RF. https://pharmacopoeia.ru/ofs-1-2-1-2-0001-15-hromatografiya/. Accessed 26 August 2022 (In Russ.).

[4] OFS.1.1.0013.15 Statistical processing of the results of chemical experiments. Available via Pharmacopoeia RF. https://pharmacopoeia.ru/wp-content/uploads/2016/11/OFS.1.1.0013.15-Statisticheskaya-obrabotka-rezultatov-eksperimenta.pdf. Accessed 26 August 2022 (In Russ.).

[5] OFS.1.1.0012.15 Validation of analytical methods. Available via Pharmacopoeia RF. https://pharmacopoeia.ru/ofs-1-1-0012-15-validatsiya-analiticheskih-metodik/. Accessed 26 August 2022 (In Russ.).

vulgaris bark and *Eleutherococcus senticosus* rhizomes and roots, rosavin and salidroside in the MPRMs and HMPs of *Rhodiola rosea*, arbutin in the MPRMs and HMPs of *Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea*, isosalipurposide in the MPRMs and HMPs of *Helichrysum arenarium.* Table 1 presents the optimal conditions for sample preparation and analysis determined by the highest concentration of extractable BAC. In all cases, the optimal extraction time from the studied range (30, 60, and 90 min) comprised 60 min.

For all the assay procedures, the linearity of the peak height dependence on the analyte concentration in the range specified in Table 2 was confirmed. Satisfactory metrological characteristics in terms of accuracy, repeatability, and intermediate precision were obtained.

Table 3 presents the quantitative content of BAC in the studied MPRMs obtained by the developed assay procedures, according to the principle of end-to-end standardization of analytical approaches in the "MPRM—phytosubstance—HMP" series.

For those cases where it was difficult to identify the individual component that makes the greatest contribution to the biological activity of a particular HPRM and HMP and/or that is most susceptible to degradation processes in the process of harvesting raw materials and their storage, procedures for identifying the total amount of BAC by the spectrophotometric method were developed. Thus, we previously proposed approaches to estimate the quantitative content of the total amount of saponins (aralosides) in the raw materials and preparations of *Aralia mandschurica* using the total amount of araloside ammonium salts as an RM (referred to as "Saparal"). For that purpose, we modified the technological scheme of Saparal manufacturing [17]. The Saparal samples obtained in the course of our studies represented an amorphous powder of a cream color without odor with the total amount of aralosides exceeding 80% (85.06 ± 1.12%) expressed in terms of the ammonium salt of aralosides A, B, and C with the weigh-average molecular weight. The yield of the finished product ranged between 62.9 and 65.0%.

We developed a procedure for quantitative determination of the total amount of *Aralia* saponins (aralosides) in raw materials and preparations by spectrophotometry of the reaction products of the analytes with concentrated sulfuric acid at 510 nm. The quantitative content was calculated using the data obtained for Saparal or (in cases where a Saparal RM was absent) using the value of the specific absorption index for Saparal upon its interaction with concentrated sulfuric acid considering the total amount of the contained aralosides of 56.0. The optimal extraction conditions were determined to be as follows: 70% ethanol; the "raw material—extractant" ratio of 1:50; extraction time 60 min. This procedure was applied to analyze the total amount of saponins in the samples of *Aralia mandschurica* roots (ranged from 9.41 ± 0.18 to 10.46 ± 0.15%) as well as in those of commercially available tinctures of *Aralia mandschurica* (ranged from 1.51 ± 0.05 to 1.72 ± 0.06%).

For raw materials and preparations of *Eleutherococcus senticosus* rhizomes and roots, we propose to replace the pharmacopoeial method for determining the total amount of eleutherosides with a procedure for determining the total amount of biologically active phenylpropanoids. This method consists in obtaining a water-alcohol

Table 1 General description of HPLC methods for the analysis of biologically active compounds in the studied medicinal plant raw materials

MPRM	Extractant	Ratio "raw material—extractant" (weight/volume)	Need for purification (filtering through an alumina layer)	Mobile phase—acetonitrile—1% acetic acid solution	Detection	Analyte	Retention time, min
Arctostaphylos uva-ursi	40% ethanol	1:50	Required	1:9	280 nm	Arbutin	4.87 ± 0.03*
Vaccinium vitis-idaea	Purified water	1:50	Required	1:9	280 nm	Arbutin	4.87 ± 0.03*
Syringa vulgaris bark	70% ethanol	1:30	Not required	15:85	266 nm	Syringin (eleutheroside B)	2.82 ± 0.08
Eleutherococcus senticosus rhizomes and roots	40% ethanol	1:50	Required	15:85	266 nm	Syringin (eleutheroside B)	2.84 ± 0.08
Rhodiolae roseae rhizomes and roots	70% ethanol	1:30	Not required	14:86	252 nm	Rosavin	12.82 ± 0.07
					278 nm	Salidroside	2.98 ± 0.07
Helichrysum arenarium flowers	70% ethanol	1:50	Not required	25:75	360 nm	Isosalipurposide	12.84 ± 0.08

Table 2 Metrological characteristics of HPLC assay procedures for biologically active compounds in the investigated medicinal plant raw materials

MPRM (BAC)	$\overline{X}$, %	$\overline{\varepsilon}$, % ($P = 95\%$, $n = 10$)	Concentration range for linearity determination (r^2)	Accuracy, %
Arctostaphylos uva-ursi (arbutin)	11.2	3.1	0.076–0.49 mg/mL (0.9994)	99.5
Vaccinium vitis-idaea (arbutin)	4.8	2.1	0.076–0.49 mg/mL (0.9994)	99.5
Syringa vulgaris bark (syringin)	5.4	3.2	0.34–0.51 mg/mL (0.9997)	98.8
Eleutherococcus senticosus rhizomes and roots (syringin)	0.089	3.3	0.34–0.51 mg/mL (0.9997)	98.3
Rhodiolae roseae rhizomes and roots (rosavin)	1.4	4.4	0.12–0.96 mg/mL (0.9973)	100.4
Rhodiolae roseae rhizomes and roots (salidroside)	2.9	4.7	0.15–1.47 mg/mL (0.99996)	99.1
Helichrysum arenarium flowers (isosalipurposide)	1.45	4.1	0.20–1.40 mg/mL (0.9964)	97.8

Designations $\overline{X}$—mean value; P—confidence interval, $\overline{\varepsilon}$—relative error of the mean value, r^2—determination coefficient

extract from the rhizomes and roots of *Eleutherococcus senticosus*, its purification by filtering through an alumina layer followed by spectrophotometric determination of the total amount of phenylpropanoids at 266 nm expressed in terms of eleutheroside B (syringin). The conducted experiments into the extraction capacity of different water–ethanol mixtures revealed the most optimal extractant with respect to phenylpropanoids to be 40% ethanol at a "raw material:extractant" ratio of 1:50 and extraction time of 60 min. The metrological characteristics of the spectrophotometric method for determining the total amount of phenylpropanoids expressed in terms of eleutheroside B (filtered through an alumina layer) include the ±4.20 and ± 6.24% relative error of the mean value of active substance quantitative determination in *Eleutherococcus senticosus* rhizomes and roots and *Eleutherococcus senticosus* liquied extract (confidence interval of 95%). The total amount of phenylpropanoids in *Eleutherococcus senticosus* rhizomes and roots ranged from 0.30 ± 0.02 to 0.37 ± 0.02%.

We also developed and validated an assay procedure for quantitative determination of the total amount of anthracene derivatives by differential spectrophotometry (absorption maximum at 412 nm), which is expressed in terms of barbaloin contained in the MPRMs and HMPs of *Aloe arborescens* Mill. During the course of the study, the following significant parameters were determined: extractant composition (40% ethanol), "raw material—extractant" ratio (1:50), and extraction time

Table 3 Content of active substances in the experimental and commercial HMPs based on the studied MPRMs

HMP	HMP type	Need for purification (filtering through an alumina layer)	Analyte	Active substance content, %
Arctostaphylos uva-ursi syrup	Experimental	Required	Arbutin	0.86 ± 0.02
Vaccinium vitis-idaea syrup	Experimental	Required	Arbutin	0.70 ± 0.02
Syringa vulgaris tincture	Experimental	Not required	Syringin (eleutheroside B)	0.45 ± 0.03
Eleutherococcus liquid extract	Commercial	Required	Syringin (eleutheroside B)	From 0.065 ± 0.003 to 0.089 ± 0.004
Eleutherococcus syrup	Experimental	Required	Syringin (eleutheroside B)	From 0.0026 ± 0.0003 to 0.0029 ± 0.0003
Rhodiolae roseae liquid extract	Commercial	Not required	Rosavin	From 0.21 ± 0.03 to 0.32 ± 0.04
			Salidroside	From 0.96 ± 0.04 to 2.75 ± 0.08
Helichrysum arenarium flowers	Commercial	Not required	Isosalipurposide	From 1.22 ± 0.03 to 1.42 ± 0.03

(60 min). The linearity of the dependence of optical density on the analyte concentration was confirmed; satisfactory metrological characteristics in terms of accuracy, repeatability, and intermediate precision were obtained. The relative error of the mean value when determining the total amount of anthracene derivatives in *Aloe arborescens* Mill. fresh leaves comprised ± 3.36% (confidence interval of 95%). In accordance with the principles of unification and harmonization of analytical approaches for MPRMs and respective HMPs, we modified the procedure developed for the analysis of raw materials to that of ex tempore *Aloe arborescens* juice and medicinal products “Aloe vera juice” (JSC Vifitech), “Aloe extract liquid,” a solution for subcutaneous administration (JSC Dalkhimpharm and JSC Vifitech). The content of the total amount of anthracene derivatives expressed as barbaloin comprised 0.50 ± 0.02% in the freshly prepared *Aloe arborescens* juice, 0.135 ± 0.006% in “Aloe vera juice,” and 0.020 ± 0.001% in “Aloe extract liquid” preparations.

On the basis of the conducted research, we formulated a concept for a systematic approach to the analysis of medicinal plant raw materials and herbal medicinal products containing BAC of aromatic and terpenoid nature (Fig. 2).

The main provisions of this concept can be formulated as follows:

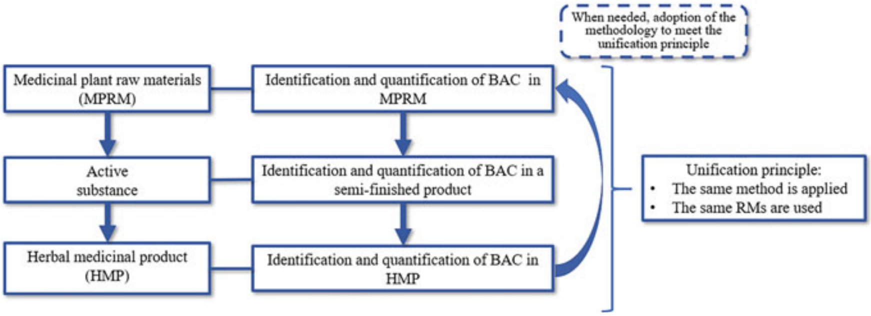

Fig. 2 Principles of the proposed systematic approach to the analysis of medicinal plant raw materials and herbal medicinal products

- when selecting an assay procedure for the quantitative determination of active substances, account should be taken of the intended use of the MPRM (RM source, BAC content, extractant type, etc.), the pharmacotherapeutic significance of the components, technological features (the possibility of isolating BAC from MPRM), and BAC stability (whether labile BACs are present);
- when selecting RMs for pharmacopoeial analytical methods, a diagnostically significant substance present in the studied plant or a substance of the same BAC class with similar physical and chemical characteristics should be used;
- the principle of unifying approaches to the identification and quantitative determination of active substances in MPRMs, pharmaceutical substances on their basis, and respective HMPs.

Conclusion

The conducted research allowed us to scientifically substantiate the use of the following RMs for qualitative and quantitative analysis: syringin (*Syringa vulgaris* bark and *Eleutherococcus senticosus* rhizomes and roots), rosavin and salidroside (*Rhodiolae roseae* rhizomes and roots), the total amount of araloside ammonium salts (*Aralia mandschurica* roots), arbutin (*Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea* leaves), a mixture of aloins A and B (*Aloe arborescens* fresh leaves).

We developed and validated assay procedures for quantitative determination of syringin in the MPRM and HMP of *Syringa vulgaris* and *Eleutherococcus senticosus*, the total amount of biologically active phenylpropanoids in the MPRM and HMP of *Eleutherococcus senticosus*, rosavin and salidroside in the MPRM and HMP of *Rhodiolae roseae*, arbutin in the MPRM and HMP of *Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea*, isosalipurposide the MPRM and HMP of *Helichrysum arenarium*, the total amount of anthracene derivatives expressed as barbaloin the MPRM and HMP of *Aloe arborescens*, the total amount of saponins (aralosides) in *Aralia mandschurica* roots.

With the purpose of unifying quality requirements and monitoring the influence of the technological process on the extraction efficiency and stability of BAC, the assay procedures developed for identifying active substances in MPRMs should also be used for analyzing both the pharmaceutical substances obtained on their basis and respective HMPs.

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Contribution of the Authors Ryazanova T.K.—conducting research, development of a methodology, formal analysis, validation, writing a draft of the article; Kurkin V. A.—development of the research concept, interpretation of ^{1}H-NMR, ^{13}C-NMR spectra, mass spectra and UV spectra of isolated BACs, revision of the text.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Reference Materials in the Field of Quality of Life

Determination of Sunflower Seed Oil Content Using Natural Sunflower Oil: Calibration of a Pulsed NMR Analyzer

Oleg S. Agafonov and Sergey M. Prudnikov

Abstract The present study substantiates the application of natural sunflower oil for the calibration of pulsed NMR analyzers, as well as for the identification and quality assessment of oilseeds and their products using the NMR method. Being the essential characteristic of sunflower seeds, the oil content is mandatory for the certification of agricultural products and their cost assessment. The first calibration option includes the measurement of oil NMR characteristics with the oil content determined using the Soxhlet exhaustive extraction. The second option involves the calibration of a NMR analyzer using sunflower oil, obtained by pressing identical cultivars and hybrids, as well as that acquired at a retail network. A comparison of the obtained calibration dependencies showed their similar nature. The application of sunflower oil for the calibration of NMR analyzers provides no increase in the oil content measurement error as compared to the first calibration option. Therefore, using natural oil for the calibration of NMR analyzers can considerably simplify the calibration process, reduce the calibration duration from 3–4 days to 3–4 h, as well as to exclude toxic solvents and additional high-value equipment from the process without essential variations in the oil content measurement error.

Keywords NMR analyzer · Calibration · Oil content · Moisture content · Reference materials · Sunflower seeds · Sunflower oil

Abbreviations

RM	Reference material
TAG	Triacylglycerol
NMR spectroscopy	Nuclear magnetic resonance spectroscopy

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Introduction

In accordance with GOST 22391-2015 [1], the oil-and-fat industry identifies the seed oil content as the main quality indicator of raw materials during their gathering and processing. The oil content comprises the content of raw fat and accompanying lipoid substances, which, together with fat, pass into the ether extract from the seeds. In accordance with GOST 10857-64 [2], the determination of this indicator is based on the method of Soxhlet exhaustive extraction. A drawback of this method consists in its duration, application of toxic chemical solvents, low productivity, and high requirements to the personnel qualification.

At present, due to the wide development of technical equipment and information technologies, instrumental methods, such as IR spectroscopy and pulsed NMR methods, which can be used to obtain information about the seed oil content, are quite common. The determination of oil content using the method of IR spectroscopy is typically performed in accordance with GOST 32749-2014 [3]. In the case of sunflower seeds, this method is unpopular due to significant errors (up to 2 abs%) and the dependence of measurement results on the seed appearance and their fineness degree. In addition, this method requires the calibration of IR analyzers using a large number of seeds with a certain value of determined indicators obtained using referee methods [4–8].

In the contemporary Russian oil-and-fat industry, the most widespread instrumental method for determining the oil content of seeds and products of their processing involves pulsed nuclear magnetic resonance (NMR). This is explained by its analytical simplicity, lack of a complex sample preparation, high accuracy (error of not more than 0.6 abs%, which is comparable in accuracy with a referee chemical method), promptness (the analysis of one sample takes < 30 s), lack of the effect caused by the operator's subjectiveness on the analytical results, as well as ease of implementation. Currently, AMV-1006M (V.S. Pustovoit All-Russian Research Institute of Oil Crops, VNIIMK, Russia) is the most common NMR analyzer used at Russian enterprises. The determination of oil content using this instrument is based on the substantiated scientific and methodological approach with the rank of 0.04 [9].

The calibration of operating NMR analyzers for the determination of oil content in sunflower seeds is carried out using GSO 3107-84[1] state reference materials [10, 11], which were used for the verification of AMV-1006M NMR analyzers until the expiration of their Type Approval Certificate in 2016.

These reference materials (RM) are made on the basis of chemically inert substances-imitators, thermally resistant, resistant to thermal oxidation and ultraviolet effects, as well as having the dielectric properties (of organosilicon liquids) [12–14] of oil NM relaxation characteristics.

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[1] GSO 3107-84 Reference materials of the approved type of oil content and moisture content of sunflower seeds (set). Available via FIF EUM. www.fgis.gost.ru/fundmetrology/registry/19/items/393823. Accessed 12 September 2022 (In Russ.).

Imitated values of indicated GSO 3107-84 were assigned using an NMR analyzer, whose calibration and testing were performed using sunflower seeds with the oil content determined by the Soxhlet method of exhaustive extraction. The drawbacks of this calibration method include its duration, high requirements to the operator's qualification, as well as the application of specially prepared traceable seeds. It should be noted that even specially prepared seeds have a scattering in the measured oil content values in individual samples, isolated from one specimen using the method of exhaustive extraction to 1 abs%. This is explained by the fact that seeds represent a natural heterogeneous object [15, 16].

Let us note that one of the key features of the NMR method comprises a functional dependence between the oil content in the analyzed seed sample and the amplitude of the NMR signal obtained from the protons of triacylglycerins (TAG) contained in the oil.

At present, the NMR method is widely used for determining the oil content of seeds and oil-containing raw materials [17–24],[2] which is explained by its application simplicity, high metrological characteristics, sample preparation simplicity, ecological safety, as well as the non-destructive nature of analysis. More than 350 enterprises of the Russian oil-and-fat industry currently use AMV-1006M NMR analyzers to quickly obtain information on the oil content at all stages of gathering, storage, and processing of oil seeds.

At the same time, the calibration of quantitative NMR analyzers represents a complex problem.

Currently, several main methods of calibrating quantitative analyzers, used for the quality assessment of lipid-containing raw materials, can be found in literature:

- using natural samples[3,4];
- using imitator RMs produced from chemically inert substances [13, 14, 24];
- using samples, obtained on the basis of natural components, e.g., oil cakes and oil (see footnote 3) [25].

In this study, we aim to scientifically and experimentally substantiate the application of sunflower oil samples in the calibration of NMR analyzers.

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[2] The minispec Oil content and moisture in seeds and nuts. Available via: URL: http://spectrante.ru/images/pdf_series/Zernovie.pdf. Accessed 12 September 2022 (In Russ.).

[3] Measurement of oil component in dried palm mesocarp. Available via: Oxford Instruments. https://nmr.oxinst.com/assets/uploads/18_Measurement_of_Oil_Content_in_Dried_Palm_Mesocarp.pdf. Accessed 12 September 2022.

[4] Measurement of oil and water in seeds according to ISO 10565. Available via Oxford Instruments. https://nmr.oxinst.com/assets/uploads/3_3344_MR_Oilseeds_App%20Note_Web.pdf. Accessed 12 September 2022.

Materials and Methods

Experiments were carried out at the Central Experimental Base of V.S. Pustovoit All-Russian Research Institute of Oil Crops (Krasnodar) in 2021–2022. Traceable sunflower seed samples of VNIIMK[5] breeding and two samples of commercially distributed oil were prepared, including the refined and deodorized oil of the “BLAGO” trademark (Russia) and non-refined oil of the “STAVROPOL’E” trademark (Russia), acquired in a retail network.

Sunflower samples were cleaned off damaged seeds and waste admixtures. The oil and moisture content of seeds was preliminarily determined using an AMV-1006M NMR analyzer in accordance with GOST 8.597-2010 [26]. The seed samples presented in the study belong to contemporary high-productive varieties. The acid number of the seed oil was determined according to GOST 31933-2012 [24, 27] using the titrimetric method with visual indication.

The determination of the oil content in seeds was carried out by the method of exhaustive extraction in four repetitions for each sample in order to reduce the measurement error, in accordance with the procedure developed based on GOST 10857-64. The duration of extraction was 24 h, at a temperature providing a number of siphonings from 7 to 10 per hour. The completeness of extraction was checked using a watch glass sample. The average value of four measurements was taken as the final result.

The oil from sunflower seeds was obtained using a Laboratoroff PR-L laboratory hand press (LLC Eltemix, Russia) with a force of 12 tons. The obtained oil was further filtered to remove seed particles, trapped in the oil during its pressing, using the FS laboratory filtering paper.

In order to construct the oil content calibration curves, from each oil sample, both obtained by pressing and acquired at a retail network, five weighments evenly distributed in the range from 2000 to 7000 g with the accuracy of 0.001 g were taken using an AND HK-50AG laboratory weights (AND, Japan).

Prior to measuring the oil proton NMR signals, the prepared samples were thermostated at a temperature of 23 ± 0.5 °C for 2 h in a TVL-K(50)B thermostat (CJSC INSOVT, Russia). The thermostating of samples comprises an important stage, since the temperature has the essential effect on the NM relaxation characteristic of oil samples [21].

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[5] Sunflower varieties of VNIIMK breeding, Russia:

UMNIK, oil content 30–35%. Available via VNIIMK. https://vniimk.ru/products/belosnezhnyy-sq/. Accessed 12 September 2022 (In Russ.).

DZhIN, oil content 44–46%. Available via VNIIMK. https://vniimk.ru/products/dzhinn/. Accessed 12 September 2022 (In Russ.).

IMIDZh, oil content 48%. Available via VNIIMK. https://vniimk.ru/about/fgup/Каталог%20ВНИИМК.pdf. Accessed 12 September 2022 (In Russ.).

SPK, oil content 46–47%. Available via VNIIMK. https://vniimk.ru/products/spk/. Accessed 12 September 2022 (In Russ.).

BELOSNEZhNYI, oil content 30–35%. Available via VNIIMK. https://vniimk.ru/products/belosnezhnyy-sq/. Accessed 12 September 2022 (In Russ.).

Measurements were conducted using typical AMV-1006M NMR analyzers of the oil and moisture content (VNIIMK, Russia). In accordance with the analytical procedure, when performing measurements by an AMV-1006M NMR analyzer, the volume of each analyzed seed weighment is equal to 25 cm^3.

For the control of NMR analyzers and primary processing of NMR signals, obtained using the studied oil samples, the own software was used [28]. The obtained data were statistically analyzed using Statistica and Excel software.

Results and Discussion

The main characteristics of sunflower seeds used in the study are presented in Table 1.

It is known [2] that, for oilseeds with a moisture content of < 8%, the intensity of NM relaxation characteristics is determined exclusively by the protons of the oil and accompanying substances. This is due to the high degree of connection between water molecules and the protein part of the seeds. The data in Table 1 show that the moisture content of the samples under study is much lower. Therefore, the resulting analytical parameter will further characterize only TAG protons.

The seed samples prepared for the analysis characterize the range of the oil content from 32.1 to 56.3%. The seeds are healthy; their acid number corresponds to the first-class sunflower and does not exceed 0.8 mg KOH/g for all samples.

At the first stage of the study, we obtained the NM relaxation characteristics of the prepared oilseeds and determined their oil content using the Soxhlet method of exhaustive extraction. The obtained calibration curve, describing the dependence of the oil mass on the amplitude of the NMR signal, is shown in Fig. 1.

The resulting curve is characterized by a high correlation coefficient of 0.9999. The main drawback of the described calibration technique is the complexity of implementation, duration, and high requirements for the qualification of personnel.

Table 2 shows the calculated values of the oil mass in sunflower seeds and the actual values obtained by the extraction method.

Table 2 shows that the maximum error in measuring the oil mass in the analyzed sample by the NMR method does not exceed 28 mg, which, in terms of the oil

Table 1 Characteristics of the studied sunflower seeds

Seed sample	Indicators		
	Oil content, %	Moisture content, %	Oil acid number, mg KOH/g
BELOSNEZhNYI	32.1	6.9	0.6
IMIDZh	41.3	5.2	0.7
DZhIN	42.5	5.5	0.6
SPK	48.2	5.3	0.8
UMNIK	56.3	4.8	0.6

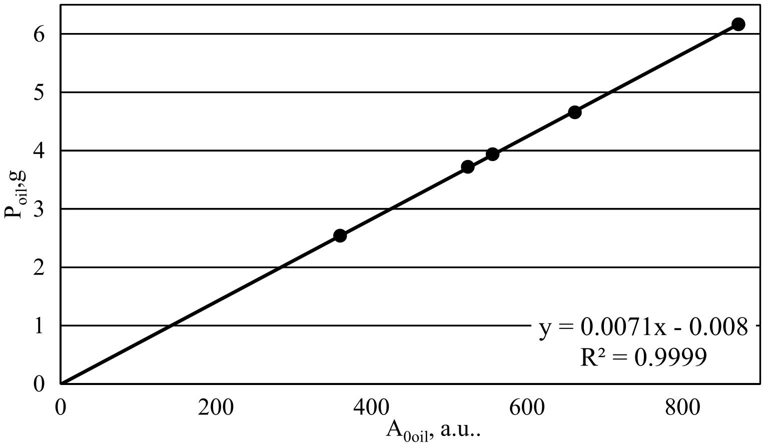

Fig. 1 Dependence of the mass of the sunflower oil, obtained by the Soxhlet extraction, on the amplitude of the NMR signal

Table 2 Calculated and actual (obtained by the extraction method) values of the oil mass in sunflower seeds

Seed sample	Oil mass in the analyzed sample (P_{oil}), g		Deviation from the actual value, g	Deviation in terms of the oil content, %
	Actual	Calculated		
1	2.540	2.539	0.001	0.02
2	3.719	3.706	0.013	0.15
3	3.936	3.932	0.004	0.04
4	4.656	4.684	– 0.028	– 0.29
5	6.164	6.178	– 0.014	– 0.13

content, taking into account the seed moisture content and the mass of the sample with a volume of 25 cm^3, is < 0.29%.

At the next stage, the possibility of using oil, obtained by pressing sunflower seeds, for the calibration of an NMR analyzer was investigated. Figure 2 illustrates the dependence of the oil mass on the amplitude of the NMR signal obtained using the oil pressed from sunflower seeds of three contemporary high-oil varieties (Dzhin, Imidzh, SPK).

According to the presented curve, the variety special features of seeds cause no significant effect on the amplitude of the oil NMR signal and, consequently, on the obtained analytical dependence. The difference between the obtained curve and that shown in Fig. 1 can be explained by the introduction of different amounts of accompanying substances in the process of oil extraction and pressing.

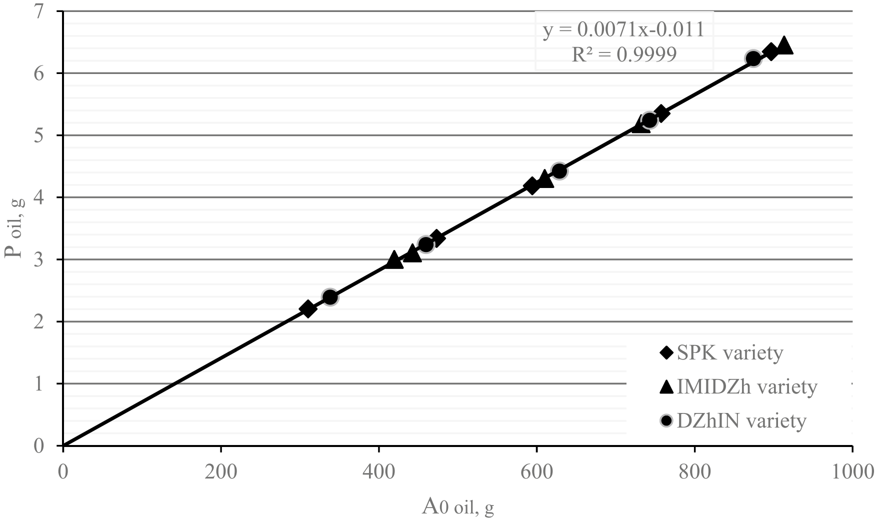

Fig. 2 Dependence of the pressed oil mass on the amplitude of the proton NMR signal

Table 3 provides the calculated values of the oil mass in sunflower seeds according to the calibration of an NMR analyzer using pressed oil.

The application of a calibration curve, obtained using pressed oil, leads to a maximum measurement error of not more than 32 mg or 0.33 abs% in terms of the oil content taking into account the seed moisture content and the mass of the sample with a volume 25 of cm^3 in the entire studied range.

At the third stage of the study, two samples of sunflower oil were used: refined and deodorized of the "BLAGO" trademark and non-refined of the "STAVROPOL'E" trademark, acquired at a retail network.

Figure 3 plots the dependence of the oil mass on the amplitude of the NMR signal, obtained using oil acquired at a retail network.

Table 3 Calculated values of the oil mass in sunflower seeds according to the calibration of an NMR analyzer using pressed oil

Seed sample	Oil mass in the analyzed sample (P_{oil}), g		Deviation from the actual value, g	Deviation in terms of the seed oil content, %
	Actual	Calculated		
1	2.540	2.543	− 0.003	− 0.03
2	3.719	3.710	0.009	0.10
3	3.936	3.936	0.000	0.00
4	4.656	4.688	− 0.032	− 0.33
5	6.164	6.182	− 0.018	− 0.16

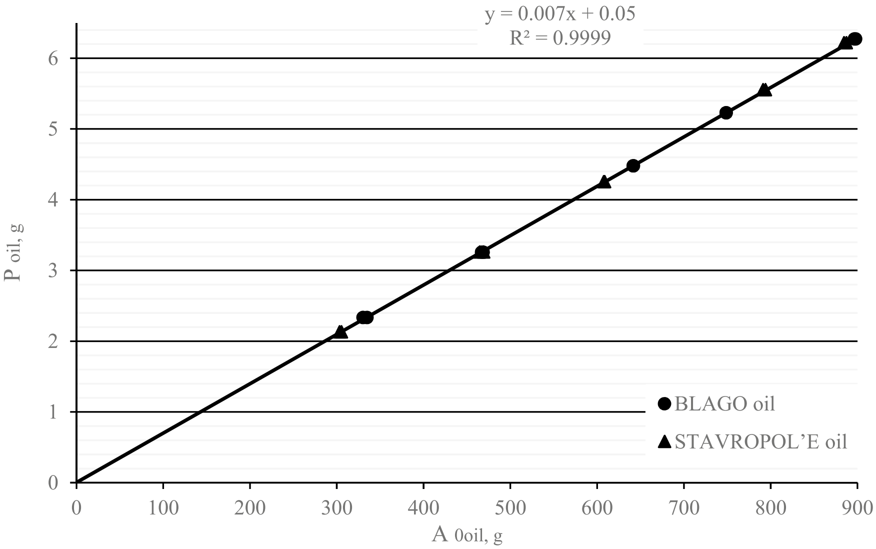

Fig. 3 Dependence of the commercially distributed oil mass on the amplitude of the proton NMR signal

As in the case of pressed oil, the obtained curve of the dependence between the mass of the analyzed oil sample and the amplitude of the oil NMR signal has a linear nature with the high correlation coefficient.

Table 4 provides the calculated values of the oil mass in sunflower seeds according to the calibration of the NMR analyzer using commercially distributed oil.

The application of a calibration curve, obtained using commercially distributed oil, leads to a maximum measurement error of not more than 28 mg or 0.35 abs% in terms of the oil content taking into account the seed moisture content and mass of the sample with a volume 25 of cm^3 in the entire studied range.

Table 4 Results of the oil mass measurement in sunflower seeds according to the calibration, obtained using commercially distributed oil

Seed sample	Oil mass in the analyzed sample (P_{oil}), g		Deviation from the actual value, g	Deviation in terms of the seed oil content, %
	Actual	Calculated		
1	2.540	2.568	– 0.028	– 0.35
2	3.719	3.719	0.000	0.00
3	3.936	3.942	– 0.006	– 0.06
4	4.656	4.683	– 0.027	– 0.28
5	6.164	6.155	0.008	0.08

At the next stage, the oil samples were studied for the temporal stability of NM relaxation characteristics. Three weighments of both refined deodorized and pressed oil were selected for a storage at a temperature of 8 °C in tightly-closed glass cups. Once a week, the samples were taken out of the refrigerator and thermostated at a temperature of 23 ± 0.5 °C for 4 h followed by threefold measurements of the oil NMR signal amplitude. The mean of three measurements was taken as the measurement result. Further, using the obtained earlier calibration equations, the oil masses of the studied samples were calculated and the deviations from the basic value were determined (Table 5).

According to Table 5, the NM relaxation characteristics of the refined oil and, consequently, the calculated values of the oil mass remain stable during the considered storage time. However, for the pressed oil samples, the measured amplitude of the NMR signal of TAG protons considerably altered and led to a decrease in the calculated oil mass value by 570 mg.

Thus, according to the results of the temporal stability study, pressed oil samples can preserve their proton NM relaxation characteristics during not more than 5–7 days, while the refined deodorized samples demonstrate the stability during not < 3 months at a temperature of 8 °C. This can be explained by the presence of a large quantity of free radicals, moisture, and other accompanying substances in pressed oil, facilitating the flow of oxidation processes, which lead to an increase in the quantity of free fatty acids and other products of oxidizing reactions.

Table 5 Results of the oil mass measurement in the analyzed samples depending on the storage time

Week	Deviation of measured oil mass values, g For the samples					
	Refined deodorized			Pressed		
	1	2	3	1	2	3
0	0.000	0.000	0.000	0.000	0.000	0.000
1	– 0.012	– 0.007	– 0.012	0.048	0.049	0.051
2	0.009	– 0.011	–0.010	0.046	0.051	0.052
3	– 0.012	0.003	– 0.013	0.080	0.084	0.089
4	0.011	–0.010	0.013	0.117	0.117	0.126
5	0.008	0.003	0.011	0.166	0.168	0.173
6	– 0.010	– 0.008	– 0.012	0.214	0.219	0.222
7	– 0.013	– 0.010	0.007	0.264	0.269	0.270
8	0.012	0.009	0.015	0.315	0.317	0.321
9	– 0.011	– 0.009	– 0.007	0.366	0.364	0.368
10	– 0.006	– 0.010	– 0.009	0.427	0.428	0.429
11	0.010	0.012	– 0.003	0.491	0.490	0.491
12	– 0.011	– 0.010	0.010	0.568	0.567	0.566

Table 6 Comparative characteristics of NMR-analyzer calibration methods for determining the oil content of sunflower seeds

Indicator	Calibration procedure		
Used materials	Traceable sunflower seeds	Pressed sunflower oil	Refined sunflower oil
Auxiliary equipment	Scales (Accuracy class—high-II), thermostat, Soxhlet extraction equipment	Scales (Accuracy class—high-II), thermostat, laboratory press	Scales (Accuracy class—high-II), thermostat
Calibration time	3 days	1 day	4 h
Personnel qualification requirements	High	Medium	Medium
Sample reusability	None	Can be reused within 3–5 days	Can be reused within 3 months

Comparative characteristics of the studied methods for calibrating an AMV-1006M NMR analyzer are presented in Table 6.

Conclusion

The conducted research shows that the use of sunflower oil, obtained in different ways, for the calibration of NMR analyzers can significantly facilitate the calibration process, reduce the calibration time from 3–4 days to 3–4 h, as well as to eliminate the use of toxic solvents and additional expensive equipment from the process without significantly increasing the error in determining the oil content of sunflower seeds by the NMR method.

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Author cotributions The authors have contributed equally.

Conflict of interest The article was prepared on the basis of a report presented at the V International Scientific Conference “Reference materials in measurements and technologies” (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized and the review procedure was carried out.

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Measurement of Carbon Isotope Ratio in Vanillin Using the CM-CRDS Method: Achieving an Expanded Uncertainty Below 0.1‰

Ian K. Chubchenko

Abstract Isotopic analysis is an effective method for quality control and detection of adulterated food products, with the elemental analyzer isotope ratio mass spectrometry (EA-IRMS) being the conventional approach, specified in relevant regulatory documents. Although measuring the carbon isotope ratio by combustion module cavity ring-down spectroscopy (CM-CRDS) has gained significant interest for isotope analysis of food products, its metrological characteristics remain understudied for analysis of the isotopic composition of vanillin. Therefore, this article focuses on the development of a measurement procedure of the carbon isotope ratio in vanillin in preparation for the international key comparison CCQM-K167 "Carbon Isotope Delta Measurements of Vanillin". The experimental part of the research was carried out using the reference installation included in the State primary standard for units of molar fraction, mass fraction, and mass concentration of components in gases GET 154-2019. The expanded uncertainty (at $k = 2$) of this measurement method was $< 0.09‰$, which is comparable to the best measurements obtained by the EA-IRMS method. The achieved results validate the applicability of the CM-CRDS method for quality control and detection of adulterated vanillin.

Keywords Metrology · Stable isotope · Isotope ratio mass spectrometry · Isotope-ratio infrared spectroscopy · CRDS · CM-CRDS · EA-IRMS · Vanillin · $^{13}C/^{12}C$ · $\delta^{13}C$ · Isotope ratio · Measurement procedure

Introduction

Isotopic analysis is an effective method for quality control and detection of adulterated food products, which is widely used in the Russian Federation for quality control and confirmation of juice product safety under TR CU

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023/2011 [1] and alcoholic products under TR EEC 047/2018 [2]. The corresponding measurement methods are regulated by GOST R 55460-2013, GOST R 55518-2013, GOST 32710-2014 [3–5], and in the Federal Information Fund under reg. no FR.1.31.2014.17273, FR.1.31.2016.24753, FR.1.31.2018.31997, FR.1.31.2013.15529, FR.1.31.2016.24962, FR.1.31.2017.28360, and FR.1.31.2012.13424.[1] In world practice, the method is used for quality control of food products, including honey [6–8], juices [9], baby food, alcoholic drinks [10], olive oil [11], cheese [12], beef [13], and vanillin [14]. In particular, isotopic analysis plays a crucial role in differentiating natural vanillin from its synthetic analog [15].

The method of elemental analyzer isotope ratio mass spectrometry (EA-IRMS) [16, 17] is widely used in the field of isotope analysis for food products and is regulated in the relevant normative documents. The expanded uncertainty (at $k = 2$) for the ratio of stable carbon isotopes $\delta^{13}C_{VPDB}$ in vanillin measured by the EA-IRMS method varies from 0.06‰ to 3.8‰ [15, 18–21, 28].

The combustion module cavity ring-down spectroscopy (CM-CRDS) method comprises a rapidly developing technique for measuring carbon isotope ratios in food products that offers a number of advantages, including the ease of operation, a high level of automation, and the low cost of equipment and consumables. A number of scientific papers [22–25] investigated the metrological characteristics of the CM-CRDS method in the analysis of the isotopic composition of food products such as honey, milk, lemon, and coconut juices. The standard uncertainty of the measurements of carbon isotope ratio in honey by the CM-CRDS method in the works [23, 26] ranged from 0.01 to 0.32‰, while for lemon juice in the work [22] from 0.02 to

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[1] FR.1.31.2012.13424 Method for measuring the 13C/12C isotope ratio of ethanol in beer and beer drinks by isotope mass spectrometry. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/282517. Accessed 11 November 2022 (In Russ.).

FR.1.31.2013.15529 The methodology establishes the procedure for determining the ratio of 18O/16O isotopes of exogenous and endogenous water in wines and musts. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/281364. Accessed 11 November 2022 (In Russ.).

FR.1.31.2014.17273 Method for measuring the ratio of carbon isotopes $^{13}C/^{12}C$ in alcoholic beverages of grape origin by isotope mass spectrometry. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/280000. Accessed 11 November 2022 (In Russ.).

FR.1.31.2016.24753 Method for measuring the oxygen isotope ratio, $^{18}O/^{16}O$ of exogenous and endogenous water in wines and musts by isotope mass spectrometry. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/298716. Accessed 11 November 2022 (In Russ.).

FR.1.31.2016.24962 Method for measuring ethanol isotope ratios in cognacs and cognac distillates by isotope mass spectrometry. FR.1.31.2016.24962 Method for measuring ethanol isotope ratios in cognacs and cognac distillates by isotope mass spectrometry. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/298746. Accessed: 11 November 2022 (In Russ.).

FR.1.31.2017.28360 Method for measuring the ratios of carbon, oxygen, hydrogen isotopes of ethanol to detect the presence of synthetic alcohol in alcoholic products, as well as in alcohol-containing food flavorings by isotope mass spectrometry. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/299163. Accessed 11 November 2022 (In Russ.).

FR.1.31.2018.31997 Method for measuring the ratio of oxygen isotopes $^{18}O/^{16}O$ of the water component of ciders and poiret by isotope mass spectrometry. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/495958. Accessed 11 November 2022 (In Russ.).

0.28‰. However, no data on the expanded uncertainty (at $k = 2$) of the measurements of the stable carbon isotope ratio $\delta^{13}C_{VPDB}$ in vanillin by the CM-CRDS method are reported in the scientific literature.

The paper [27] describes the methods to address several factors that contribute to the measurement uncertainty budget for substances and materials by the CM-CRDS method, including the impact of the CO_2 feed concentration and the influence of the dilution gas on the readings of the CM-CRDS analyzer. In order to reduce the adverse effects, correction factors are proposed in the work [27]. In addition, the sample masses are selected to ensure that the volume fractions of carbon dioxide in the gas mixtures, formed following the combustion of the test and reference materials and entering the analyzer, remain identical, while the same dilution gas is used. However, the development of a technique for measuring the carbon isotope ratio in vanillin by the CM-CRDS method was beyond the scope of this work [27].

This study introduces a method for measuring the carbon isotope ratio in vanillin by the CM-CRDS method, achieving an expanded uncertainty (at $k = 2$) of < 0.1‰. The indicated value of the expanded uncertainty was selected on the basis of the accuracy analysis of the EA-IRMS method.

The objectives of the study were as follows: to determine the factors that contribute to the measurement uncertainty budget of the carbon isotope ratio in vanillin and to eliminate or minimize their adverse effects if possible; to develop a procedure for determining the carbon isotope ratio in vanillin by CM-CRDS; to validate the metrological characteristics of the procedure in international key comparisons.

The following methodology can be used to detect the adulteration of vanillin in the food industry.

Materials and Methods

The measurement methods were developed in preparation for the international key comparison CCQM-K167 "Carbon Isotope Delta Measurements of Vanillin" organized by the international workshop on isotope ratio measurements of the Consultative Committee for Amount of Substance (CCQM) of the International Bureau of Weights and Measures (BIPM), held from 2019 to 2022. A high-purity sample of natural vanillin, prepared by the National Research Council of Canada (NRC) as part of the international comparison CCQM-K167 was used for analysis.

The experimental part was carried out using the reference installation included in the State primary standard for units of molar fraction, mass fraction, and mass concentration of components in gases GET 154-2019,[2] which includes a Picarro G2131i cavity ring-down spectrometer (CRDS) (referred to as the analyzer) and a

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[2] GET 154-2019 State Primary Standard of Units of Mole Fraction, Mass Fraction and Mass Concentration of Components in Gas and Gas Condensate Environments: Custodian Institute VNIIM im. D. I. Mendeleev. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1365155. Accessed 11 November 2022 (In Russ.).

Picarro combustion module (CM) (referred to as the combustion module) (Fig. 1). The CM-CRDS method involves instantaneous combustion of a sample in the oxidation reactor of the combustion module when oxygen is introduced to the nitrogen flow, the purification of the resulting gas mixture from impurities (NO_2, SO_2, H_2O, and excess oxygen) in the combustion module, and the analysis of the isotopic composition of the formed carbon dioxide by a cavity ring-down spectrometer. The external appearance of the reference setup is shown in Fig. 2.

The oxygen of ultra-high purity (volume fraction of oxygen of at least 99.999% according to TU 2114-001-05798345-2007 “Oxygen in liquid and gaseous form of ultra-high purity”) was used as an oxidizing gas (referred to as oxygen). Gaseous nitrogen (volume fraction of nitrogen of at least 99.999%, referred to as nitrogen) was used as a carrier gas. The traceability of the $\delta^{13}C_{VPDB}$ certified value to the

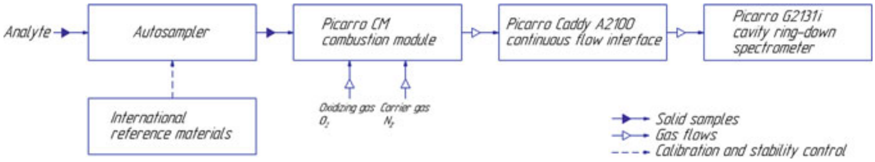

Fig. 1 Functional diagram of reference installation

Fig. 2 External appearance of reference setup

international reference delta scale VPDB is achieved by using certified reference materials CIPM/104-26[3] approved by the decision of the BIPM. To calibrate the reference installation, the following international reference materials (RMs) were used: UME CRM 1312 (honey, certified $\delta^{13}C_{VPDB}$ value of − 24.095‰, combined standard uncertainty of 0.039),[4] IAEA-CH-3 (cellulose, certified $\delta^{13}C_{VPDB}$ value of − 27.724‰, combined standard uncertainty of 0.041),[5] IAEA-600 (caffeine, certified $\delta^{13}C_{VPDB}$ value of − 27.771‰, combined standard uncertainty of 0.043),[6] IAEA-CH-7 (polyethylene, certified $\delta^{13}C_{VPDB}$ value of − 32.151‰, combined standard uncertainty of 0.050).[7] NBS22 (mineral oil, certified $\delta^{13}C_{VPDB}$ value of − 30.03‰, combined standard uncertainty of 0.04)[8] was used for accuracy control. The samples were weighed using laboratory electronic scales MV 210-A manufactured by CJSC "Sartogosm" in St. Petersburg (referred to as laboratory scales).

The measurements were carried out according to the methodology developed at D. I. Mendeleyev Institute for Metrology over a one-month period in 2020. The final report of the international key comparisons CCQM-K167, which confirmed the obtained result, was published in the International Key Comparison Database (KCDB) in 2022.

Equipment Preparation

All equipment was prepared for operation in accordance with the operation manuals. Subsequently, a series of tests described below were carried out to verify their serviceability and to achieve optimal metrological characteristics. The detailed substantiation of these tests, along with the factors contributing to the measurement uncertainty budget, can be found in the "Results and Discussion" section.

Prior to commencing the operation on the reference installation, nitrogen and oxygen were connected to the combustion module, and the inlet pressure was adjusted. The gas leakage test was carried out in accordance with the CM manual, and the standard nitrogen and oxygen flow rates were configured. However, during the operation, it was observed that the set oxygen flow rate varied over time. Therefore, to

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[3] 104th meeting of the CIPM (Session I), 9 to 10 March 2015. Available via BIPM. https://www.bipm.org/en/committees/ci/cipm/104-_1-2015. Accessed 11 November 2022.

[4] UME CRM 1312 Certificate of the Reference Material Honey (unadulterated). Available via. https://rm.ume.tubitak.gov.tr/sertifika/ume_crm_1312_certificate.pdf. Accessed 11 November 2022.

[5] IAEA-CH-3 Cellulose. Available via. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-CH-3.aspx. Accessed 11 November 2022.

[6] IAEA-600 Caffeine. Available via: https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-600.aspx. Accessed 11 November 2022.

[7] IAEA-CH-7 Polyethylene. Available via. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-CH-7.aspx. Accessed 11 November 2022.

[8] NIST RM 8539 NBS 22 oil (carbon and hydrogen isotopes in oil). Available via. https://www.sigmaaldrich.com/RU/en/product/sial/nistrm8539. Accessed 11 November 2022.

ensure efficient oxidation and improve the reproducibility of the measurement results, the oxygen flow rate was set anew prior to each series of measurements (approximately once every 5 h). In accordance with the operation manual, the combustion module was switched to the heating mode of the oxidation reactor. The heating of the combustion module required at least 12 h. The operating parameters of the combustion module in the software were as follows: "Sample delay"—18 s, "Sample stop"—10 s, "Oxygen stop"—50 s, and "Run time"—900 s. The working parameters for processing the measurement results were preset in Picarro Peak Integration Software: "Pre-trigger time"—10 s, "Post-trigger time"—120 s, "Threshold ratio above baseline"—2000%, "Sample run time"—900 s.

In the initial test, the background values of carbon dioxide in nitrogen were measured in order to minimize the influence of carbon dioxide present in nitrogen on the analyzer readings. To achieve this, the Picarro Caddy A2100 continuous flow interface (Fig. 1) was switched from the "atmospheric air" position to the "combustion module" position. Nitrogen was passed through the combustion module without heating the oxidation reactor to the analyzer. The test was considered successful if the analyzer readings for the $^{12}CO_2$ component were below 0.1 ppm.

In the second test, the background values of carbon dioxide in nitrogen flowing through the heated combustion module were measured in order to minimize the impact of residual carbon in the oxidation reactor on the analyzer readings. To achieve this, the combustion module was switched to the heating mode of the oxidation reactor following the instructions provided in the operation manual. The heating of the combustion module required a minimum of 12 h. During the heating process, residual carbon in the oxidation reactor was oxidized to carbon dioxide and, mixed with nitrogen, was supplied to the analyzer. The test was considered successful if the analyzer readings for the $^{12}CO_2$ component were < 0.2 ppm.

In the third test, the background values of carbon dioxide in the blank sample were measured in order to minimize the impact of carbon dioxide in oxygen on the analyzer readings. To achieve this, no sample was loaded into the autosampler, and the measurement process was initiated using a portion of oxygen that oxidized the remaining organic contaminants in the reactor. The test was considered successful if the analyzer readings for the $^{12}CO_2$ component were < 2.5 ppm.

In the fourth test, the background values of carbon dioxide in the tin crucible were measured in order to minimize the effect of residual carbon in the tin crucible on the readings of the analyzer. To achieve this, the empty tin crucible was loaded into the autosampler. The measurement was initiated, with a portion of oxygen supplied to the oxidation reactor, which oxidized the remaining organic contaminants in the reactor and tin crucible. The test was considered successful if the analyzer readings for the $^{12}CO_2$ component were < 25 ppm.

In the fifth test, the linearity of the analyzer with respect to carbon dioxide concentrations was examined in order to identify and address the influence of analyzer nonlinearity on the measurement result. To carry out this test, gas mixtures with different concentrations but identical isotopic compositions of carbon dioxide were introduced into the analyzer through the Picarro Caddy A2100 gas-distributing system. The gas mixtures containing a gas mixture of carbon dioxide in nitrogen GSO

10768-2016, cylinder No. 66902 (0.45099%) (referred to as GSO 10768-2016), and nitrogen were prepared using the mixed gas generator GGS-R, Reg. No. in Federal Information Fund 62151-15 (referred to as generator). The linearity of the analyzer was tested in the range of carbon dioxide volume fractions from 2000 to 4000 ppm. The test was considered successful if the deviation of the measured values from the linear dependence was < 0.1 ppm.

In the sixth test, the focus was on evaluating the stability of the analyzer to identify and address any potential impact of instability on the measurement results. To carry out this test, the same gas mixture (0.2% CO_2/N_2) obtained by diluting the GSO 10768-2016 with nitrogen in the generator was injected 10 times into the analyzer through the Picarro Caddy A2100 gas-distributing system. The test was considered successful if the standard deviation of the results was $< 0.05‰$.

In the seventh test, the combustion efficiency of samples at various temperatures in the combustion module was examined in order to evaluate and, if necessary, eliminate the effect of incomplete combustion of the sample on the measurement result. For this task, six IAEA-600 samples were loaded into the autosampler. Three samples were burned at 980 °C, while the remaining three were burned at 1100 °C. The test was considered successful if the difference in arithmetic mean values for different temperatures was $< 0.1‰$.

If the above conditions were violated, the tests were repeated. In case the conditions continued to be unsatisfactory, the factors leading to inadequate results were identified and eliminated.

In order to verify the combustion efficiency of the sample in the combustion module, two tests, namely the seventh and the third, were performed during the measurements, with the third test being carried out prior to each series of measurements.

Sample and Reference Material Preparation

0.74 to 0.88 mg of vanillin extracted from one test sample was weighed in tin crucibles on a laboratory scale. Reference materials IAEA-600, IAEA-CH-3, IAEA-CH-7, UME CRM 1312, and NBS22 were processed using the same procedure. The masses of IAEA-600 samples ranged from 0.85 to 1.06 mg, of IAEA-CH-3—from 1.02 to 1.28 mg, of IAEA-CH-7—from 0.54 to 0.65 mg, of UME CRM 1312—from 1.32 to 2.31 mg, and of NBS22—from 0.55 to 0.73 mg. The masses were selected to ensure that the volume fractions of carbon dioxide in the gas mixtures generated following the combustion of the test and reference materials differ by no more than 300 ppm and ranged from 2500 to 3500 ppm.

The tin crucibles with samples were tightly compressed into a cube shape to minimize the content of atmospheric air within the crucible. For each series of measurements, two samples of each reference material and one test sample of vanillin were prepared.

Measurements

During laboratory measurements, the following conditions were carefully controlled:

- air temperature between 22 and 25 °C,
- atmospheric pressure between 99 and 103 kPa,
- relative humidity of no more than 60%.

Samples packed in tin crucibles were placed in the autosampler, which automatically delivered them to the oxidation reactor in the combustion module heated to 980 or 1100 °C, where they were oxidized to CO_2, H_2O, NO_x, and SO_x in a flow of nitrogen (100 ml/min) and oxygen (30 ml/min). The oxidation reactor consisted of a hollow quartz heat-resistant glass tube filled with chromium oxide, copper wire, and silvered cobalt oxide separated by silica wool interlayers. Chromium oxide was used to facilitate the sample oxidation. The gas mixture of nitrogen, oxygen, and the oxidation products (CO_2, H_2O, NO_x, SO_x) was passed through the copper wire to remove NO_x and oxygen, prior to the contact with the silvered cobalt oxide to remove SO_x. The resulting gas mixture of CO_2, H_2O, and nitrogen was directed through the H_2O removal trap, i.e., a glass hollow tube filled with magnesium perchlorate and quartz wool, prior to entering a Picarro Caddy A2100 gas distribution system containing two expansion volumes in the form of Swagelok steel cylinders (50 ml) and further to the analyzer for measurements.

Each measurement series presented in Table 1 comprised a sequence of measurements of the following samples: Blank test—Control sample (Acetanilide, Thermo Fisher Scientific)—Reference material 2—Reference material 1—Test sample—Reference material 2—Reference material 1.

Data Processing

The stable carbon isotope ratio for the analyzed vanillin sample ($\delta^{13}C_{VPDB}$, ‰) was calculated using the following formula:

$$\delta^{13}C_{VPDB} = \frac{X_1^c - X_2^c}{X_1^{mes} - X_2^{mes}}\left(X_t^{mes} - X_1^{mes}\right) + X_1^c \qquad (1)$$

where X_1^c is the value of the carbon isotope ratio listed in the certificate of RM 1 (of the two specified in the "Materials and Methods" section),

X_2^c is the value of the carbon isotope ratio listed in the certificate of RM 2 (of the two specified in the "Materials and Methods" section),

X_t^{mes} is the measured value of the carbon isotope ratio in the test sample, obtained by introducing a correction associated with the nonlinearity of the analyzer for carbon dioxide concentrations (refer to the fifth test in "Equipment Preparation" section), to the analyzer reading X_A:

Table 1 Measurement results of carbon isotope ratio $\delta^{13}C_{VPDB}$ in vanillin

Vanillin sample number	Vanillin sample mass, mg	Measured carbon isotope ratio $\delta^{13}C_{VPDB}$ in vanillin, ‰	Reference materials	Temperature in oxidation reactor, °C
1	0.760	– 25.75	IAEA-600 and IAEA-CH-3	980
2	0.800	– 25.80	IAEA-600 and IAEA-CH-3	980
3	0.800	– 25.79	IAEA-600 and IAEA-CH-3	980
4	0.790	– 25.78	IAEA-600 and IAEA-CH-3	980
5	0.880	– 25.84	IAEA-600 and IAEA-CH-3	980
6	0.850	– 25.85	IAEA-600 and IAEA-CH-3	980
7	0.850	– 25.83	IAEA-600 and IAEA-CH-3	980
8	0.860	– 25.87	IAEA-600 and IAEA-CH-3	980
9	0.860	– 25.81	IAEA-600 and IAEA-CH-3	1100
10	0.870	– 25.89	IAEA-600 and IAEA-CH-3	1100
11	0.870	– 25.83	IAEA-600 and IAEA-CH-3	1100
12	0.850	– 25.74	IAEA-CH-7 and UME CRM 1312	980
13	0.870	– 25.86	IAEA-CH-7 and UME CRM 1312	980
14	0.870	– 25.81	IAEA-600 and IAEA-CH-3	1100
15	0.840	– 25.79	IAEA-600 and IAEA-CH-3	1100
16	0.860	– 25.81	IAEA-600 and IAEA-CH-3	1100
17	0.740	– 25.82	IAEA-600 and IAEA-CH-3	1100
18	0.750	– 25.82	IAEA-600 and IAEA-CH-3	1100
19	0.740	– 25.76	IAEA-600 and IAEA-CH-3	1100

$$X_t^{mes} = X_t - 0.0002 * (3000 - Y_t) \tag{2}$$

where X_t is the analyzer reading when feeding test sample,

Y_t is the measured value of the volume fraction of carbon dioxide in the test sample,

X_1^{mes} is the measured value of the carbon isotope ratio in RM 1, obtained by introducing corrections related to the drift and nonlinearity of the analyzer for carbon dioxide concentrations (refer to the fifth test in the "Equipment Preparation" section), to the analyzer reading:

$$X_1^{mes} = X_1^n + \frac{1}{3}\left(X_1^m - X_1^n\right) \tag{3}$$

$$X_1^n = X_{1n} - 0.0002 * \left(3000 - Y_{1n}\right) \tag{4}$$

$$X_1^{mes} = X_{1m} - 0.0002 * \left(3000 - Y_{1m}\right) \tag{5}$$

X_{1n} is the analyzer reading when RM 1 is injected prior to feeding a test sample,

X_{1m} is the analyzer reading when RM 1 is injected following feeding a test sample,

Y_{1n} is the measured value of the carbon dioxide volume fraction when RM 1 is injected prior to feeding test sample,

Y_{1m} is the measured value of the carbon dioxide volume fraction when RM 1 is injected following feeding test sample,

X_2^{mes} is the measured value of carbon isotope ratio in RM 2, obtained by introducing corrections associated with drift and nonlinearity of the analyzer for carbon dioxide concentrations (refer to the fifth test in "Equipment Preparation" section), to the analyzer reading:

$$X_2^{mes} = X_2^n + \frac{2}{3}\left(X_2^m - X_2^n\right) \tag{6}$$

$$X_2^n = X_{2n} - 0.0002 * \left(3000 - Y_{2n}\right) \tag{7}$$

$$X_2^m = X_{2m} - 0.0002 * \left(3000 - Y_{2m}\right) \tag{8}$$

X_{2n} is the analyzer reading when RM 2 is injected prior to feeding a test sample,

X_{2m} is the analyzer reading when RM 2 is injected following feeding a test sample,

Y_{2n} is the measured value of the carbon dioxide volume fraction when RM 2 is injected prior to feeding a test sample,

Y_{2m} is the measured value of the carbon dioxide volume fraction when RM 2 is injected following feeding test sample,

Measurement Accuracy Control

The periodic accuracy control was carried out using the international standard sample NBS22 as the control sample.

The deviation of the carbon isotope ratio measured by this technique in the control sample $\delta^{13}C^{mes}_{VPDB}$ from the $\delta^{13}C^{cert}_{VPDB}$ value, specified in the certificate, must satisfy the condition

$$\left|\delta^{13}C^{mes}_{VPDB} - \delta^{13}C^{cert}_{VPDB}\right| \leq 0.1\ ‰ \tag{9}$$

If Condition (9) is unsatisfied, the test procedure is repeated. In case of repeated failure to meet the conditions, the factors leading to unsatisfactory results are identified and addressed.

Results and Discussion

The measurement results of the carbon isotope ratio in vanillin using the developed method are presented in Table 1.

Each measured value of the carbon isotope ratio $\delta^{13}C_{VPDB}$ in vanillin is calculated by Eq. (1).

The sources of uncertainty in measurements are shown in Fig. 3. The factors contributing to the measurement uncertainty budget are marked in bold and underlined on the diagram, while other factors are minimized or eliminated.

The first and second tests, described in the "Equipment Preparation" section, provided the means to determine the value of the factor "Background values of carbon dioxide in the dilution gas and oxidizing gas." The third test helped to minimize the factor "Purity of quartz reactor." The fourth test allowed the value of the factor "Background values of carbon dioxide in the tin crucible" to be identified.

To address the "Combustion efficiency" factor, the seventh test was carried out. To eliminate the factors "Oxidizing gas and dilution gas flow rate" and "Oxidizing gas flow rate," the standard nitrogen flow rate was adjusted, and the oxygen flow rate was recalibrated prior to each series of measurements.

The "Environment" factor was excluded from the measurement uncertainty budget, since the temperature in the measuring cell of the analyzer was maintained at 45.55 ± 0.01 °C in automatic mode, the pressure was 18,667 ± 20 Pa, and the room temperature was maintained within the range of 22–25 °C. To address the factor "Purity of oxidizing gas and dilution gas," the purity of oxidizing gas and dilution gas was analyzed using GET 154-2019 described in the "Materials and Methods" section. The factors "Purity and homogeneity of test sample" and "purity and homogeneity of RM" were excluded from the measurement uncertainty budget, since high purity and homogeneity are ensured by the manufacturers of the samples.

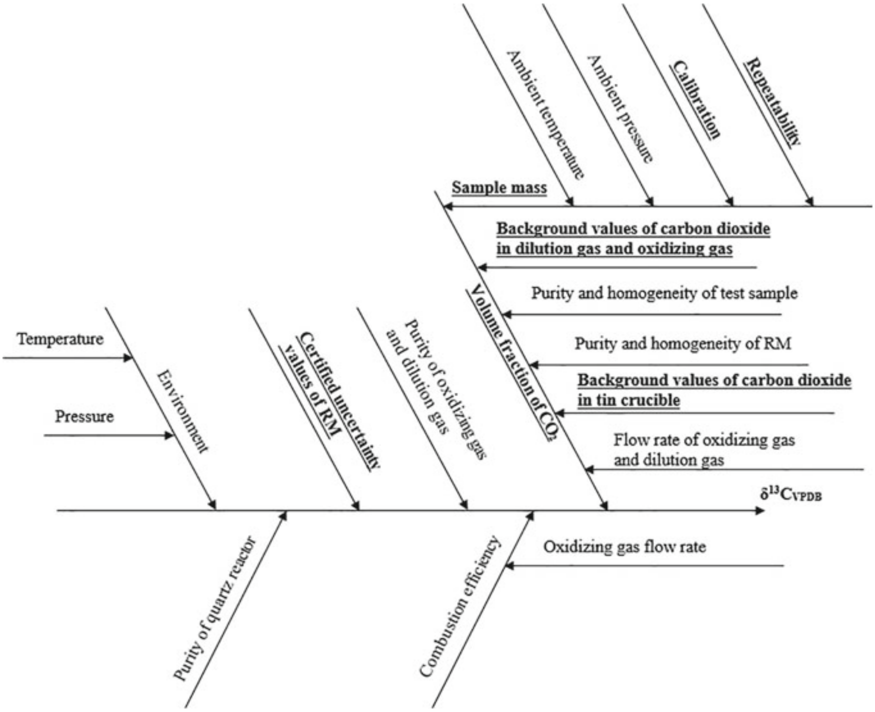

Fig. 3 Sources of measurement uncertainty

The source of uncertainty "Reproducibility of measurements" included the following factors: "Calibration" and "Repeatability."

The uncertainty budget of measurements of carbon isotope ratio $\delta^{13}C_{VPDB}$ in vanillin is presented in Table 2.

The primary source of uncertainty in the uncertainty budget was attributed to the certified uncertainty values of the used reference materials.

The linearity of the analyzer for carbon dioxide concentrations, verified in the fifth test, has no contribution to the uncertainty budget, since corrections (4), (5), (7),

Table 2 Uncertainty budget of measurements of carbon isotope ratio $\delta^{13}C_{VPDB}$ in vanillin

Type of uncertainty	Source of uncertainty	Value, ‰
Type A	Reproducibility of measurements	0.01
Type B	Certified uncertainty values of reference materials	0.04
	Background values of carbon dioxide in tin crucible, dilution gas and oxidizing gas	0.01
Absolute combined standard uncertainty		0.045
Expanded uncertainty (at $k = 2$)		0.09

and (8) were applied to the measurement results using the dependence established in the fifth test and shown in Fig. 4.

The analyzer instability examined in the sixth test is excluded from the uncertainty budget, since the measurement result was corrected for drift of readings using Eqs. (3) and (6).

The developed procedure was used to carry out the measurements of the carbon isotope ratio $\delta^{13}C_{VPDB}$ in vanillin within the framework of the international key comparisons CCQM-K167 [28]. A comparison of the measurement results with those obtained by other participants is presented in Table 3 and Fig. 5. The values measured by the participants are shown in the column $\delta^{13}C_{VPDB}$. The result obtained by the method developed in this work is denoted as "VNIIM," with the combined standard uncertainty indicated in the column u(x) being 0.04‰ (obtained at FSUE "VNIIM named after D. I. Mendeleyev"). The expanded uncertainty (at $k = 2$) of measurements u(x) obtained using the developed method equaled 0.09‰. The DoE values show the deviation of the measurement result from the Key Comparison Reference Value. The expanded uncertainty U_{95}DoE was calculated by the organizer of comparisons using the Bayesian method. Despite the fact that the participants used the EA-IRMS method which was generally considered to be more accurate in comparison with the CM-CRDS method, the values of measurement uncertainty u(x), U(x), and U_{95}DoE obtained by the authors are among the lowest values.

Figure 5 shows the DoE values obtained by each participant in the key comparisons, which show the deviation of the measurement result from the reference value. For the result obtained using the developed technique, the DoE equals 0.023‰, which is significantly lower than the declared expanded uncertainty of 0.09‰. This confirms the high accuracy and precision of the measurement result obtained by the authors.

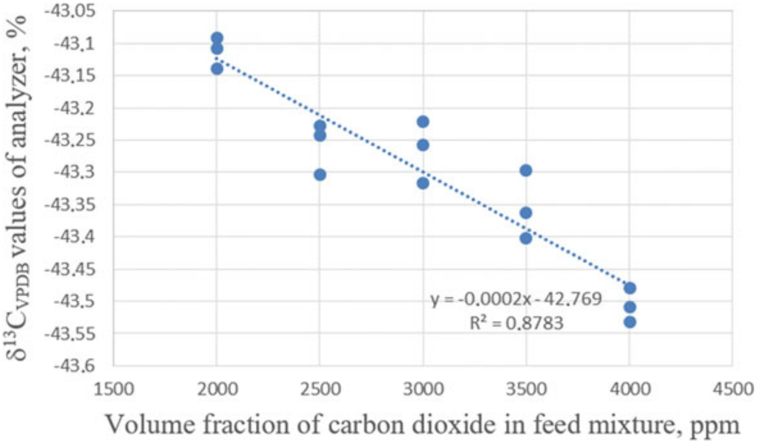

Fig. 4 Dependence of analyzer readings of $\delta^{13}C_{VPDB}$ on the volume fraction of carbon dioxide

Table 3 Results of the international key comparison CCQM-K167 “Carbon Isotope Delta Measurements of Vanillin” [23]

Institution	Country	Method	$\delta^{13}C_{VPDB}$, ‰	u(x), ‰	U(x), ‰	DoE	U_{95}DoE
INMETRO	Brazil	EA-IRMS	–25.96	0.056	0.11	–0.127	0.178
JSI	Slovenia	EA-IRMS	–25.87	0.06	0.11	–0.037	0.180
NRC	Canada	EA-IRMS	–25.86	0.03	0.06	–0.027	0.146
NMIA	Australia	EA-IRMS	–25.833	0.041	0.083	0.000	0.156
UME	Turkey	EA-IRMS	–25.82	0.08	0.16	0.013	0.170
NIM	China	EA-IRMS	–25.812	0.048	0.096	0.021	0.166
VNIIM	Russia	CM-CRDS	–25.81	0.04	0.09	0.023	0.154
LGC	United Kingdom	EA-IRMS	–25.72	0.05	0.11	0.113	0.166

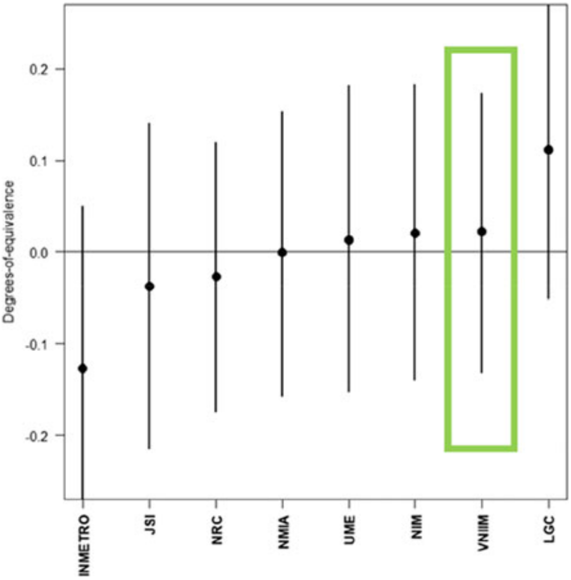

Fig. 5 Results of international key comparison CCQM-K167 “Carbon Isotope Delta Measurements of Vanillin” [23]

All participants of the key comparisons carried out the instrument stability test similar to the fifth test (“Equipment preparation” section). All participants except JSI completed the impurity content test for the carrier gas, similar to the first test. An instrument linearity test, similar to the sixth test, was performed by JSI, LGC, NMIA, and NRC. JCI and LGC performed the peak center adjustment test. LGC and NRC performed the EA-IRMS leak test. NIM carried out the following tests prior to each series of measurements: sensitivity evaluation, peak shape test, repeatability, and blank sample analysis, similar to the third test outlined in the “Equipment Preparation” section.

Table 4 Reference materials used in international key comparisons CCQM-K167 "Carbon Isotope Delta Measurements of Vanillin" [23]

Reference material	JSI	VNIIM	NRC	LGC	UME	NMIA	INMETRO	NIM
LGC 1712				+				
USGS66				+				
IAEA-CH-6			+	+	+	+		+
BCR-657								+
USGS65	+		+					
UME 1312		+						
IAEA-CH-3		+						+
USGS40				+				+
IAEA-600		+	+					+
NBS 22			+		+		+	+
IAEA-CH-7		+				+		+
USGS61			+					
USGS64	+			+				
LGC 1711				+				

The sample masses of vanillin prepared by the participants ranged from 0.069 to 0.88 mg. The average sample mass for the participants was 0.41 mg, which is lower than the average mass for our method (0.81 mg).

The international reference materials used by the participants of the key comparisons are listed in Table 4.

The same RMs used in our developed method, namely IAEA-CH-3, IAEA-600, and IAEA-CH-7, were used by three institutions: NIM, NMIA, and NRC.

The successful participation in the international key comparisons allowed FSUE "VNIIM named after D.I. Mendeleyev" to be the first laboratory in the Russian Federation to have its calibration and measurement capabilities (CMC)[9] listed in the international KCDB BIPM database for isotope ratio measurements.

A comparative analysis of the results obtained in the international key comparisons CCQM-K167 confirms the accuracy and declared uncertainty of the measurement results obtained using the developed methodology.

Therefore, the aim of the study has been achieved: a method for measuring the carbon isotope ratio in vanillin by CM-CRDS with an expanded uncertainty (at $k = 2$) of < 0.1 ‰ was developed. To achieve the goal, the following tasks were addressed: identification of factors contributing to the measurement uncertainty budget, implementation of procedures for equipment and sample preparation, establishment of measurement accuracy control procedures, and development of processing procedures for measurement results.

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[9] CMC Quick search. Available via CIPM MRA database (KCDB). https://www.bipm.org/kcdb/cmc/quick-search?includedFilters=&excludedFilters=&page=0&keywords=VNIIM+ isotope. Accessed 11 November 2022.

The results of the international key comparisons CCQM-K167 confirmed the feasibility of measuring the carbon isotope ratio in vanillin by the developed CM-CRDS method with an expanded uncertainty (at $k = 2$) of 0.09 ‰, which corresponds to the best measurements performed by the EA-IRMS method.

Further research will focus on the development of procedures for measuring carbon isotope ratios using the CM-CRDS method in other substances and materials.

Conclusion

In the course of this study, a procedure for measuring the carbon isotope ratio in vanillin by the CM-CRDS method was developed, achieving an expanded uncertainty (at $k = 2$) of < 0.1‰. It is demonstrated that the accuracy of the CM-CRDS method is comparable to that of the EA-IRMS method.

In order to achieve this goal, the following tasks were addressed. The factors contributing to the measurement uncertainty budget were determined, with part of them being eliminated or minimized. A measurement procedure was developed, including the preparation of equipment and samples, measurement procedure, accuracy control, and data processing. The results of international key comparison CCQM-K167 confirmed the metrological characteristics of the measurement procedure, in particular, the expanded uncertainty (at $k = 2$) of 0.09‰.

The obtained metrological characteristics of the developed measurement method are comparable to the most accurate measurements of the carbon isotope ratio in vanillin using the EA-IRMS method, as well as in other food products (honey, milk, lemon, and coconut juices) using the CM-CRDS method.

The practical significance of this result lies in the confirmation of the feasibility of using the CM-CRDS method for quality control and the detection of adulterated vanillin.

The traceability of the measurement results to the internationally recognized delta scale of the isotope ratio is ensured by the use of appropriate international reference materials, which guarantees the recognition of the measurement results at the international level.

Further research will focus on the development of techniques for measuring the carbon isotope ratio by the CM-CRDS method in other substances and materials analyzed in the food industry, including quality control and safety confirmation of juice products under TR CU 023/2011 and alcohol products under TR EEC 047/2018.

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Conflict of Interests The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022) as well as at the EGU General Assembly 2020 in online format (May 4–8, 2020) [29]. The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Development of Isotopic Reference Materials for ^{13}C-Urea Breath Tests

Ian K. Chubchenko

Abstract Over 30 standards and certified measurement techniques are currently used in Russia to determine carbon, oxygen, and hydrogen isotope ratios. The techniques implemented in these documents are based on foreign reference materials (RMs). Similar domestically-produced RMs are not available, which makes the use of these techniques limited in the near future. This study aims to develop a certified RM (CRM) for the isotopic composition of carbon dioxide for ^{13}C-urea breath tests. The following objectives were formulated: to develop a technique for obtaining RMs; to manufacture an RM pilot batch; to carry out type approval tests. The RM was manufactured by gravimetric mixing of carbon dioxide of two different isotopic composition from different sources with synthetic air. The mixing ratio was calculated using a specially developed technique. The RM stability was tested by an accelerated aging procedure. Following type approval tests, the developed CRM was included in the Federal Information Fund for Ensuring the Uniformity of Measurements under the registration number GSO 11999-2022. The practical significance of the obtained results is related to the possibility of using the developed CRM for improving the accuracy and validity of ^{13}C-urea breath tests. Compared to the analogs, the developed CRM demonstrates a higher chemical purity.

Keywords Metrology · Stable isotope · Isotope-ratio mass spectrometry · Isotope-ratio infrared spectroscopy · Isotopic reference materials · Isotopic composition reference materials · Isotopic breath test · ^{13}C-urea breath test

Introduction

Isotopic analysis is currently gaining increased popularity in various domains, including the food industry for quality control and safety assurance of juice products according to the Technical Regulation of the Customs Union 023/2011 [1] and alcoholic products according to Technical Regulations of the Eurasian Economic

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Union 047/2018 [2], in healthcare for diagnosis and treatment of peptic ulcer disease according to the Order of the Ministry of Health of the Russian Federation No. 611n of June 10, 2021, determination of liver functional status, and doping control of athletes according to WADA[1] recommendations, in customs control to confirm the safety and correctness of imported products certification, in meteorology and atmospheric monitoring to analyze the carbon footprint, hydrological cycle, and geochemical carbon cycle, in the oil and gas industry for prospecting and exploration, in forensics to determine the origin of substances and materials, in scientific research to study climate history and adjustment of fundamental physical constants. About 200 measuring instruments (hereinafter referred to as MIs) of element isotopic composition are used in the Russian Federation, which are graduated, calibrated, and verified with the help of about 2000 certified reference materials (hereinafter referred to as CRMs).

Today, more than 30 standards and certified measurement techniques are used in the Russian Federation to determine carbon, oxygen, and hydrogen isotope ratios. The techniques implemented in these documents are based on the application of foreign CRMs (IAEA, NIST, IRMM, USGS, etc.). Similar domestically-produced CRMs are not available, which makes the prospects of the widespread use of these techniques highly limited in the near future. Therefore, the development of domestic CRMs for isotopic composition presents an extremely important research task.

The characteristic of isotopic composition recommended by IUPAC[2] is the delta value of the isotope ratio [4], e.g., the carbon isotope ratio delta value $\delta^{13}C_{VPDB,}$ which is the relative deviation of the isotope ratio value from the zero of the international reference delta scale VPDB. In accordance with BIPM[3] CIPM/104-26 [5], the traceability of isotope ratio delta values to the international reference isotope ratio delta scale is ensured by using the appropriate international CRMs specified in CIPM/104-26. The most popular among them are CRMs for the isotopic composition of carbon, oxygen, and hydrogen in liquid, solid, and gaseous media. Thus far, more than 20 hydrogen, 40 carbon, 55 oxygen, and 20 nitrogen CRMs have been developed [5]. However, more than 70% of the CRMs have been expended and are not available for purchase according to the IAEA[4] list of available CRMs.

The work [6] described a procedure for manufacturing the CRM—GSO 11,037–2018[5]—for carbon isotopic composition in carbon dioxide gas mixtures. This procedure implies mixing isotopically pure gases $^{12}CO_2$ and $^{13}CO_2$ in a predetermined

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[1] WADA—World Anti-Doping Agency. Available via. https://www.wada-ama.org/en. Accessed 20 August 2022.

[2] IUPAC—International Union of Pure and Applied Chemistry. Available via IUPAC. https://iupac.org/. Accessed 20 August 2022.

[3] BIPM—Bureau international poids et mesures. Available via BIPM. https://www.bipm.org/en/. Accessed 20 August 2022.

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[5] GSO 11037-2018 Reference materials of carbon isotope composition in carbon dioxide gas mixtures (ISUDU-VNIIM-ES). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/389216. Accessed 20 August 2022 (In Russ.).

calculated proportion with oxygen and nitrogen by a gravimetric technique in order to obtain the target volumetric fraction of carbon dioxide-12 and carbon dioxide-13. The tolerance ranges of the certified values are as follows: the carbon dioxide-12 ($^{12}CO_2$) volume fraction—from 0.010 to 99.9%; the carbon dioxide-13 ($^{13}CO_2$) volume fraction—from 0.010 to 1.10%; and the carbon isotopic composition characteristic $\delta^{13}C_{VPDB}$—from −4 to −55‰.

The authors in [7] presented the process of preparing an RM for the isotopic composition of carbon dioxide, which involves gas sampling from different sources, removal of water vapor from the sampled gases, and their sealing in 0.25 dm^3 glass ampoules. After sealing, the ampoules are placed in an oven at 70 °C for 24 h in order to accelerate possible isotopic exchange reactions. According to the CRM certificate available on the IAEA website, the VSMOW2[6] CRM fabrication process involves mixing of three selected freshwater samples obtained from Lake Bracciano, Italy, Lake Galilee, Israel, and a well located near Cairo, Egypt, in appropriate proportions. The δ^2H value of one of the starting materials was changed by a hydrogen diffusion exchange reaction between the starting and deuterated water. The paper [8] described the preparation process of IAEA-603[7] CRM, which required 80 kg of Carrara marble crushed and sieved to obtain about 15 kg of fractions with grain sizes of 200 to 500 μm. The selected fraction was homogenized and split into 31 RM samples.

It can be seen that the production of RMs for isotopic composition involves, as a rule, collecting samples from different natural origins, mixing them if necessary, correcting the isotopic composition of one of the elements by isotopic exchange reactions, purifying to remove impurities, degassing and demineralization (for liquid RMs), crushing and selecting a certain fraction (for solid RMs), drying, homogenization by mixing, and packaging in sealed ampoules.

Homogeneity is conventionally examined by analyzing randomly selected RM samples from a batch. Stability is examined according to ISO Guide 35-2015 [9] by comparison with previously prepared stable RMs. Although the method of mixing of isotopically pure substances is typically used, the as-obtained RMs may not exhibit sufficient chemical purity. In addition, in the case of carbon dioxide, it is impossible to obtain a gas mixture with a given ratio of oxygen isotopes corresponding to the oxygen isotopic composition of exhaled air, alcoholic and food products, and greenhouse gases. The cost of isotopically pure substances is significantly higher than that of pure substances of different isotopic compositions.

The main problem consists in the lack of gas CRMs for the isotopic composition of carbon dioxide, which can be used to calibrate, verify, and graduate instruments used in ^{13}C-urea breath tests. In this test, a patient exhales the first air sample into an isotope analyzer, then takes an aqueous solution of urea enriched with the ^{13}C carbon isotope, and exhales the second air sample into the isotope analyzer. If *H.*

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[6] VSMOW2 Vienna Standard Mean Ocean Water 2 (Hydrogen and Oxygen Isotopes in Water). Available via Merck. https://www.sigmaaldrich.com/RU/en/product/sial/nist8535a. Accessed 20 August 2022.

[7] IAEA-603—reference material calcite ($CaCO_3$). Available via IAEA. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-603.aspx. Accessed 20 August 2022.

pylori is present in the stomach, the isotopic compositions of the first and second samples will be significantly different [10].

At present, the measurement accuracy of such tests is verified as follows: the operator fills two breath collection bags with exhaled air before performing the test, analyzes them, and compares the results. If the result difference is less than a certain value, the test is considered successful. The drift and linearity of the analyzer by the concentration and CO_2 isotope ratio is not assessed. Traceability to the international isotope ratio delta scale is absent. According to the experience of metrological studies of isotope analyzers, the negligence of these factors can lead to significant errors [11–13].

This work is aimed at developing a CRM for the isotopic composition of carbon dioxide for use in ^{13}C-urea breath tests. To achieve this end, the following objectives were formulated: to develop a technique for developing RMs; to manufacture an RM pilot batch; to carry out type approval tests.

Materials and Methods

The material used for CRM preparation included an artificial gas mixture consisting of synthetic air and carbon dioxide, having isotopologues $^{12}C^{16}O_2$, $^{13}C^{16}O_2$, and $^{12}C^{18}O^{16}O$ in its composition. Depending on the components and their content, the mixture was pressurized from 0.1 to 15 MPa in a gas cylinder. The CRM was produced by gravimetric mixing of two carbon dioxides of different isotopic compositions from different sources with synthetic air.

Prior to gravimetric mixing, the impurities in the initial pure gases—nitrogen, oxygen, and carbon dioxide—were analyzed. For this purpose, oxygen was fed into an installation based on a gas chromatograph TGA 1326 and MAESTRO GC. The main analyzed impurities were krypton, argon, hydrogen, carbon (II) oxide, carbon (IV) oxide, methane, nitrogen, and water. Nitrogen was fed into an installation based on a gas chromatograph TGA 1326 and Agilent-7890B, as well as electrochemical gas analyzers Delta F310-H0050M and Delta F310E-S10000. The main analyzed impurities were argon, hydrogen, carbon monoxide (II), carbon monoxide (IV), methane, water, and oxygen. Carbon dioxide was fed into an installation based on a gas chromatograph CHROMOS GC 1000 and Agilent-7890B. The main analyzed impurities were hydrogen, carbon monoxide (II), methane, nitrogen, water, and oxygen. In addition, carbon dioxide from two different sources was fed into an isotope ratio mass spectrometer (hereinafter referred to as IRMS), of the isoprime precision model, to measure the isotopic composition. The measured ratios of stable carbon isotope $\delta^{13}C$ and oxygen $\delta^{18}O$, as well as the measured impurity values in the initial pure gases, were used to calculate the mixing ratio of the components comprising the CRM.

The determination of the mass of all components to obtain the required isotopic composition values was performed using a specially developed technique, consisting of the calculation of isotopic distributions of $^{12}C^{16}O_2$, $^{13}C^{16}O_2$, and $^{12}C^{18}O^{16}O$ in the starting components, using the previously measured ratios of stable carbon and oxygen isotopes. The calculation of the mixing ratio of two carbon dioxide gas sources using the given isotope ratios was performed as follows.

The carbon isotope ratio ^{13}C/^{12}C ($13R$) was determined from the measured $\delta^{13}C$ values in the two carbon dioxide sources:

$$13R = 0.01118 \cdot \left(\frac{1 + \delta^{13}C}{1000}\right), \tag{1}$$

The oxygen isotope ratios ^{18}O/^{16}O ($18R$) and ^{17}O/^{16}O ($17R$) were determined from the measured $\delta^{18}O$ values in the two sources:

$$17R = 0.0003931 \cdot \left(\frac{1 + \delta^{18}O}{1000}\right)^{0.52}, \tag{2}$$

$$18R = 0.00208835 \cdot \left(\frac{1 + \delta^{18}O}{1000}\right), \tag{3}$$

The isotopic abundance of carbon-12 ($12X$) was determined from the calculated ^{13}R isotope ratios:

$$12X = \frac{1}{1 + 13R}, \tag{4}$$

The isotopic abundances of oxygen-16 ($16X$) and oxygen-18 ($18X$) were determined from the calculated ^{17}R and ^{18}R isotope ratios:

$$16X = \frac{1}{1 + 17R + 18R}, \tag{5}$$

$$18X = 16X \cdot 18R \tag{6}$$

The isotopic abundances of ^{12}C^{16}O^{16}O ($626X$), ^{13}C^{16}O^{16}O ($636X$), and ^{12}C^{18}O^{16}O ($628X$) were determined from the calculated isotopic abundances:

$$626X = 12X \cdot 16X \cdot 16X, \tag{7}$$

$$636X = (1 - 12X) \cdot 16X \cdot 16X, \tag{8}$$

$$628X = 12X \cdot 16X \cdot 18X, \tag{9}$$

Similarly, the abundances of isotopologues ^{12}C^{16}O$_2$, ^{13}C^{16}O$_2$, and ^{12}C^{18}O^{16}O in the target gas mixture were calculated based on the given δ^{13}C and δ^{18}O values.

Using the calculated abundances of isotopologues ^{12}C^{16}O$_2$, ^{13}C^{16}O$_2$, and ^{12}C^{18}O^{16}O in the two carbon dioxide sources and the target gas mixture, the Y

mixing ratio of the two carbon dioxide sources to obtain the target gas mixture was found:

$$Y = \frac{626X_{mix} - 626X_{cyl1}}{626X_{cyl2} - 626X_{mix}}, \tag{10}$$

where $626X_{mix}$ is the abundances of isotopologue $^{12}C^{16}O_2$ in the target gas mixture, $626X_{cyl1}$ is the abundances of isotopologue $^{12}C^{16}O_2$ in the first carbon dioxide source, $626X_{cyl2}$ is the abundances of isotopologue $^{12}C^{16}O_2$ in the second carbon dioxide source.

For the gravimetric mixing of CRM components, the equipment included in GET 154–2019[8] was used. After preparation, the CRM was reloaded to the IRMS for isotopic composition measurements for CRM validation purposes. At the same time, for the IRMS calibration international RMs (hereinafter referred to as IRMs) NBS18, IAEA-603, IAEA-610, IAEA-611, and IAEA-612,[9] representing different natural forms of calcium carbonate $CaCO_3$, were used. In order to feed the IRMs to the IRMS, $CaCO_3$ was reacted with orthophosphoric acid H_3PO_4, which was prepared according to the procedure described in [14].

For this purpose, 220–380 μg of IRM was placed in a 4 cm^3 glass vial. After thoroughly purging the vial with high-purity helium to remove atmospheric air, orthophosphoric acid was injected such that the acid completely covered the IRM. The reaction of interaction between IRM and orthophosphoric acid was carried out at 25 ± 0.1 °C for 24 h in accordance with the recommendations [15]. After the reaction was completed, the obtained CO_2 in a helium flow was transferred to the IRM. A total of 120 vials of IRMs, 24 vials of each IRM, were prepared and submitted to the IRMS. The measurements were performed for 8 days. The samples were fed in the following order: IAEA-610 (3 vials)—NBS18 (3 vials)—IAEA-603 (3 vials)—Analyzed gas (10 vials)—IAEA-611 (3 vials)—IAEA-612 (3 vials). Eight repetitions of this sequence over 8 days were performed. Within RM stability and homogeneity control, 40 measurements were carried out in 4 days after the accelerated aging according to RMG 93–2015 [16]. The point of the accelerated aging method was to amplify the factors causing the trend of the certified characteristic

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[8] GET 154-2019 State Primary Standard of the Unit of Mole Fraction and Mass Concentration of Components in Gas and Gas Condensate Environments: Custodian Institute D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1365155. Accessed 20 August 2022 (In Russ.).

[9] NBS18 reference material calcite ^{13}C and ^{18}O. Available via IAEA. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/NBS18.aspx. Accessed 20 August 2022.

IAEA-603 reference material calcite ($CaCO_3$). Available via IAEA. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-603.aspx. Accessed 20 August 2022.

IAEA-610 reference material carbonate ^{13}C. Available via IAEA. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-610.aspx. Accessed 20 August 2022.

IAEA-611 reference material carbonate ^{13}C. Available via IAEA. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-611.aspx. Accessed 20 August 2022.

IAEA-612 reference material carbonate ^{13}C. Available via IAEA. https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/IAEA-612.aspx. Accessed 20 August 2022.

Fig. 1 General view of reference material heating

by heating the RM. The Vant-Goff formula was used to calculate the heating time. The duration of accelerated aging was 6 days at 80 °C. The pressure in the cylinder during the heating was increased from 9 to 11 MPa. The heating was accomplished with a thermocouple and a heating cable as shown in Fig. 1.

Results and Discussion

Following the methodology described above and based on the analysis of existing techniques, RMs for the isotopic composition of carbon dioxide for use in ^{13}C-urea breath tests were developed.

A pilot batch of seven pressurized RM cylinders of two types was produced: an aluminum cylinder of Luxfer and an aluminum cylinder of NPO POISK. Both types of cylinders were equipped with brass valves and had a volume of 1 dm^3. A general view of the RM is presented in Fig. 2.

The results of the RM certification and homogeneity test are presented in Table 1. In total, 20 measurements of RMs from each cylinder were performed. Table 1 shows the average X_1^R, X_2^R values of 10 measurements.

Fig. 2 General view of the pilot batch of reference materials

The results of the accelerated aging CRM stability tests in the two above-mentioned types of cylinders are presented in Table 2. In total, 44 measurements were performed for each cylinder. Table shows the average X_1^R, X_2^R values of 22 measurements.

The results of $\delta^{13}C$ and $\delta^{18}O$ measurements of mixture No. 3 before and after heating are presented in Figs. 3 and 4. The measurement results before and after heating the cylinder are presented to the left and to the right of the vertical line, respectively.

A total of 44 measurements were performed in eight days. The standard deviation (SD) was 0.13‰ and 0.24‰ for $\delta^{13}C$ and $\delta^{18}O$, respectively. The difference between the mean values of the measurement results before and after the heating was less than the SD of the measurement results and was less than 0.1‰, which confirms the stability of the developed CRMs.

Tests for the type approval of the developed CRMs were finalized. The CRM was registered in the Federal Information Fund for Ensuring the Uniformity of Measurements (hereinafter referred to as FIF EUM) as GSO 11999-2022. The metrological characteristics of the developed CRMs are presented in Table 3.

The expanded uncertainty of the developed CRMs satisfies the requirements for the accuracy of the ^{13}C-urea breath test [17, 18]. The test is considered positive when the difference in isotopic composition between the samples exceeds 4‰, negative when it is less than 3‰, and intermediate (threshold) when it is between 3‰ and 4‰ [10].

Table 1 Measurement results for the delta value of carbon δ^{13}C and oxygen δ^{18}O isotope ratios

Mixture No.	Certified characteristic	Certified value, ‰	Resulting value of the certified characteristic, ‰				U***, ‰
			July 28, 2022		August 12, 2022		
			X_1^R*	$\lvert X_1^R - X^C$**$\rvert$	X_2^R	$\lvert X_2^R - X^C\rvert$	
1	δ^{13}C	– 5.0	– 4.91	0.09	– 4.69	0.09	0.4
	δ^{18}O	– 12.01	– 11.62	0.39	– 11.77	0.24	0.4
2	δ^{13}C	– 27.5	– 27.17	0.33	– 27.18	0.33	0.4
	δ^{18}O	– 29.33	– 29.55	0.22	– 29.41	0.08	0.4
3	δ^{13}C	– 43.35	– 43.20	0.15	– 43.11	0.24	0.4
	δ^{18}O	– 24.95	– 24.56	0.39	– 25.08	0.13	0.4
4	δ^{13}C	– 5.0	– 4.69	0.31	– 5.04	0.04	0.4
	δ^{18}O	– 12.01	– 11.77	0.24	– 11.71	0.30	0.4
5	δ^{13}C	– 27.5	– 27.38	0.12	– 27.21	0.29	0.4
	δ^{18}O	– 29.33	– 29.21	0.13	– 29.15	0.18	0.4
6	δ^{13}C	– 43.35	– 43.24	0.11	– 43.16	0.19	0.4
	δ^{18}O	– 24.95	– 24.61	0.34	– 25.19	0.24	0.4
7	δ^{13}C	– 43.35	– 43.47	0.12	– 43.41	0.06	0.4
	δ^{18}O	– 24.95	– 24.83	0.12	– 24.78	0.17	0.4

* X^R, the value of the certified characteristic

** X^C, the certified value of the characteristic

*** U, the value of the expanded uncertainty at the coverage factor $k = 2$ assigned to a given RM type (from RM type description)

Metrological characteristics of the developed CRMs were compared with foreign CRMs. The metrological characteristics of CRMs produced by the National Institute of Standards and Technology (hereinafter referred to as NIST), USA are presented in Table 4.

As can be seen from Table 3, the expanded uncertainty values of the developed CRMs are higher than those of CRMs produced by NIST. However, the latter cannot be used for the calibration of MIs used for ^{13}C-urea breath tests due to their insufficient volume of 0.001 dm^3. The volume of the developed CRMs exceeds 10 dm^3.

In comparison with the previously developed GSO 11037-2018, GSO 11999-2022 exhibits a higher chemical purity. In addition, the developed preparation technique makes it possible to obtain a gas mixture with a given ratio of oxygen isotopes corresponding to the oxygen isotopic composition of exhaled air, alcoholic and food products, and greenhouse gases. The cost of the developed CRMs is significantly lower than that of isotopically pure GSO 11037-2018.

Given the above metrological characteristics, the research aim was successfully achieved. CRMs for the isotopic composition of carbon dioxide for use in ^{13}C-urease breath tests was developed. Moreover, the developed CRMs can be used for

Table 2 Results of the RM stability test

Mixture No.	Cylinder type	Certified characteristic	Resulting value of the certified characteristic				U***
			March 17, 2022 (before heating)		May 6, 2022 (after heating)		
			X_1^{R}*	$\lvert X_1^{R} - X^{C}$**$\rvert$	X_2^{R}	$\lvert X_2^{R} - X^{C}\rvert$	
3	Luxfer aluminum cylinder, brass valve, volume 1 dm^3	Delta value of carbon isotope ratios $\delta^{13}C$, ‰	– 43.09	0.26	– 42.95	0.4	0.4
		Delta value of oxygen isotope ratios $\delta^{18}O$, ‰	– 24.98	0.03	– 24.89	0.06	0.4
7	NPO POISK aluminum cylinder, brass valve, volume 1 dm^3	Delta value of carbon isotope ratios $\delta^{13}C$, ‰	– 43.43	0.08	– 43.39	0.04	0.4
		Delta value of oxygen isotope ratios $\delta^{18}O$, ‰	−24.83	0.12	– 24.88	0.07	0.4

* X^R, the value of the certified characteristic

** X^C, the certified value of the characteristic

*** U, the value of the expanded uncertainty at the coverage factor $k = 2$ assigned to a given RM type (from RM type description)

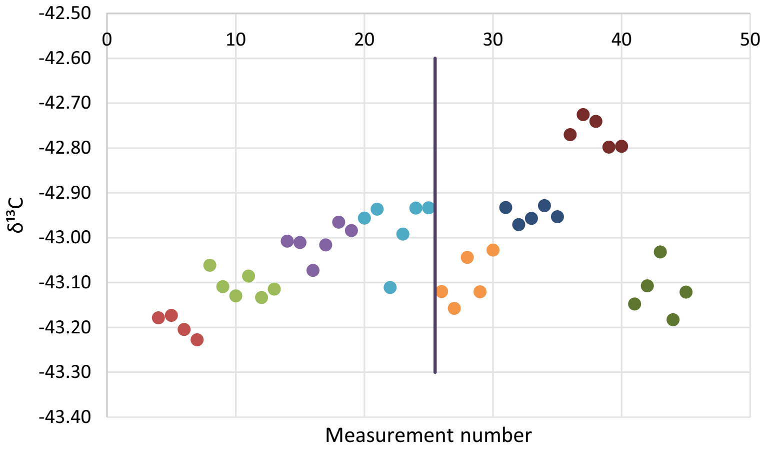

Fig. 3 Measurement results of the delta value of carbon isotope ratios $\delta^{13}C$

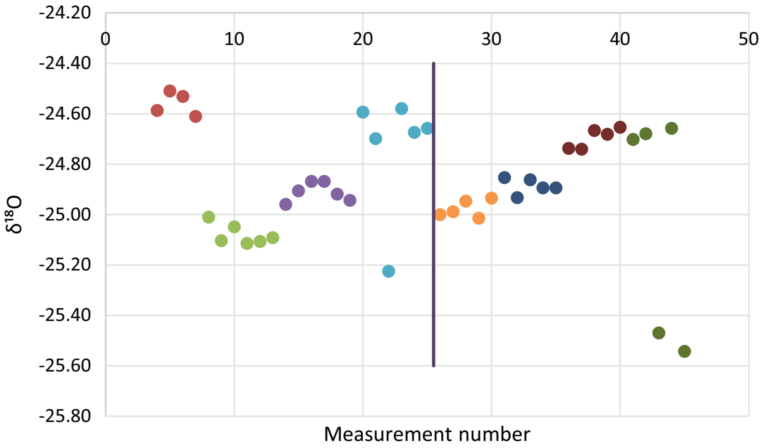

Fig. 4 Measurement results of the delta value of oxygen isotope ratios δ^{18}O

Table 3 Standardized metrological characteristics of the developed CRMs

Certified characteristic, measurement units	Tolerance range of certified values	Expanded uncertainty (U)* tolerance values at $k = 2$ and $P = 0.95$
Delta value of carbon isotope ratios δ^{13}C, ‰	−50 to −5	0.4
Delta value of oxygen isotope ratios δ^{18}O, ‰	−30 to −5	0.4

The tolerance range of the relative deviation from the nominal values of certified characteristics:
± 2.0‰ for the characteristic "delta value of isotope ratios";
± 20% for the characteristic "components molar fraction."
* Numerically equal to the tolerance range of the absolute error at the confidence level of $P = 0.95$

Table 4 Measurement results for the delta value of carbon δ^{13}C and oxygen δ^{18}O isotope ratios in CRMs produced by NIST

CRM	Delta value of carbon isotope ratios δ^{13}C, ‰	Expanded uncertainty value δ^{13}C at the coverage factor $k = 2$, ‰	Delta value of oxygen isotope ratios δ^{18}O, ‰	Expanded uncertainty value δ^{18}O at the coverage factor $k = 2$, ‰
CRM 8562	– 3.76	0.06	8.45	0.22
CRM 8563	– 41.56	0.12	– 23.72	0.22
CRM 8564	– 10.45	0.08	0.19	0.20

the calibration of ^{IRMS} in the fields of gas, chemical, and food industries, oil refining, healthcare, atmospheric monitoring, and scientific research.

Further studies will be focused on the development of CRMs of carbon, oxygen, and hydrogen isotope ratios in liquid, solid, and gaseous media, particularly for the purpose of foreign RM substitution.

Conclusion

CRMs for the isotopic composition of carbon dioxide that can be used in ^{13}C-urea breath tests were developed. This aim was achieved by progressively solving the following problems: development of a technique for obtaining RMs, production of a pilot RM batch, and carrying out RM type approval tests. The obtained CRM was registered in the FIF EUM as GSO 11999-2022.

The developed CRMs can improve the accuracy and validity of ^{13}C-urea breath tests. Although the existing CRMs produced by the National Institute of Standards and Technology, USA, are characterized by lower uncertainty values, they cannot be used for the calibration of MIs used for ^{13}C-urea breath tests due to their insufficient volume. The expanded uncertainty of the developed CRMs satisfies the requirements for the accuracy of the ^{13}C-urea breath test. The developed technique can be used to produce RMs with any isotopic composition across a wide range of isotope ratios, which is important for the calibration, verification, and graduation of the respective MIs. Compared to the previously developed GSO 11037-2018, the developed CRMs exhibits a higher chemical purity. In addition, the developed preparation technique makes it possible to obtain a gas mixture with a given ratio of oxygen isotopes corresponding to the oxygen isotopic composition of exhaled air, alcoholic and food products, and greenhouse gases. The cost of the developed CRMs is significantly lower than that of isotopically pure CRMs.

The traceability of the certified RM values to the international reference isotope ratio delta scale is ensured by using the appropriate international CRMs, which ensures international recognition of measurement results.

Further studies will be focused on the development of CRMs of carbon, oxygen, and hydrogen isotope ratios in liquid, solid, and gaseous media, particularly for the purpose of foreign RM substitution. The developed technique of gravimetric RM preparation from pure substances of different isotopic compositions will be used to obtain materials with a wide range of isotopic composition certified values.

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On Developing Metrological Assurance to Identify and Quantify the Content of Non-infectious Food Protein Allergens of Animal or Plant Origin in Food Products

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Abstract As specified in Technical Regulations of the Customs Union 022/2011 “Food products in terms of their labeling”, allergen presence in food composition is subject to mandatory control. Creating a metrological assurance system to identify and quantify the content of non-infectious food protein allergens of animal or plant origin in foods includes developing and certifying identification and quantification techniques. Other objectives are performing round-robin comparative tests (proficiency testing), creating and testing reference materials for type approval. Within this research framework, we developed and certified methods to identify and quantify the content of non-infectious food protein allergens of plant (gluten, peanuts, nuts, soybeans) and animal (cow’s milk, chicken eggs, fish, seafood) origin using reagent kits for enzyme-linked immunosorbent assay produced by XEMA LLC. The samples included all types of foods and objects related to requirements for foods, countertop wipe samples obtained during in-process inspection. Quantitative techniques were certified according to the legislative requirements for measurement assurance using imported food allergen reference materials. The lower sensitivity limits (detection limits) for the presence or absence of antigens by allergen protein mass ranged from

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0.001 ppb to 5 ppm. The developed methods ensure food safety when establishing product compliance with the legislation on technical regulation.

Keywords Food allergy · Allergens · Measurement technique · Test system · Enzyme-linked immunosorbent assay

Introduction

Today, allergy is commonly understood as manifestations of the immune system hypersensitivity to an allergen (antigen) upon repeated contact with it. In case of food allergies, such a reaction occurs when consuming foods in which the main allergens are proteins, both simple and complex (for example, glycoproteins), less often polypeptides (haptens). The latter combine with food proteins and acquire the property of immunogenicity [1]. The ability of a food protein to act as an allergen depends on the presence of an "epitope" in its composition. This is the part of an allergen macromolecule which is recognized by the immune system [1]. Thus, epitopes determine specificity and induce antibody responses [1, 2]. In accordance with the International Food Code [3, 4], the most allergenic foods include cow's milk, chicken eggs, wheat, peanuts, nuts, soybeans, fish, and seafood (crustaceans and mollusks). These products constitute the so-called "big eight allergens".

Both mild (urticaria) and severe allergic reactions, such as fulminant and life-threatening food anaphylaxis, can develop in response to the consumption of these foods. In some cases, a minimal amount of the allergen, for example, when inhaling peanut smell or when allergens are ingested with breast milk, can trigger a systemic reaction. Some food allergens are able to change their antigenic properties when foods are cooked. Moreover, enzymes (and after entering the body, digestive fluids) affect them. For instance, protein denaturation when a product is heated makes some products lose their allergenicity while others, on the contrary, become more allergenic [1, 5–7]. For example, roasting peanuts increases their allergenicity. Conversely, some cow's milk and chicken egg proteins are destroyed and lose this property.

If there is an allergic reaction to any food, patients should go to a health facility. Based on a patient's account of their medical history (anamnesis), doctors clarify and identify the "guilty" food product. After that they suggest the patient undergo skin tests, which give a quick result at a minimum cost. However, the obtained results are often false positive due to cross-reactivity [8]. Subsequently, the patient must stick to a certain strict diet that completely excludes food containing the allergen and to buy foodstuffs labeled as allergen-free. In accordance with the requirements of Technical Regulations of the Customs Union TR CU 022/2011 [9], "components, including food additives, flavorings, dietary supplements, whose use may cause allergic reactions or is contraindicated in certain disease types, are specified in the composition of foods regardless of their quantity." Thus, the presence of allergens in the composition of food products is subject to mandatory control. At the same time, it should also be considered that the unintentional allergen presence in a food product can result from allergen cross-contamination throughout the supply chain, i.e. while storing, transporting, and producing it [10].

With advances in immunology, food allergen detection methods are being improved. While developing such techniques, attention is given to their sensitivity and specificity to facilitate more accurate detection of a particular allergen. Currently, there is an array of methods of analysis. They can be conveniently divided into four large groups: mass spectrometry, nucleic acid-based methods, biosensors, and immunoassay methods [8].

Mass spectrometry is a method of identifying and studying substances. It is based on the ionization of atoms (molecules) in the composition of a substance and the subsequent registration of the mass spectrum of the resultant ions. A significant difference between mass spectrometry and other analytical spectral methods, for example, optical methods, is that they detect radiation or energy absorption by molecules or atoms, while mass spectrometry directly detects substance particles themselves [11, 12]. The method has a fairly high sensitivity. However, it is expensive due to the cost of the equipment for analysis.

Nucleic acid-based methods are analytical methods based on the detection of genes encoding the target antigen. These methods have an important advantage over antigen detection since they identify the gene, i.e. a more stable molecule, even after exposure to such factors as high temperature, fermentation, acidity, and so on. Among these methods, the polymerase chain reaction (PCR) is the most common technique [13]. Limitations include the effect of heat treatment on the result due to detectable DNA destruction, difficulties in discriminating between different food products (e.g., eggs and chicken contain the same genetic material), and the inability to detect certain allergens at low concentrations (for example, egg and milk).

Biosensors are combination devices consisting of a biochemically or biologically active component (or a biological component, i.e. an enzyme, an antibody, an antigen, a microorganism, a biological membrane) and an electronic transducer. The advantage of biosensors is high biocomponent specificity, as well as its ability to perform recognition without additional energy input (temperature increase, applied potential, and so on) [14, 15]. Limitations of using biosensors are associated with their slow reaction rate, low reproducibility and stability, the difficulty in obtaining bioorganic materials of constant composition, sensitivity to high and low temperatures, bactericidal contamination.

Immunoassay diagnostic methods are based on antigen–antibody interaction. Among immunoassay methods, the enzyme-linked immunosorbent assay (ELISA) is the most widely used method [8] as it is sensitive and inexpensive. There are competitive and non-competitive ELISAs. The difference between the two methods is that at the first stage of the competitive method, not only the antigen itself is present, but also its analog. Thus, two antigens compete for binding to antibodies. The non-competitive ELISA lacks the analog of the target allergen. Thus, there is no competition for binding to the antibody. Moreover, this assay has a higher analytical sensitivity and is simple to perform. Currently, ELISA is an internationally recognized method. It is the most frequently used assay for the routine detection of allergens in food products [3].

XEMA LLC (Moscow) has developed reagent kits that enable identification and quantification of the content of non-infectious food protein allergens of animal or plant origin in samples of all types of food products and objects related to the requirements for foods, as well as wipe samples obtained from countertops during in-process inspection. According to the current legislation in the field of technical regulation (No. 184-FZ On technical regulation [16]), certified measurement techniques must be used to assess product compliance with the mandatory requirements.

Since 2016, the specialists of the UNIIM, a branch of the D. I. Mendeleev All-Russian Research Institute for Metrology (VNIIM), have been working on the metrological assurance of the enzyme-linked immunosorbent assay using the available instrumental and reference base [17, 18]. The cumulative experience resulted in a consistent methodological approach to the development of a metrological assurance complex for measurements performed using ELISA kits [19]. The complex includes the following types of work: (1) developing and certifying identification and quantification methods; (2) conducting interlaboratory comparative tests (proficiency testing); (3) creating and testing standard reference materials for the composition of allergens for type approval. These studies have significantly contributed to ensuring food safety, improving people's quality of life, and detecting adulterated food products. However, the issue of quantitative determination of allergens is still relevant.

This study aims to develop and certify methods for identifying and quantifying the content of non-infectious food protein allergens of animal or plant origin in samples of all types of food products. Moreover, the investigation also includes objects related to the requirements for foods, as well as countertop wipe samples obtained during in-process inspection.

Materials and Methods

Description of the Enzyme-Linked Immunosorbent Assay (ELISA)

The reagent kits manufactured by XEMA LLC use a non-competitive (sandwich) ELISA. It has the highest level of sensitivity and specificity due to the use of a pair of matching antibodies [20]. The test sample is applied to the microplate wells whose surface is coated with specific capture antibodies against the allergen. The antigen from the sample binds to the antibodies on the well surface. The microplate is washed to remove unbound antigen. Peroxidase-labeled secondary antibodies to the same antigen are applied to the wells. After repeated washing, the enzyme activity is manifested by adding a chromogen-substrate mixture, a stopping solution. Then it is subjected to optical density measurement on a spectrophotometer at a wavelength of 450 nm. The spectrophotometer blank solution is set to zero. Figure 1 shows the diagram of non-competitive (sandwich) ELISA.

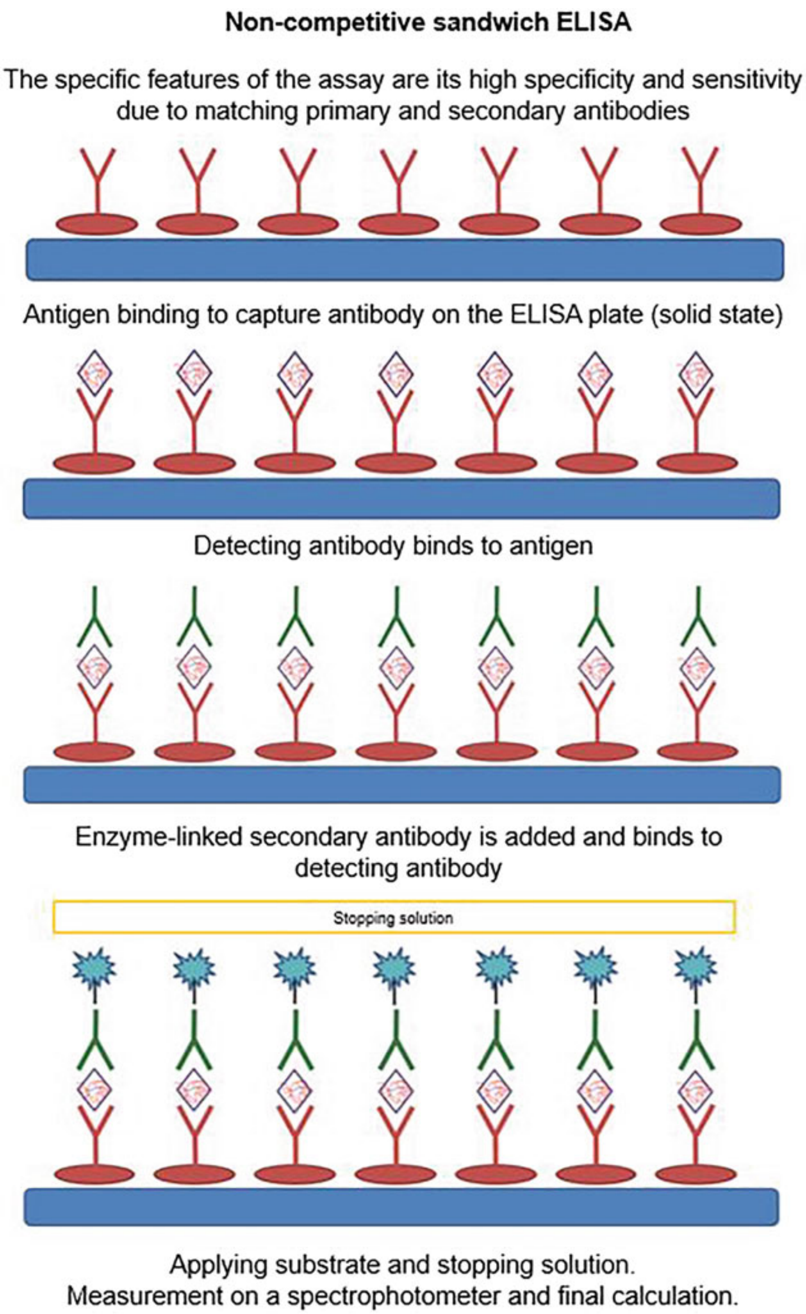

Fig. 1 Schematic diagram of non-competitive (sandwich) enzyme-linked immunosorbent assay

The technique for measuring the allergen mass fraction in samples is based on the dependence of the optical density of the test solution at a wavelength of 450 nm on the allergen mass fraction. To build a calibration curve, we used calibration solutions containing the allergens under study. They are included in the reagent kit manufactured by XEMA LLC. The calibration curve plot represents a linear proportional relationship described by a first order polynomial.

The Certification Procedure of Measurement Techniques

A microplate spectrophotometer (Multiskan FC, Thermo Scientific), a non-automatic Sartorius CPA225D balance (accuracy class I), a laboratory centrifuge CM 6MT (ELMI), automatic dispensers (Biohit), and a set of volumetric glassware were used to certify the methods of quantifying the content of non-infectious food protein allergens of animal or plant origin in food products (measurement techniques).

Working samples of flour, grain, bran, and pasta were used as samples to evaluate the accuracy of the gluten content measurement technique. The measurement validity indicators were evaluated by the calculation-experimental method for assessing the quality indicators of the analysis technique (as a characteristic of the error of its components) according to Appendix C of the RMG (CIS Standardization Recommendations) 61-2010 [21].

In order to determine the applicable scope of the measurement techniques, we analyzed the OKVED (RCPEA) 2 groups (All-Russian classifier of products by type of economic activity). The most characteristic members from each group were selected as samples for evaluation. Admittedly or presumably, they did not contain the allergens under study and totaled 34 working samples. These samples of food products and raw foods included barley, sesame seeds, brown rice, minced chicken breast fillet, minced beef, minced pork, frozen cooked shrimps, frozen cod fillet, frozen cooked mussels, canned tomatoes, canned pineapple, frozen chanterelles, lactose-free milk containing 0.5% fat, Rossiysky cheese, cottage cheese containing 9% fat, rye flour, oat bran, buckwheat flour, multi-grain hardtack cookies, sponge cakes, couscous grain, milk chocolate, boiled chicken egg, baby formula, chocolate pudding, whey protein isolate, instant oatmeal porridge, oat milk, rice milk with a coconut flavor, almond milk, vanilla flavoring, food supplement “Clerici Dry Animal Enzyme (chymosin/pepsin)”, corn starch, as well as wipe samples obtained from countertops.

Samples of products and food raw materials were taken in accordance with the GOSTs for the analyzed type of food products, or other regulatory and/or technical documents on sample taking in force at enterprises. Next, we prepared sample extracts in accordance with the instructions for ELISA test kits.

The indicators of measurement technique precision were evaluated according to the results of the interlaboratory experiment involving five laboratories. The experiment was performed in compliance with GOST R 5725-2-2002 [22]. We assessed the accuracy indicators of quantitative measurement techniques employing the standard addition method in accordance with the RMG 61-2010 regulations. The solutions used to introduce additives were prepared by diluting a well-known weighed sample of imported food allergen reference materials:

- pure substance of soybean trypsin inhibitor (Trypsin inhibitor from Glycine max (soybean), Product No: T9128, Batch No. SLBR5919V) in dry form 1.0 g with a mass fraction of the standard substance of 100.00%, manufactured by Sigma Aldrich;
- pure substance of the sum of the αs1, αs2, β, and κ casein fractions (Casein from cow's milk, powder, CAS No. 9000-71-9, Product No: C3400, Batch No: SLBZ2763) in dry form 1.0 g with a mass fraction of the standard substance of 91.23%, manufactured by Sigma Aldrich.

Additionally, performing the experiment we established the detection limits for the techniques employed to identify non-infectious food protein allergens of animal or plant origin in food products, as well as in wipe samples obtained from countertops.

Results and Discussion

In 2018–2022, the collaboration between XEMA LLC and UNIIM, the affiliated branch of the D. I. Mendeleyev all-Russian Institute for Metrology, continued to provide metrological assurance of quality control and food safety [17, 18]. We developed, certified, and tested the applicability of a technique for measuring the gluten content in grain products by enzyme-linked immunosorbent assay using the GLIADIN-ELISA reagent kit manufactured by XEMA LLC. Moreover, we prepared a series of documents entitled "Methods for identifying and quantifying the content of non-infectious food protein allergens of animal or plant origin in samples of all types of food products and objects related to the requirements for food products, as well as wipe samples obtained from countertops during in-process inspection using ELISA reagent kits manufactured by XEMA LLC".

The technique for measuring the gluten content in processed grains by ELISA using the GLIADIN-ELISA reagent kit manufactured by XEMA LLC was devised during elaborating GOST 33838-2016 [23, 24].

According to the Codex Alimentarius Commission [25], gluten is defined as a protein fraction of wheat, rye, barley, oats, or their hybrids, as well as derivatives from this protein fraction. Some people can have an intolerance to gluten. Gluten consists of two fractions: prolamins and glutelins. The gluten content directly depends on the content of the prolamin fraction.

The measurement range of the gluten mass fraction is from 2.0 to 100.0 ppm (mg/kg) inclusive. Table 1 gives the metrological characteristics of the measurement technique.

Appendix A contains a technique for a qualitative sample analysis of the presence or absence of gluten using an immunochromatographic test strip from the GLUTEN XEMATest Kit Manufactured by XEMA LLC.

Table 1 Values of the accuracy, correctness, repeatability, and reproducibility indices of the technique for measuring the gluten mass fraction

Repeatability index (relative standard deviation of repeatability), σ_{r_0}, %	Reproducibility index (relative standard deviation of the reproducibility of a single measurement result), σ_{R_0}, %	Correctness index (residual systematic error bounds at confidence coefficient $P = 0.95$), $\pm\,\delta_c$, %	Accuracy index (relative error bounds at confidence coefficient $P = 0.95$), $\pm\,\delta$, %
10.0	15.0	6.6	30.0

The GLUTEN XEMATest is based on a pair of monoclonal antibodies. It detects gliadin (the main component of gluten) and prolamin glycoproteins (hordein and secalin) related to it. These two make up about 50% of the total gluten content with high sensitivity and specificity, which also hods true after prolonged thermal food processing.

Table 2 shows the sensitivity of the GLUTEN XEMATest (detection limit) and the detection range in the extract prepared according to the procedure described in the technique (mass/volume ratio of 1:10) for various prolamins.

The results of interlaboratory comparative tests aimed to determine the quality indicators of pasta enabled us to confirm the applicability of the qualitative and quantitative analytical methods for detecting gluten in processed grain products. These tests were performed in accordance with GOST ISO/IEC 17043-2013 [26]. We have also begun developing a reference material [27]. The measurement technique can be used to confirm the compliance of products with the mandatory requirements established in the technical regulation of the Customs Union TR CU 027/2012 [28].

The first document of the series "Methods for identifying and quantifying the content of non-infectious food protein allergens of plant origin in samples of all types of food products and objects related to the requirements for food products, as well as wipe samples obtained from countertops during in-process inspection using ELISA reagent kits manufactured by XEMA LLC" includes the following methods for the qualitative and quantitative analysis of non-infectious food protein allergens of plant origin:

1. *The qualitative screening method for detecting the presence or absence of soybean trypsin inhibitor antigen in test samples using the SOYA XEMATest reagent kit.*

Table 2 Characteristics of the qualitative determination of the presence or absence of gluten

Cereal grain	Prolamin	Limit of detection, mg/kg	Range of detection, mg/kg
Wheat	Gliadin	2	2–2000
Barley	Hordein	2	2–40,000
Rye	Secalin	3	3–200,000
Oats	Avenin	200	200–400,000

The methodology applies to samples of all types of food products and objects related to the requirements for food products arising during their production (manufacturing), storage, shipment (transportation), sale, and disposal, as well as wipe samples taken from countertops during in-process inspection. It establishes a procedure for a qualitative screening of test samples for the presence or absence of soybean trypsin inhibitor antigen using an immunochromatographic test strip from the SOYA XEMATest kit manufactured by XEMA LLC.

Soybean trypsin inhibitors (hereinafter referred to as STI) are proteins present in soybean seeds in an amount of up to 25% of the total protein mass [29]. These proteins interfere with food digestion processes by suppressing the activity of trypsin and chymotrypsin, causing allergic reactions. The Kunitz trypsin inhibitor whose isoforms have molecular mass in the range of 20–25 kDa, as well as the Bauman–Birk inhibitor with a molecular mass of 8 kDa are the main inhibitors which are identified and well-studied. The disulfide bonds in the structure of the inhibitors ensure the high stability of the molecule under various influences.

The SOYA XEMATest is based on the immunochromatographic principle. It uses a combination of monoclonal antibodies to detect STI. The target antigen is bound by specific antibodies attached to the stained microparticles. This complex then migrates to the test line where it binds to another specific antibody, forming a colored line indicating a positive result. The lower detection limit of the STI antigen in the extract is about 1 mg/kg (ppm). The upper detection limit is about 2000 mg/kg (ppm).

Using the SOYA XEMATest is recommended to conduct a qualitative screening analysis of the test samples for the presence or absence of the STI antigen before employing quantitative methods for measuring the STI mass fraction. If a positive result is obtained, it is concluded that the STI antigen is present in the test sample.

2. *The technique for measuring the mass fraction of soybean trypsin inhibitor in samples of all types of food products and objects related to the requirements for food products, as well as wipe samples taken from countertops during in-process inspection, by enzyme-linked immunosorbent assay using the Soybean trypsin inhibitor-ELISA reagent kit manufactured by XEMA LLC* [30].

The measurement range of the STI mass fraction is 0.5 to 25.0 μg/kg (ppb) inclusive. The detection limit is 0.1 μg/kg (ppb). Table 3 gives the metrological characteristics of the measurement technique.

Table 3 Values of the accuracy, correctness, repeatability, and reproducibility indices of the technique for measuring the STI mass fraction

Repeatability index (relative standard deviation of repeatability), σ_{r_0}, %	Reproducibility index (relative standard deviation of the reproducibility of a single measurement result), σ_{R_0}, %	Correctness index (residual systematic error bounds at confidence coefficient P = 0.95), $\pm\ \delta_c$, %	Accuracy index (relative error bounds at confidence coefficient $P = 0.95$), $\pm\ \delta$, %
13	18	19	40

3. *The technique for the qualitative determination of the presence or absence of non-infectious food protein allergens of plant origin using reagent kits manufactured by XEMA LLC.*

Table 4 gives the numbers of reagent kits produced by XEMA LLC, the list of allergens with the corresponding target antigens, as well as the lower sensitivity limits (limits of detection) for the presence or absence of antigens (LOD) by allergen protein mass in extracts prepared according to the procedure described in the methodology.

The second document in the series (Part 2) [31] includes the following techniques for the qualitative and quantitative analysis of non-infectious food protein allergens of animal origin:

1. The methodology of qualitative screening analysis for the presence or absence of milk antigen in test samples using the Milk XEMATest Reagent Kit.

The methodology applies to samples of all types of food products and objects related to the requirements for food products arising during their production (manufacturing), storage, shipment (transportation), sale, and disposal, as well as wipe

Table 4 Characteristics of the qualitative determination of the presence or absence of non-infectious food protein allergens of plant origin

Kit number	Allergen	Target antigen	LOD, mg/kg (ppm)
K371P	Pecan	*Ca i1*—specific fragment of pecan *2S albumin*	0.4
K371W	Walnut	*Jug r4*—specific fragment of walnut *11 S globulin*	5.0
K372	Hazelnut	*Cor a 9*—specific fragment of hazelnut *11S globulin*	0.003
K373	Peanut	*Ara h1*—peanut 7 *S vicilin-like globulin*	0.005
K374	Almond	*Pru du 6*—specific fragment of almond *11 S globulin*	0.006
K375	Macadamia	*Mac i1 and Mac i2*—specific fragments of macadamia *11 S globulin*	1.0
K376	Cashew	*Ana o1*—specific fragment of cashew *11 S legumin*	1.0
K377	Pistachio	*Pis v2*—specific fragment of pistachio *11 S legumin*	1.0
K378	Brazil nut	*Ber e2*—specific fragment of Brazil nut *11 S globulin*	1.0
K385	Lupine	*Lup a5*—specific fragment of lupine *profiling*	0.32
K389	Sesame	*Ses i2*—specific fragment of sesame *2S albumin*	0.14
K392	Celery	*Api g1*—specific fragment of celery *PR-10* pathogenesis-related protein	0.4
K395	Mustard plant	*Sin a1*—specific fragment of mustard plant *2S albumin*	0.10

samples taken from countertops during in-process inspection. It establishes a procedure for a qualitative screening of test samples for the presence or absence of milk using an immunochromatographic test strip from the Milk XEMATest kit manufactured by XEMA LLC.

The Milk XEMATest is based on the immunochromatographic principle. It uses a combination of monoclonal antibodies to detect milk antigens. The lower detection limit of milk antigens in the extract is about 1 mg/kg (ppm).

Using the Milk XEMATest is recommended to conduct a qualitative screening analysis of the test samples for the presence or absence of milk antigens before employing quantitative methods for measuring total casein mass fraction. If a positive result is obtained, it is concluded that milk antigen is present in the test sample.

2. The technique for measuring the total casein mass fraction in samples of all types of food products and objects related to the requirements for food products, as well as wipe samples taken from countertops during in-process inspection, by enzyme-linked immunosorbent assay using the Milk ELISA reagent kit manufactured by XEMA LLC.

Caseins are a group of proteins found in cow's milk along with whey proteins. Both whey and casein proteins can cause food anaphylaxis. Currently, the allergenicity of caseins cannot be eliminated using known methods such as heating, chemical and enzymatic (for example, curdling) treatment. Caseins are subdivided into α_{s1}, α_{s2}, β, and κ fractions (19–25 kDa). Their ratio is relatively constant and amounts to 37, 13, 37, 13%, respectively. Among these, β casein is the largest contributor to food allergy. It consists of 209 amino acids and has 12 genetic variants, i.e. A1, A2, A3, B, C, D, E, F, G, H1, H2 and I, differing in structure [32, 33].

The total casein mass fraction measured by the method is the total content of caseins of the four fractions (αs1, αs2, β, and κ). The measurement range of the total casein mass fraction is 0.5 to 25.0 μg/kg (ppb) inclusive. The detection limit is 0.1 μg/kg (ppb). The coefficient of cross-reacting derivatives (cross-sensitivity) of the sum of the αs1, αs2, β, and κ casein fractions is 100%. γ casein fraction is less than 0.1%. The values of the accuracy indicators in the relative form of the measurement technique coincide with the values given in Table 3.

3. The technique for the qualitative determination of the presence or absence of non-infectious food protein allergens of animal origin using reagent kits manufactured by XEMA LLC.

Table 5 gives the numbers of reagent kits produced by XEMA LLC, the list of allergens with the corresponding target antigens, as well as the lower sensitivity limits (limits of detection) for the presence or absence of antigens (LOD) by allergen protein mass in extracts prepared according to the procedure described in the methodology.

At present, specialists from UNIIM, the affiliated branch of the D. I. Mendeleyev all-Russian Institute for Metrology and XEMA LLC, are developing methods for preparing homogeneous and stable food reference materials for interlaboratory comparative tests (proficiency testing) in order to introduce (check the efficiency) of the developed methods for the qualitative and quantitative analysis of non-infectious

Table 5 Characteristics of the qualitative determination of the presence or absence of non-infectious food protein allergens of animal origin

Kit number	Allergen	Target antigen	LOD, mg/kg (ppm)
K350	Crustaceans	Specific fragment of crustacean tropomyosin	0.22
K355	Mollusks	Specific fragment of mollusk tropomyosin	2.0
K363	Fish	Specific fragment of fish tropomyosin	0.10
K360X	Egg	Specific fragment of avian egg white ovalbumin	0.5

food protein allergens of plant and animal origin. We also plan to involve laboratories of various food industry enterprises, as well as the reference centers of the Federal Service for Veterinary and Phytosanitary Supervision (Rosselkhoznadzor) and the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor).

Currently, the Federal Information Fund of Measurement Assurance (FIF OEI) does not have reference materials for the composition of food allergens necessary to control the identification and quantification results. In this regard, the next stage in the development of the metrological assurance complex aimed at identifying and quantifying the content of food allergens will be designing and testing for type approval of reference materials for the composition of the “big eight allergens” [3, 6] as the most reliable ways to control the results obtained.

Conclusion

In the course of the study, we developed and certified methods for identifying and quantifying the content of non-infectious food protein allergens of plant (gluten, peanuts, nuts, soybeans) and animal (cow’s milk, chicken eggs, fish, seafood) origin in samples of all types of food products and objects related to the requirements for food products, as well as wipe samples obtained from countertops during in-process inspection using ELISA reagent kits manufactured by XEMA LLC.

For gluten, soybeans and cow’s milk, the following analysis scheme is proposed: 1) qualitative screening of the samples under study for the presence or absence of the target antigen; 2) quantitative evaluation of the antigen content in the samples with a positive screening test result. It is planned to implement this scheme to detect other food allergens.

We have established the detection limits of the methods used to identify non-infectious food protein allergens of animal or plant origin in food samples and countertop wipe samples. Moreover, the techniques for measuring the mass fraction of soybean trypsin inhibitor and total casein provided in the developed documents were certified for compliance with the requirements of Federal Law No. 102-FZ [34] and GOST R 8.563-2009 [35].

We have also revealed the need for developing reference materials for the composition of food allergens to control the identification and quantification results.

The developed methods for identifying and quantifying the content of non-infectious food protein allergens of plant and animal origin make it possible to ensure the safety of food products when establishing product compliance with the requirements of TR CU 022/2011 and TR CU 027/2012.

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Measurement Techniques for the Composition of Air Environments: Development and Application

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Abstract Accurate and reliable measurements of the composition of air environments (workplace air, ambient air, industrial air emissions) form a solid basis for decision making in the area of environmental quality. This article deals with the problem of developing and testing techniques for measuring air quality parameters. The disadvantages of the existing techniques are identified. A methodology for creating appropriate techniques is proposed. Procedures for developing reference materials (RMs) for internal and external quality control of measurement results are described, along with approaches to estimating the metrological characteristics of measurement techniques. The process of applying measurement techniques is specified. It is shown that techniques for measuring air quality parameters may be divided for two basic stages, i.e., the stage of analytical sampling and the stage of measuring the required indicators in this sample. This approach expands the possibilities of developing and certifying such techniques, thereby increasing the reliability of measurement results. Using the developed approach, the authors have successfully developed 12 techniques for measuring air quality parameters and two certified reference materials (CRMs). Works in this direction are continued.

Keywords Measurement technique · Air environment composition indicators · Workplace air · Ambient air · Industrial emissions · Measurement error · Measurement uncertainty · Accuracy assurance · Reference material

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Introduction

Air quality is an essential condition for public health and environmental safety, both in Russia and globally [1–3]. Air pollution due to industrial emissions is associated with adverse health effects for employees at industrial enterprises [4, 5].

Lead, nickel, and chromium are referred to as highly-toxic hazard substances [6, 7] posing a severe risk of allergic and oncological diseases. Chronic intoxication with nickel or chromium compounds damages nasopharynx and lungs, leads to the appearance of malignant neoplasms and allergic lesions in the form of dermatitis and eczema. Continuous inhalation of chromium (VI) compounds increases the risk of lung, nasal, and sinus cancer. Lead dust may cause neurological, gastrointestinal, blood, and kidney diseases.

Prolonged exposure to copper results in eye, nose, and mouth irritation, producing headache, dizziness, vomiting, and diarrhea. Titanium excess is found among people working in chemical and metallurgical plants. Exposure to titanium dust is manifested in coughing, chest pain, and cramps, leading to breathing problems. Inhalation of silicon dioxide is associated with a significant threat to human health [8]. Found in the air in the form of aerosol, silicon dust causes irritation of the upper respiratory tract and bronchus, as well as gastrointestinal tract diseases.

Harmful substances in the atmosphere are responsible for climate change and greenhouse effects [9], thus negatively affecting all natural processes and phenomena. Therefore, measures should be taken to reduce the presence of harmful substances by purifying industrial emissions [10], imposing stricter requirements for cars, etc. One essential measure consists in the monitoring of pollutants emitted into the atmosphere. Reliable information on the air composition and characteristics of air environments (workplace air (WA), industrial air emissions (IAE), ambient air (AA)) should underpin the decision-making process in the sphere of public health. The solution of these tasks require:

- improved measurement techniques to reliably and accurately monitor the composition of air environments;
- a methodology for developing such techniques;
- characterization and calibration techniques;
- procedures for implementation and application of the developed techniques;
- development and application of certified reference materials (CRMs) of approved types, appropriate to the analytical samples selected for measurements.

Reasons for the Development of New Techniques for Measuring the Composition of Air Environments

In the Russian Federation, new Sanitary Regulations and Standards SanPiN 1.2.3685-21 [7] were approved in 2021. This document formulates requirements for allowable concentrations of harmful substances in AA and WA environments, which are presented in Table 1.

The requirements for industrial air emissions are defined in relation to specific enterprises and are regulated by regional levels of executive power.

In accordance with the Federal Law of the Russian Federation No. 102-FZ [11], the implementation of activities in the field of environmental protection refers to the sphere of state regulation of the uniformity of measurements, with such measurements being subject to mandatory metrological requirements. New requirements for measurement ranges of harmful substances in air environments and for their measurement accuracy are introduced in the List of measurements related to the state regulation of ensuring the uniformity of measurements, approved by the Government of the

Table 1 Excerpt from SanPiN 1.2.3685-21 for the chromium mass concentration

Measurement object	Substance	Maximum allowable concentrations (mg/m^3)
Ambient air	Chromium/expressed as chromium (VI) oxide	0.0015
Workplace air	Chromium hydroxide sulfate/ expressed as chromium (III)/ (chromium sulfate basic)	0.06/0.02
	Chromium-2,6-dihydrogen phosphate/by chromium (III)/ (chromium phosphate monosubstituted)	0.06/0.02
	Chromium (VI) trioxide (chromium trioxide; chromic anhydride)	0.03/0.01
	di-Chromium trioxide/by chromium (III)/(di-chromium trioxide), chromium oxide	3/1
	Chromium trifluoride/by fluorine/(chromium fluoride)	2.5/0.5
	Chromium trichloride hexahydrate/by chromium (III)	0.03/0.01
	Chromium phosphate (chromium orthophosphate)/ (chromium phosphate trisubstituted)	2
	Chromic acid salts/expressed as chromium (VI)	0.03/0.01

Russian Federation Decree of November 16, 2020 No. 1847 (List of measurements) [12] (see Table 2).

For example, the permissible error for measurements of the mass concentrations of organic and inorganic substances in WA in the new List of measurements was increased from 25 to 35%. In addition, the specified List of measurements establishes a permissible error of 10% in measuring the air flow rate when taking WA samples in the measurement range (in dm^3/min) from 0.1 to the value established in the certified measurement technique. An additional requirement is formulated for the mass concentration of harmful substances in WA: "a selective measurement of the harmful substance concentration must be provided in the presence of associated components at ≤ 0.5 of the maximum allowable concentration (MAC), (mg/m^3)."

According to paragraph 72 of SanPiN 2.1.3684-21, "Sanitary and epidemiological measures shall be taken with respect to sources of exposure causing chemical, physical, and biological effects exceeding 0.1 of the MAC (Safe Reference Levels of Impact (SRLI)) and (or) the Maximum acceptable limit (MAL)…" [13]. In accordance with the Federal Law 247-FZ [14], in 2020, obsolete measurement techniques that did not satisfy modern requirements were abolished.

In accordance with [11], in the sphere of state regulation, the traceability of measurement results must be ensured; similar requirements are set for laboratories in paragraph 6.5 of GOST ISO/IEC 17025-2019 [15]. Measurement results should correspond to the established measurement accuracy characteristics, which are established through the concept of measurement error in the List of measurements. At the same time, paragraph 7 of GOST ISO/IEC 17025-2019 [15] suggests that laboratories should establish the uncertainty of their measurement results.

Since measurements of the content of airborne components belongs to the sphere of the state control for measurement uniformity assurance, the accuracy control of measurement results (internal and external) should be performed using CRMs (if available), which are appropriate for the selected analytical samples.

The metrological characteristics of the majority of existing techniques (more than 80%) cannot be established using reference gas mixtures or microflow sources. In this case, the laboratory techniques used to measure the composition of air environments can be divided into two stages: analytical sampling and the analytical stage [16]. For such techniques, an analytical sample taken directly at the place of air sampling is represented by the components absorbed from the air environment onto an appropriate absorbent (filter, sorbent, absorbent solution), rather than a sample of the tested air environment.

Methodology for Developing Measurement Techniques

Figure 1 outlines a structural scheme of the proposed methodology for developing measurement techniques.

Let us consider the stages shown in Fig. 1 in greater detail.

Table 2 Excerpt from the list of measurements related to the state regulation of the uniformity of measurements, approved by the Government of the Russian Federation from November 16, 2020 No. 1847 [12]

Measurement object	Accuracy standards
Ambient air	Permissible measurement error limits of mass concentrations of organic and inorganic substances in ambient air δn = ± (10–25)%
Industrial air emissions	Permissible measurement error limits of mass concentrations of organic and inorganic substances in industrial air emissions δn = ± (8–25)%
Workplace air	Permissible measurement error limits of mass concentrations of organic and inorganic substances in workplace air for single measurements (single sampling) δn = ± (15–35)%

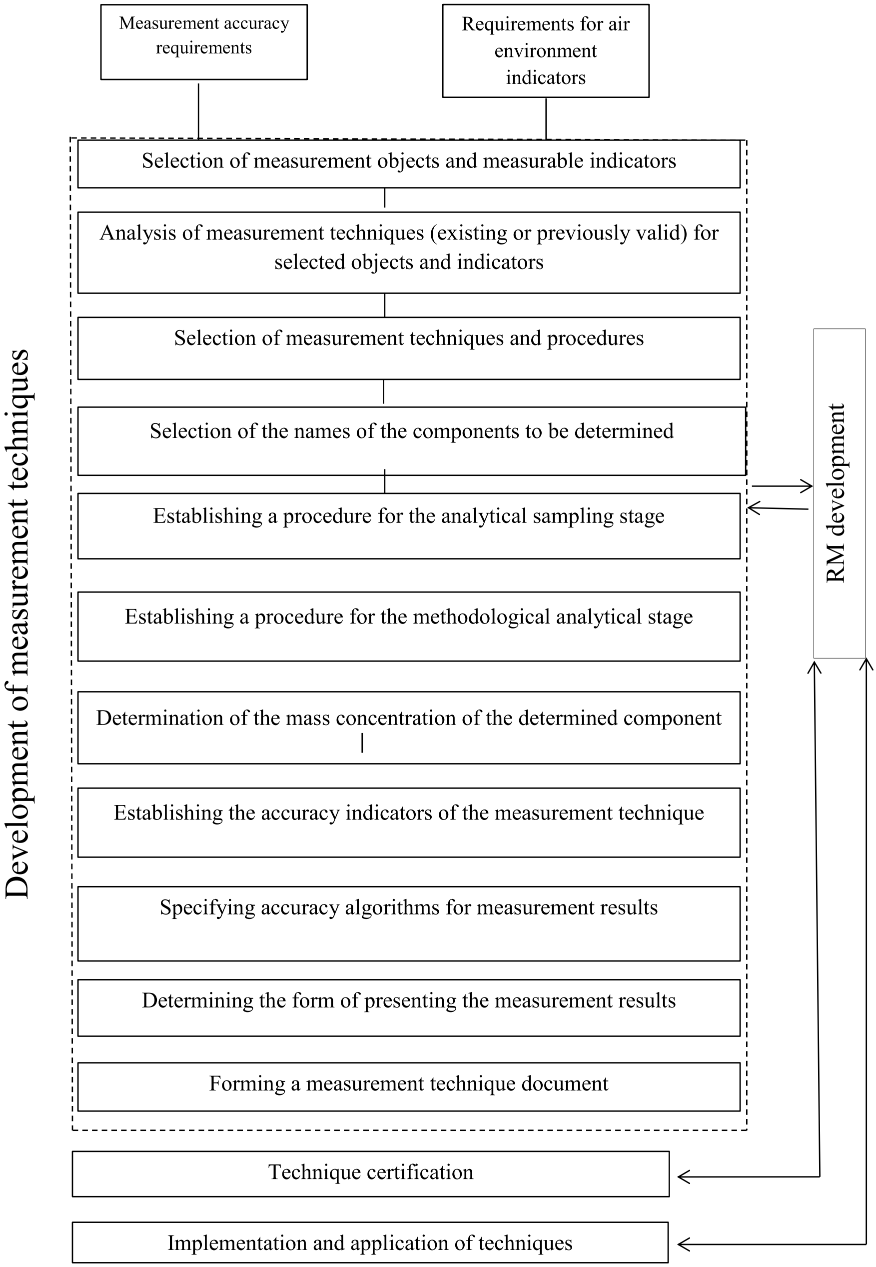

Fig. 1 A structural scheme of development of measurement techniques for air environment composition

Selection of Measurement Objects and Measurable Indicators

Since measurement procedures for different air environments (WA, IAE, and AA) are fundamentally identical, a decision to select one measurement technique for all environments should be taken, with specification of the sampling procedure for each environment. The measured parameters are selected from those most relevant in laboratories.

Analysis of Measurement Techniques (Existing or Previously Valid) for Selected Objects and Indicators

The conducted analysis of existing measurement techniques revealed their following shortcomings:

(1) the names of the defined components often do not correspond to SanPiN, which leads to inconsistency between the parties involved;
(2) some techniques lack information about the sampling rate and the volume of sampled air;
(3) incomplete description of the procedure used for obtaining measurement results and the shelf life of reagent solutions;
(4) misspellings in the techniques often lead to incorrect results;
(5) the applied chemical reactions do not allow determining the content of a particular compound; as a result, the technique determines the total content of the same anions (or cations) from different compounds contained in the analytical sample;
(6) the interfering influences that lead to over- or underestimation of the measurement results are not taken into account;
(7) the isokineticity of gas flows should be regarded during measurements, which is almost impossible;
(8) no CRMs are used for constructing a calibration curve (CC) and for quality control of measurement results, which contradicts [11];
(9) accuracy control is most often limited to verifying the CC stability.
(10) the measurement range does not always meet the requirements of statutory instruments, SanPiN 1.2.3684-21 in particular [13];
(11) absence of information about the errors or uncertainty characteristics of measurement results.

Below, some examples of such techniques are presented.

MU 4945-88 [17]

(1) The information on how a sample is taken is absent, including whether a filter (and what kind) or an absorbent solution (what kind) is used. No information is given about the speed and duration of air environment aspiration.
(2) Procedures for preparing solutions, samples for calibration and accuracy control, etc., are absent.
(3) The description of the measurement procedure starts with the words "1–5 ml of a sample solution…". The question arises at what stage this solution appeared—during sampling or during the preparation of the sample for measurements.

MUK 163777 [18]

(1) According to the description, it is assumed that the sample volume from one absorption solution depends on the content of ammonia in the sample. For this purpose, "1 and 5 ml are taken to conduct two parallel measurements of the ammonia mass concentration." As a result, some executors sum up the results obtained using different aliquots.
(2) The formula for obtaining the final measurement result (see formula 1) is given in paragraph 4 of the measurement technique.

The ammonia concentration X in mg/m^3 of air is calculated by the formula:

$$X = \frac{G V_1}{V V_{CT}}, \tag{1}$$

where G is the amount of ammonia found in the analyzed solution volume, μg;

V is the total sample volume, ml;

V_1 is the sample volume taken for analysis, ml;

V_{CT} is the air volume sampled for analysis, reduced to standard conditions according to the formula

$$V_{CT} = V_t \frac{(273 + 20) P}{(273 + t) 101.33} = V_t K_1,$$

where V_t is the air volume measured at t°C and a pressure of 101.33 kPa.

The formula presented in [18] is incorrect. The authors propose to swap the volume of the sample taken for analysis and the total volume of the sample. Then, when calculating the ammonia mass concentration according to the formula, the measurement result is reduced by several times.

Selection of Measurement Techniques and Procedures

The main methods currently used to analyze the air environment composition are the following [19–21]. These are: photometry and spectrophotometry; atomic absorption spectrometry; titrimetry; potentiometry; voltammetry (polarography); gas chromatography or high-performance liquid chromatography. When developing the techniques, the most accessible methods for laboratories were selected—photometry and titrimetry.

According to [18], the measurement procedure consists of two stages (analytical sampling and analytical stage), which makes it possible to describe the stage of taking an analytical sample for each air environment separately, and then—to measure the content of the component in the analytical sample in accordance with a single procedure.

Selection of the Names of the Components to Be Determined

The names of the components to be determined are selected with reference to the names established in the Sanitary Regulations and Standards, based on the possibility of determining the selected indicators by the established measurement method and the possible influence of interfering components on the measurement results of the indicators in analyzed samples.

Establishing the Necessary Measurement Ranges of the Components to Be Determined

The establishment of the measurement ranges of the component to be determined is based on the requirements of the current statutory instruments (SIs) and regulatory documents (RDs) (such as, for example: List of measurements [12], SanPiN 1.2.3685-21 [7], SanPiN 2.1.3684-21 [13], etc.). This takes into account the measurement ranges of the components in the previously developed measurement techniques, since the developers of techniques do not have information about all regional SIs.

In addition, the following parameters are set for each stage: the volume of the aspirated air environment for the first stage and the measurement range of the mass of the analyzed component in the analytical sample for the second stage.

Establishing a Procedure for the Analytical Sampling Stage

At this stage of the procedure, the equipment, reagents, and materials necessary for taking an analytical sample are selected.

The analytical sampling stage should be described in detail for each air environment separately, taking into account the requirements of the current SIs and RDs. At the same time, it is necessary to pay special attention to the indication of the required aspiration rate and time, the volume of the aspirated air environment. When selecting these parameters, the capabilities of the measuring instruments (MIs) used for sampling are also considered. The MI errors must comply with the requirements of SIs and RDs.

When obtaining an analytical sample, the component to be determined is absorbed into the appropriate absorbent solution or absorbed onto the selected filter (sorbent) during the air environment sampling process.

In addition, at the analytical sampling stage, the atmospheric pressure, the temperature of the air environment in relation to the sampling point, and the pressure or vacuum at the inlet of the sampling device (for industrial emissions) are determined.

Establishing a Procedure for the Methodological Analytical Stage

The procedure for establishing the CC (when using the spectral method) is selected based on the following steps:

(1) selection of equipment, reagents, materials, and CRMs required to establish a CC. If available, CRMs of approved types for the composition of the determined component solution, traceable to state standards. It is allowed to use other MIs, auxiliary equipment, reagents, materials, and RMs, provided that their metrological, technical, and qualification characteristics are not worse than those specified in the technique;
(2) setting the CC range;
(3) establishing procedures for preparing calibration samples, taking into account the shelf life of each solution;
(4) establishing a procedure for creating a CC, considering the linear dependence of the MI signal on the mass of the analytical sample component to be determined;
(5) establishing the number of parallel signals when creating a CC and their permissible range;
(6) determining a formula for the relationship between the analytical sample component mass and the MI signal;
(7) establishing a standard and procedure for verifying the CC linearity.
(8) establishing a standard and procedure for verifying the CC stability.

Establishing a Measurement Procedure for an Analytical Sample

When preparing the analytical sample for measurements, including establishing a procedure for preparing solutions (including an analytical sample solution) and their shelf lives, procedures are established for measuring the prepared analytical sample and obtaining the measurement result according to the analytical stage of the technique.

If necessary, additional aliquoting and/or dilution of the prepared analytical sample shall be provided. In this case, the measurement result of the component mass taking into account the aliquoting and/or dilution factor must fall within the measurement range for the analytical stage of the technique.

Based on the aspirated air volume and the determined component mass in the analytical sample, the mass concentration of the measured component is calculated. To this end, the aspirated air volume, adjusted to normal (for AA and IAE) or standard (for WA) conditions, taking into account the atmospheric pressure during sampling, air temperature in the sampling point, the pressure or underpressure value at the sampling device inlet (for industrial emissions), is used.

Establishing the Accuracy Indicators of the Measurement Technique

The accuracy indicators are defined in the form of error and uncertainly characteristics of measurement results. For the analytical sampling stage, the error characteristic is established by the computational or computational-experimental method using Annex B of RMG 61-2010 [16, 22]. The uncertainty value is established using GOST 34100.3-2017/ISO/IEC Guide 98-3:2008 [23].

For the analytical stage, the accuracy value (both in the form of an inaccuracy characteristic and in the form of an uncertainty value) is established by applying the algorithm using the samples for evaluation presented in paragraph 5 of RMG 61-2010 [22]. In this case, the samples for evaluation are used, as adequate as possible to the analyzed analytical samples.

The accuracy indicators of the measurement technique are based on the superposition of the accuracy indicators of the analytical sampling stage and the analytical stage.

Specifying Accuracy Algorithms for Measurement Results

Since the measurement technique is divided into two stages, accuracy verification should be conducted for each stage separately.

The quality of the analytical sampling stage is controlled by calibration of all MIs used at this stage and periodic visual control of the sampling procedure. Intralaboratory quality control of measurement results (in this case—for the analytical stage of the technique) in accordance with RMG 76-2014 [24] consists of two components: operational control and control of the stability of measurement results.

Algorithms used to verify the stability of measurement results are not specified in the technique. Their selection depends on the specifics of a particular laboratory: the frequency of measurements, the variety of measurement conditions, the range of practical measurements, etc.

The measurement quality of the analytical stage of the technique is assured using an algorithm based on control samples according to paragraph 5.5 of RMG 76-2014 [24]. Control samples include those adequate to the analytical samples and similar to the samples used when evaluating the metrological characteristics of this measurement stage.

Determining the Form of Presenting the Measurement Results

In accordance with RMG 93-2015 [25], the presentation of measurement results in documents that imply their further use should include the accuracy indicator consisting of no more than two significant digits. The measurement result must end with a digit of the same place as the accuracy indicator.

Forming a Measurement Technique Document

The document for the measurement technique is prepared in accordance with GOST R 8.563-2009 [26].

Technique Characterization

According to [11], in the state regulation of measurement uniformity assurance, measurement techniques must be characterized, i.e., examined and approved for compliance with the established metrological requirements for measurements. Characterization of measurement techniques is carried out in accordance with the Order of the Ministry of Industry and Trade of December 15, 2015 No. 4091 [27].

The certified techniques are included in the Federal Information Fund for Ensuring the Uniformity of Measurements (FIF EUM),[1] in which they are assigned a registration number.

Implementation and Application of Techniques

The developed measurement techniques are applied in sanitary, ecological, and analytical laboratories, or other departments performing environmental control and/or air quality monitoring. Such techniques can be applied in other testing and analytical laboratories specializing in similar research.

According to paragraph 7.2.1.5 of GOST ISO/IEC 17025-2019 [17], "before implementing the selected techniques in operation, the laboratory shall confirm that it can properly apply the selected techniques, ensuring the required performance. Records of verification shall be stored."

Thus, in order to use the selected technique, the laboratory should:

- select (purchase) equipment, materials, reagents, and CRMs that meet the requirements of the technique;
- implement the measurement procedure;
- confirm that the measurement accuracy values do not exceed those specified in the technique (e.g., by implementing internal control procedures, see RMG 76-2014 [24]) or establishing its own accuracy values not exceeding the accuracy values of the measurement technique (e.g. according to RMG 76-2014, Annexes A, B [24]);
- record the obtained results in a document, e.g., a technique implementation report (note that certified measurement techniques are not subject to validation, i.e., changes in the components to be determined, measurement ranges, or application scope);
- establish a quality control system for measurement results.

To ensure confidence in the measurement results and prevent obtaining low-quality data regarding the air environment, the laboratory shall (according to GOST ISO/IEC 17025-2019 [15]) monitor the reliability of its results through internal control and, if possible, comparison with the results obtained in other laboratories (participation in interlaboratory comparisons, ILC), in accordance with GOST ISO/IEC 17043-2013 [28].

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology. Accessed 4 August 2022 (In Russ.).

Development and Application of CRMs Adequate for Analytical Samples

Existing CRMs [19, 20] are unable to solve the problem of internal and external quality control of air environment measurements. The development of CRMs adequate for analytical samples is a challenge for the analytical community. An approach to creating such samples is described in [29].

Practical Implementation of the Proposed Methodology

Since 2020, UNIIM specialists (UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology) have successfully used the methodology described in the present article to develop 12 techniques for measuring air environments. The developed techniques were characterized to confirm their compliance with the established metrological measurement standards. The techniques were certified using CRMs of approved types with the description of control procedures for each of the two stages of the technique. The analytical stage is verified using control samples adequate to the analytical samples.

In order to ensure requirements for obtaining objective and reliable information used for public life, health and environmental protection purposes, the measurement techniques have been included in the FIF EUM.

The developed techniques are presented in Table 3.

UNIIM uses samples that are adequate to analytical samples to certify the developed techniques for air environment analysis. Since 2014, the ILC provider UNIIM has been carrying out ILCs using such sample simulators. UNIIM specialists have developed GSO 11278-2019[2] and GSO 11277-2019.[3]

The measurement techniques developed at UNIIM are used not only to determine the composition of air environments composition, but also to characterize RMs and samples for quality testing. This increases the metrological level of the work performed. The developed CRMs are used for the internal quality control of measurement results and for conducting ILCs.

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[2] GSO 11278-2019 Reference materials of the mass concentration of manganese deposited on the AFA-XA filter from the air (V-Mn-03 SO UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/583284. Accessed 4 August 2022 (In Russ.).

[3] GSO 11277-2019 Reference materials of the mass concentration of iron deposited on the AFA-XA filter from the air (In Fe 02 CO UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/583285. Accessed 4 August 2022 (In Russ.).

Table 3 Measurement techniques for the composition of air environments developed at UNIIM

Code and name of the measurement technique, FIF EUM registration number	Component to be determined	Measurement range, mg/m^3		± δ[1], % (U$_0$[2], %)
M-222-1/2020 Technique for measuring the mass concentrations of iron and iron expressed as iron (III) oxide in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2020.37587	Iron	AA	From 0.025 to 4.0 inclusive	25 (25)
		WA	From 0.17 to 267 inclusive	
		IAE	From 1.0 to 2.0·10^3 inclusive	
	Iron expressed as iron (III) oxide	AA	From 0.036 to 5.7 inclusive	25 (25)
		WA	From 0.24 to 380 inclusive	
		IAE	From 1.44 to 2.8·10^3 inclusive	
M-222-2/2020 Technique for measuring the mass concentrations of manganese and manganese expressed as manganese (IV) in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2020.37584	Manganese	AA	From 0.005 to 2.7 inclusive	25 (25)
		WA	From 0.015 to 180 inclusive	
		IAE	From 0.7 to 1.4·10^3 inclusive	
	Manganese expressed as manganese (IV) oxide	AA	From 0.008 to 4.3 inclusive	25 (25)
		WA	From 0.024 to 280 inclusive	
		IAE	From 1.2 to 2.2·10^3 inclusive	
M-222-3/2020 Technique for measuring the mass concentration of dust (suspended substances) in workplace air, ambient air, and industrial air emissions by the gravimetric method, FR.1.31.2020.37773	Dust (suspended substances)	AA WA IAE	From 0.1 to 4000 inclusive	25 (25)
M-222-4/2020 Technique for measuring the mass concentration of sulfuric acid in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2020.38606	Sulfuric acid	AA	From 0.2 to 5.0 inclusive	23 (23)
		WA	From 0.5 to 25.0 inclusive	
		IAE	From 0.5 to 30.0 inclusive	

(continued)

Table 3 (continued)

Code and name of the measurement technique, FIF EUM registration number	Component to be determined	Measurement range, mg/m^3		$\pm \delta^1$, % (U_0^2, %)
M-222-5/2020 Technique for measuring the mass concentration of hydrochloric acid in workplace air, ambient air, and industrial air emissions by the turbidimetric method, FR.1.31.2021.39027	Hydrochloric acid	AA WA IAE	From 0.10 to 3000 inclusive	25 (25)
M-222-6/2021 Technique for measuring the mass concentration of ammonia in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2021.39683	Ammonia	AA	From 0.02 to 500.0 inclusive	24 (24)
		WA	From 0.2 to $2\cdot10^3$ inclusive	
		IAE	From 0.1 to $3\cdot10^3$ inclusive	
M-222-7/2021 Technique for measuring the mass concentrations of total chromium, chromium (VI), chromium (III), and total chromium expressed as chromium trioxide (chromic anhydride) in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2021.40211	Chromium (VI)	WA	From 0.011 to 9.0 inclusive	24 (24)
		AA IAE	From 0.01 to 9.0 inclusive	
	Chromium (III)	WA	From 0.011 to 9.0 inclusive	25 (25)
		AA IAE	From 0.01 to 9.0 inclusive	
	Total chromium	WA	From 0.011 to 18.0 inclusive	25 (25)
		IAE	From 0.01 to 18.0 inclusive	
		AA	From $0.75\cdot10^{-4}$ to 18.0 inclusive	
	Total chromium expressed as chromium trioxide (chromic anhydride)	WA IAE	From 0.02 to 34.6	25 (25)
		AA	from $1.4\cdot10^{-4}$ to 34.6 inclusive	
M-222-8/2021 Technique for measuring the mass concentration of oxide nitrogen (IV) in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2021.40860	Oxide nitrogen (IV) (nitrogen dioxide)	WA	From 1.0 to 100 inclusive	24 (24)
		AA	From 0.02 to 100 inclusive	
		IAE	From 0.02 to 1000 inclusive	

(continued)

Table 3 (continued)

Code and name of the measurement technique, FIF EUM registration number	Component to be determined	Measurement range, mg/m^3		± δ[1], % (U_0[2], %)
M-222-9/2021 Technique for measuring the total mass concentrations of caustic alkalis (expressed as sodium hydroxide) in workplace air, ambient air, and industrial air emissions by the titrimetric method, FR.1.31.2022.41864	Caustic alkalis expressed as sodium hydroxide	WA	From 0.20 to 1000 inclusive	21 (21)
		AA IAE	From 0.20 to 1500 inclusive	
M-222-12/2021 Technique for measuring the mass concentrations of phenol in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2022.42311	Phenol	AA WA IAE	From 0.001 to 100 inclusive	22 (22)
M-222-13/2021 Technique for measuring the mass concentrations of formaldehyde in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2022.42312	Formaldehyde	AA WA IAE	From 0.005 to 200 inclusive	22 (22)
M-222-14/2022 Technique for measuring the mass concentrations of amorphous silicon dioxide and crystalline silicon dioxide in workplace air, ambient air, and industrial air emissions by the photometric method, FR.1.31.2022.43372	Amorphous silicon dioxide	AA	From 0.0020 to 100 inclusive	25 (25)
		WA IAE	From 1.00 to 100 inclusive	
	Crystalline silicon dioxide	AA	From 0.0020 to 100 inclusive	25 (25)
		WA IAE	From 1.00 to 100 inclusive	

[1] Relative error limits (P = 0.95)

[2] Relative expanded uncertainty k = 2

Conclusion

This article describes the reasons for the development of new techniques for measuring the composition of air environments. A methodology for developing such techniques is designed and implemented in practice. The questions of certifying measurement techniques and their implementation are discussed, along with the development and application of CRMs adequate to the analytical samples used for measurements. The process of practical implementation of the described procedures is described.

UNIIM specialists continue works on the development of new measurement techniques and new CRMs, which could be used by laboratories to provide reliable information regarding air environment composition.

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Primary Reference Measurement Procedures in the Food Industry: Usage Experience and Development Prospects

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Abstract This article reviews the usage experience and development prospects of four state primary reference measurement procedures (SPRMPs) created to provide metrological support of measurements in the food and agricultural industries. The SPRMPS certified in 2016–2019 include those of the mass fraction of fat, ash, and carbohydrates in food products and raw materials; of the mass fraction of crude fat (oil content) in oilseeds and related products. The mass fraction of fat, crude fat, and oil content is measured using the Randall method; the mass fraction of ash is determined by the thermogravimetric method; the mass fraction of carbohydrates (total sugar) is defined by iodometric titration. The considered SPRMPs were used to develop reference materials (RMs) for the composition of dairy and meat, egg powder, starch, baby food, oilseeds, related products, and compound feeds. SPRMPs implementation required extending the range of the manufactured RMs for new product groups. High-precision procedures for measuring the mass fraction of fat in liquid dairy products, fish, pastry, confectionery, and pasta products, as well as the mass fraction of lactose in liquid dairy products were developed and certified to describe the characteristics of dairy, pollock fillets, and wheat crackers composition.

Keywords Primary Reference Measurement Procedures · Traceability · Certified reference materials · Food products · Food raw materials · Nutritional value

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Abbreviations

SPRMP	State primary reference measurement procedure
CRMs	Certified reference materials
ILCs	Interlaboratory comparisons
PRMP	Primary reference measurement procedure
RMP	Reference measurement procedure
RMs	Reference materials
FIF EUM	Russian Federal Information Fund for Ensuring the Uniformity of Measurements

Introduction

According to the minutes of the Consultative Committee on the Quantity of Material (CCQM) meeting [1], the primary measurement method is *"a method having the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be written in terms of the SI, and whose results are, therefore, accepted without reference to a standard of the quantity being measured."* The methods potentially related to primary methods of analysis include isotope dilution mass spectrometry, coulometry, and gravimetry, among others [2]. In 2007, the concepts of a "primary reference measurement procedure" and "reference measurement procedure" (PRMP and RMP) were included in the International Vocabulary of Metrology (VIM) (ISO/IEC Guide 99:2007) [3]. The appearance of PRMPs and RMPs is primarily due to the need of building a metrological hierarchy, the top of which is PRMPs, which helps obtain quantity measurement results without their traceability to the unit's standard of the same magnitude, and which is used to control the correctness of measurement results obtained by other methods. PRMPs are developed and approved for metrological support of the so-called "method-dependent" quantities determined by empirical measurement methods [4]. PRMPs and RMPs have become the most widespread in the world metrological practice related to bioanalysis and clinical diagnostics. Standards have been developed that establish requirements for RMPs in the field of laboratory medicine, for example, ISO 15195:2018 [5]. Discussions are underway on the possibility of recognizing the calibration and measurement capabilities of National Metrological Institutes for operationally-defined quantities by including them in the database of the International Bureau of Weights and Measures (BIPM)[1] [6–9].

In the Russian Federation, PRMPs and RMPs were introduced into the state regulation of ensuring the uniformity of measurements in 2014 as part of adopting

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[1] BIPM—Bureau International des Poids et Mesures. Available via BIPM. https://www.bipm.org. Accessed 26 August 2022.

254-FZ [10]. The procedure for PRMPs and RMPs certification, as well as the scope of their application were established by the Order of the Ministry of Industry and Trade of the Russian Federation No. 4091 [11]. The most effective application of PRMPs appeared in developing metrological support for the food and agricultural industries to ensure metrological traceability of measurements of generalized and hard to formalize indicators of the nutritional value of food products and food raw materials, such as fat, ash, and carbohydrates.

In 2016–2019, the following PRMPs were developed and certified as state PRMPs:

- SPRMP of the mass fraction of fat in food products and food raw materials[2];
- SPRMP of the mass fraction of ash in food products and food raw materials[3];
- SPRMP of the mass fraction of carbohydrates in food products and food raw materials[4];
- SPRMP of the mass fraction of crude fat (oil content) in oilseeds and products based on them.[5]

The results of the SPRMPs development are presented in [12–15].

This study aims to generalize and analyze the experience of using and prospects for the development of SPRMPs for metrological support of measurements in the food industry.

Materials and Methods

In the developed SPRMPs, the measurement of the mass fraction of fat is conducted using the Randall method, based on the quantitative extraction of free and chemically or mechanically bound fat from product samples with the subsequent weighing of the fat. In the case of determining total fat in dairy, meat, dry egg products, and baby food, this method is implemented with a preliminary stage of hydrolysis. When determining crude fat (oil content) in oilseeds and products based on them, grains of cereals and legumes, compound feeds, and feed components, preliminary hydrolysis is not performed.

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[2] FR.PR1.31.2019.00001 State primary reference measurement procedure of the mass fraction of fat in food products and food raw materials M.241.01/RA.RU.311866/2018. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/6/items/595556. Accessed 26 August 2022 (In Russ.).

[3] FR.PR1.31.2019.00002 State primary reference measurement procedure of the mass fraction of ash in food products and food raw materials M.241.02/RA.RU.311866/2018. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/6/items/595557. Accessed 26 August 2022 (In Russ.).

[4] FR.PR1.31.2019.00005 State primary reference measurement procedure of the mass fraction of carbohydrates in food products and food raw materials. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/6/items/1057023. Accessed 26 August 2022 (In Russ.).

[5] FR.PR1.31.2019.00009 State primary reference measurement procedure of the mass fraction of crude fat (oil content) in oilseeds and products based on them. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/6/items/1057069. Accessed 26 August 2022 (In Russ.).

The measurement of the mass fraction of ash is carried out using a thermogravimetric method, including sample preparation, charring, ashing of the sample of the product, and subsequent gravimetric measurement of the mass of the mineral residue after ashing.

The measurement of the mass fraction of carbohydrates (total sugar) is carried out using the method of iodometric titration, based on the oxidation of reducing sugars in an alkaline medium with iodine and titration of unspent iodine with a solution of sodium thiosulfate.

Evaluation of the SPRMPs metrological characteristics and high-precision measurement techniques is conducted considering the sources of uncertainty included in the measurement equations, contributions from methodological parameters, heterogeneity of sample material, and operator influence using the algorithms set out in ISO 5725-5:1998 [16], RMG 61-2010 [17], and the EURACHEM/CITAC Guide [4].

The study of metrological reference samples (RMs) of food products and food raw materials composition is carried out in accordance with the ISO Guide 35:2006 [18].

Interlaboratory comparison tests (ILCs) to determine quality and nutritional values are conducted in accordance with the ISO/IEC 17043:2010 [19].

Results and Discussion

The SPRMPs of the mass fraction of fat, ash, and carbohydrates in food products and food raw materials, the SPRMP of the mass fraction of crude fat (oil content) in oilseeds and products based on them are intended for high-precision measurements, evaluating the correctness of measurement results obtained using other measurement procedures (methods), including those used to confirm the compliance of the products with the mandatory requirements established in the technical regulations, as well as for the calibration of measuring instruments and determination of the RMs characteristics. The measurement objects and metrological characteristics of the SPRMPs are presented in Table 1.

The SPRMPs in conjunction with the State Primary Measurement Standard for the Units of Mass Fraction and Mass (Molar) Concentration of Water in Solid and Liquid Substances and Materials GET 173-2017 [20, 21] and the State Secondary Measurement Standard for the Units of Mass Fraction and Mass (Molar) Concentration of Components in Solid and Liquid Substances and Materials on the Basis of the Volumetric Titrimetric Method GVET 176-1-2010 [22] were used to characterize the RMs of food products and raw materials composition with certified characteristics that determine nutritional value: mass fractions of moisture, protein (crude protein), fat (crude fat, oil content), ash, and carbohydrates (lactose) [23–25].

When implementing the SPRMPs, the need to expand the range of the manufactured RMs for new product groups was identified. Using measurement base of the SPRMPs, high-precision measurement procedures of the mass fraction of fat

Table 1 Measurement objects and metrological characteristics of SPRMPs

FIF EUM No.	Measurement objects	Measured value	Measuring range, %	Absolute expanded uncertainty (k = 2), %
FR.PR1.31.2019.00001	Dairy, grain-dairy, grain, meat, dry egg products, incl. for baby food	Mass fraction of fat	0.1–80	0.08–0.80
FR.PR1.31.2019.00009	Grain and legumes seeds, seeds of oilseeds and associated products, compound feed, and feed components	Mass fraction of crude fat (oil content)	0.2–70	0.15
FR.PR1.31.2019.00002	Grain, grain products, starch, sugar, dairy products, low-lactose, lactose-free, incl. for baby food, compound feed, and feed components	Mass fraction of ash	0.1–40	0.003–0.30
FR.PR1.31.2019.00005	Dairy, grain, grain-dairy, low-lactose and lactose-free products, incl. for baby food	Mass fraction of reducing sugars in terms of lactose monohydrate	0.5–80	0.25
		Mass fraction of non-reducing sugars in terms of sucrose	0.5–50	0.30
		Mass fraction of carbohydrates (total sugar)	1–80	0.40

in liquid dairy products,[6] fish,[7] pastry, confectionery, and pasta products[8] using the Randall method, as well as the mass fraction of lactose in liquid dairy products using

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[6] FR.1.31.2020.36184 Measurement procedure of the mass fraction of fat in liquid dairy products using the Randall method. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/971941. Accessed 26 August 2022 (In Russ.).

[7] FR.1.31.2021.39190 Measurement procedure of the mass fraction of fat in fish using the Randall method M.241.0159/RA.RU.311866/2020. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/1386024. Accessed 26 August 2022 (In Russ.).

[8] FR.1.31.2022.43775 Measurement procedure of the mass fraction of fat in samples of bakery, confectionery and pasta using the Randall method M.241.0074/RA.RU.311866/2022. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/1401182. Accessed 26 August 2022 (In Russ.).

Table 2 High-precision measurement procedures using the equipment for implementing SPRMPs

FIF EUM No.	Measurement objects	Measured value	Measuring range, %	Absolute expanded uncertainty (k = 2), %
FR.1.31.2020.36184	Drinking milk	Mass fraction of fat	0.5–6	0.05
	Drinking cream		9–42	0.06
FR.1.31.2020.36183	Drinking milk	Mass fraction of lactose	4–5.5	0.08
	Drinking cream		3–8	
FR.1.31.2021.39190	Fresh, chilled, boiled, freeze-dried fish	Mass fraction of fat	0.5–20	0.25
FR.1.31.2022.43775	Pastry, confectionery, and pasta products	Mass fraction of fat	0.5–50	0.25

the iodometric titration method[9] were developed and certified. The measurement objects and metrological characteristics of high-precision measurement procedures on equipment for the SPRMPs implementation are presented in Table 2.

The measurement procedures indicated in Table 2 are used to determine the following RMs characteristics: GSO 11504-2020/GSO 11505-2020[10] [26], GSO 11687-2021,[11] GSO 11962-2022.[12] The RMs metrological characteristics are presented in Tables 3 and 4.

In addition to the SPRMP of the mass fraction of carbohydrates in food products and food raw materials, the development of measurement procedures of the mass fraction of the main substance in mono- and disaccharides using the methods of iodometric titration and material balance ("100% minus the impurities") was carried

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[9] FR.1.31.2020.36183 Measurement procedure of the mass fraction of lactose in liquid dairy products using the iodometric titration method. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/971924. Accessed 26 August 2022 (In Russ.).

[10] GSO 11504-2020/GSO 11505-2020 Reference materials for the composition of dairy products (set MS-1 RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1211469. Accessed 26 August 2022 (In Russ.).

[11] GSO 11687-2021 Reference materials for the composition of freeze-dried pollock fillets (RP-1 RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1386484. Accessed 26 August 2022 (In Russ.).

[12] GSO 11962–2022 Reference materials for the composition of wheat crackers (HB-1 RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1402489. Accessed 26 August 2022 (In Russ.).

Table 3 Metrological characteristics of GSO 11504-2020/GSO 11505-2020 of dairy products composition: drinking milk (MS-1-1), drinking cream (MS-1-2)

Certified characteristic	RMs Index	Interval of certified values, %	Absolute expanded uncertainty (k = 2), %
Mass fraction of fat	MS-1-1	0.50–6.00	0.05
	MS-1-2	9.00–42.00	0.06
Mass fraction of protein	MS-1-1	2.00–4.00	0.04
	MS-1-2	1.50–3.50	0.06
Mass fraction of dry substances	MS-1-1	8.0–15.0	0.1
	MS-1-2	20.0–55.0	
Mass fraction of lactose	MS-1-1	4.00–5.00	0.08
	MS-1-2	3.00–8.00	

Table 4 Metrological characteristics of GSO 11687-2021, GSO 11962-2022

RM	Certified characteristic	Interval of certified values, %	Absolute expanded uncertainty (k = 2), %
GSO 11687-2021 of freeze-dried pollock fillets composition	Mass fraction of moisture	1.0–10.0	0.2
	Mass fraction of nitrogen*	11.20–15.84	0.08
	Mass fraction of protein*	70.0–99.0	0.5
	Mass fraction of fat*	1.0–15.0	0.4
GSO 11962-2022 of wheat rusks composition	Mass fraction of moisture	4.00–15.00	0.15
	Mass fraction of nitrogen*	1.00–5.00	0.08
	Mass fraction of protein*	6.0–30.0	0.5
	Mass fraction of fat*	1.5–15.0	0.2

* Values are given in terms of dry matter

out, followed by the creation of the following RMs: GSO 11820-2021,[13] GSO 11839-2021/GSO 11843-2021,[14] GSO 11884-2022,[15] GSO 11885-2022,[16] GSO 11886-2022[17] [15, 27].

Table 5 shows the distribution of RMs by the main groups of food products specified in the Technical Regulations of the Customs and Eurasian Economic Union [28–35]. Along with RMs certified with the use of SPRMPs and high-precision procedures, Table 5 also lists the previously developed RMs widely used in practice with certified values of the mass fraction of moisture, set at GET 173-2017,[18] and the mass fraction of nitrogen and protein, set at GVET 176-1-2010[19]: GSO 8989-2008,[20] GSO 8990-2008,[21] GSO 9564-2010[22] [36], GSO 10148-2012[23] [37], GSO

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[13] GSO 11820-2021 Reference materials for the composition of lactose monohydrate (lactose RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1395358. Accessed 26 August 2022 (In Russ.).

[14] GSO 11839-2021/GSO 11843–2021 Reference materials for the composition of mono- and disaccharides (a set of carbohydrates RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1395634. Accessed 26 August 2022 (In Russ.).

[15] GSO 11884-2022 Reference materials for the composition of arabinose (arabinose RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1397882. Accessed 26 August 2022 (In Russ.).

[16] GSO 11885-2022 Reference materials for the composition of mannose (mannose RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1397883. Accessed 26 August 2022 (In Russ.).

[17] GSO 11886-2022 Reference materials for the composition of sucrose (sucrose RM UNIIM). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1397884. Accessed 26 August 2022 (In Russ.).

[18] GET 173–2017 State primary measurement standard for the units of mass fraction and mass (molar) concentration of water in solid and liquid substances and materials: Custodian Institute D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397857. Accessed 26 August 2022 (In Russ.).

[19] GVET 176–1–2010 State secondary measurement standard for the units of mass fraction and mass (molar) concentration of components in solid and liquid substances and materials on the basis of the volumetric titrimetric method: Custodian Institute UNIIM - Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. (In Russ.).

[20] GSO 8989–2008 Reference materials for the mass fraction of moisture of grain of the 1st categoration. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/391196. Accessed 26 August 2022 (In Russ.).

[21] GSO 8990–2008 Reference materials for the mass fraction of moisture of grain of the 2nd categoration. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/391195. Accessed 26 August 2022 (In Russ.).

[22] GSO 9564–2010 Reference materials for the mass fraction of moisture in grain processing products. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/390621. Accessed 26 August 2022 (In Russ.).

[23] GSO 10148–2012 Reference materials for the mass fraction of moisture in dry dairy products. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/390034. Accessed 26 August 2022 (In Russ.).

9563-2010[24] [22], and GSO 9734-2010[25] [38]. The candidate materials for the RMs development for excluded food groups are also provided here.

Figure 1 shows data on the RMs production using SPRMPs and high-precision measurement procedures using the equipment for implementing SPRMPs for various product groups, which demonstrate an annual increase in demand for manufactured RMs. In 2019–2022, 69, 106, 274, and 428 copies of RMs were released, respectively. The most popular RMs are the ones of dairy products composition. Over the past year, there has also been a significant increase in demand for the RMs of composition of meat products, starch, cereals, and grain-dairy cereals for baby food.

The RMs and ILCs samples analyzed using SPRMPs, provide metrological traceability of the assigned values of mass fractions of moisture, protein, fat, and ash in implementing qualification testing programs of laboratories in the field of measuring quality indicators of milk and dairy products, starch and starch products, and egg powder. A total of 7 rounds were held, the total number of participants was 121.

Using the RMs, certified with SPRMPs and high-precision measurement procedures, tests were conducted for type approval, and verification methods were developed for express analyzers of food products and food raw materials (Table 6).

The manufactured RMs of food products and food raw materials composition also significantly simplified and sped up the verification procedure for the previously approved types of express analyzers, for example, Lactane milk quality analyzers[26] and Infratec food analyzers.[27] Previously, due to the lack of appropriate RMs, such analyzers were verified using working food samples analyzed by standardized methods. The need to establish certified values of the mass fractions of components in working samples required the verification organizations to have additional specialized premises, test equipment, qualified personnel, the use of a large volume of reagents, and significant time costs.

Currently, the express analyzers of food products and food raw materials are checked annually using the developed RMs. In total, more than 190 verifications were carried out in 2020–2022 (Fig. 2).

Thus, the presented data demonstrate the high efficiency of using SPRMPs to ensure metrological traceability of measurements in the food and agricultural industry. At the same time, there is an evident necessity of carrying out the work on developing SPRMPs for excluded product groups and their further implementation

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Table 5 Distribution of RMs by food product groups [28–35]

Technical regulations	Product group	Reference materials	FIF EUM No.
TR TS 015/2011	Grain for consumption and feeding	Wheat, barley, rye, soy, compound feed, oilseed processing products	GSO 8989-2008, GSO 8990-2008, GSO 9734-2010, GSO 11268-2019/GSO 11270-2019, GSO 11279-2019/GSO 11283-2019
TR TS 021/2011	Flour and cereal products	Wheat flour, durum wheat, rye baking, rye-wheat, wheat-rye, corn; wheat groats, buckwheat, oatmeal, millet ground, barley, rice, corn, semolina; wheat bran, rye; wheat grits; soy flour*	GSO 9564-2010
	Starch products	Corn starch, potato starch	GSO 11338-2019/GSO 11339-2019
	Sugar	Sucrose, lactose monohydrate, galactose, glucose anhydride, lactulose, fructose, arabinose, mannose	GSO 11820-2021, GSO 11839-2021/GSO 11843-2021, GSO 11884-2022, GSO 11885-2022, GSO 11886-2022
	Pastry, pasta products	Wheat rusks	GSO 11962-2022
	Confectionery	Chocolate*, dessert paste*	RMs missing
	fruit and vegetable products	Tomato powder*, nut flour*	RMs missing
	Eggs and their processed products	Egg powder	GSO 11271-2019
TR TS 024/2011	Oilseed raw materials	Sunflower, soy, mustard, rapeseed, flax, cotton	GSO 11284-2019/GSO 11289-2019
TR TS 027/2012	Specialized food products	Dry rice, buckwheat, corn, multi-slag porridge	GSO 11144/GSO 11147-2018
	Specialized food products components	Soy protein isolate*	RMs missing
TR TS 033/2013	Milk and dairy products	Skimmed milk powder, whole milk powder, sour cream FD**, cottage cheese FD, dry cheese, drinking milk, drinking cream	GSO 9563-2010, GSO 10148-2012, GSO 11086-2018/GSO 11091-2018, GSO 11399-2019, GSO 11504-2020/GSO 11505-2020

(continued)

Table 5 (continued)

Technical regulations	Product group	Reference materials	FIF EUM No.
	Dairy-based baby food products	Dry milk mixture for baby food, dry rice milk porridge, buckwheat, corn, multi-slag	GSO 11088-2018, GSO 11127-2018/ GSO 11130-2018
TR TS 034/ 2013	Meat and meat products	Beef FD, pork FD	GSO 11274-2019, GSO 11275-2019
EAEU TR 040/2016	Fish and fish products	Pollock fillet FD	GSO 11687-2021
EAEU TR 051/2021	Poultry and its processing products	Poultry FD, canned food*	GSO 11276-2019

* Candidate materials for the RMs development

** FD-freeze drying

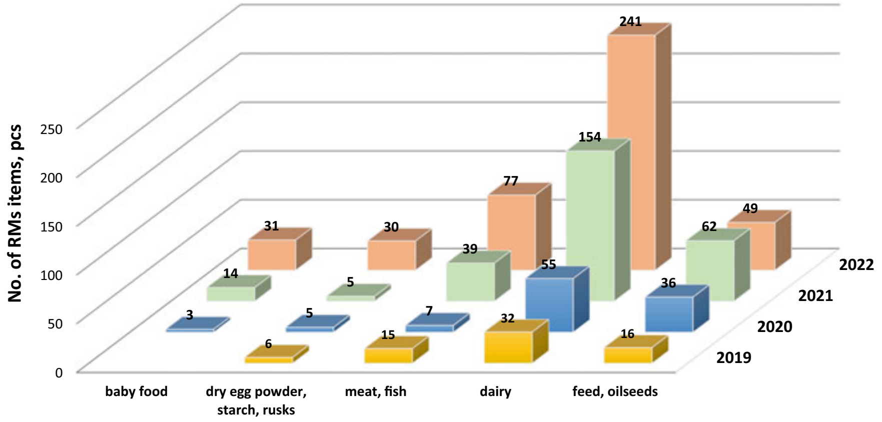

Fig. 1 Production of RMs of food products and raw materials composition using SPRMPs and high-precision measurement procedures

for metrological support of measurements of nutritional values, during which the following tasks should be solved:

(1) analyzing technical regulations, standardized measurement methods, measuring equipment; preparing development plans for RMs for excluded groups of food products; justifying the scope and measurement ranges of SPRMPs, establishing the requirements for SPRMPs accuracy indicators, providing a margin of accuracy in relation to the standardized measurement procedures and working measuring instruments by 2–3 times, at least;

(2) determining measurement parameters and ranges of nutritional values using independent physical and chemical methods (high-performance liquid chromatography, spectrometric analysis, thermogravimetric analysis with mass

Table 6 Measuring instruments tested for type approval using RMs

Measuring instrument no. in FIF EUM	Measuring instruments name and type	Manufacturer
72990-18	Foodscan 2 Food Analyzer	FOSS Analytical A/S, Denmark
77391-20	INFRANEO grain and grain products analyzers	Chopin Technologies, France
78730-20	LactoScope FT-A Milk and Dairy Analyzers	PerkinElmer Analytical Solution B. V., Netherlands
79313-20	MIRA analyzers of milk and dairy products	Bruker Optik GmbH, Germany
80513-20	MilkoScan FT3 Milk and Dairy Analyzers	FOSS Analytical A/S, Denmark
81771-21	Infracont infrared analyzers	Infracont Instruments Ltd., Hungary
85700-22	GrainSense infrared analyzers	GrainSense Oy, Finland
86418-22*	Milkotester Milk Analyzers	Milkotester LTD, Bulgaria

* Tests for type approval carried out in All-Russian Scientific Research Institute for Metrological Service

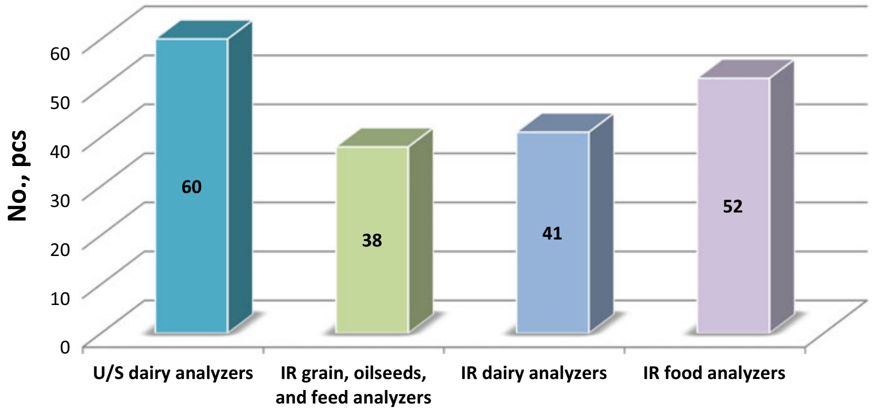

Fig. 2 Information on the no. of analyzers verifications using RMs, certified using SPRMPs and high-precision measurement procedures

spectrometric detection), as well as mathematical methods of experiment planning and data processing (sequential search, dichotomy, multifactorial experiment, regression and dispersion analysis);

(3) evaluating SPRMPs metrological characteristics considering the sources of uncertainty included in the measurement equations, contributions from methodological parameters, heterogeneity of sample material and operator influence

using algorithms set out in ISO 5725-5:1998 [16], RMG 61-2010 [17], and the EURACHEM/CITAC Guide [4].

(4) confirming the highest accuracy and equivalence of the SPRMPs by conducting comparisons with metrological institutes of other countries, Russian scientific branch institutes, the organization of the ILCs with the participation of Russian testing laboratories with subsequent processing according to the algorithm [39], analysis of other countries' CRMs produced by the USA National Institute of Standards and Technology (NIST)[28] and the European Institute for Reference Materials and Measurements (IRMM)[29];

(5) developing and conducting tests for the approval of the RMs type, representing previously uncovered groups of food products (confectionery, fruit, and vegetable products, components for specialized food products, canned food).

The solution of the tasks set will make it possible to complete the development of SPRMPs and RMs, covering the main groups of food products specified in the Technical Regulations of the Customs and Eurasian Economic Union, to implement metrological traceability of measurements of the mass fraction of fat, ash, and carbohydrates in food products and food raw materials.

Conclusion

The analysis of using the SPRMP_S of the mass fraction of fat, ash, and carbohydrates in food products and food raw materials, as well as the SPRMP of the mass fraction of crude fat (oil content) in oilseeds and products based on them, has shown.

- the high efficiency of using SPRMPs to ensure metrological traceability of measurements in the food and agricultural industry;
- the need for developing and implementing new SPRMPs.

The action plan for the development of new SPRMPs and RMs is presented in order to cover the main groups of food products presented in the Technical Regulations of the Customs and Eurasian Economic Union.

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Author Contributions Sergeeva A. S.—development of the research concept, data curation, writing a draft version of the article, proofreading, and editing the text of the article; Kasilyunas A. V.—development of methods, validation; Parfenova E. G.—conducting research, implementation of developments; Medvedevskikh S. V.—development of methodology, implementation of formal analysis

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference “Reference Materials in Measurement and Technology” (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Bismuth Determination by Controlled-Potential Coulometry: Developing a Highly Accurate Procedure Based on GET 176

Veniamin M. Zyskin and Alena V. Sobina

Abstract In this work, we develop a procedure for reproducing the units of bismuth mass fraction in metallic bismuth and those of bismuth (III) mass concentration in bismuth nitrate solutions by controlled-potential coulometry based on the GET 176–2019 State primary standard of mass (molar, atomic) fraction units and mass (molar) concentration of components in liquid and solid substances and materials based on coulometry. The results obtained can be used when manufacturing certified reference materials (CRMs) for the composition of high-purity bismuth and CRMs for the composition of solutions of bismuth (III) ions directly traceable to GET 176–2019. These CRMs may find application in pharmacological, metallurgical, and nuclear industries.

Keywords State primary standard · GET 176 · Certified reference material · CRM · Controlled-potential coulometry · Mass fraction · Bismuth · Traceability · Standard uncertainty · Bismuth mass concentration

Introduction

Bismuth and its compounds find wide application in metallurgical, chemical, and nuclear industries, as well as in physics, medicine, and pharmacopeia [1–3]. Bismuth alloys with cadmium, lead, and zinc provide an ideal basis for obtaining substances with a melting point below 100 °C [4, 5]. Bismuth compounds are used for the production of magnetoelectric, high-temperature ferroelectric, thermoelectric, and superconducting materials [6–8]. Bismuth alloys with manganese, chromium, indium, or europium allow high-quality powerful and durable permanent magnets to be manufactured [9, 10]. Compounds with gallium, iodine, and germanium are in demand

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as ionizing radiation detectors in devices for computer tomography, nuclear physics, and geology [11, 12]. Bismuth compounds are part of medical drugs used for the treatment of gastrointestinal and oncological diseases; antiseptic preparations or wound healing agents; contrasting agents for fluoroscopy [13–16]. Preparations containing organic bismuth compounds are among the few agents effective against *Helicobacter pylori*, a type of bacteria causing gastric and duodenal ulcers [17–19].

The mass fraction of bismuth in alloys, concentrates, and chemical compounds is determined, as a rule, by a photometric method with the use of thiourea according to GOST 28407.1-89 (under the bismuth content of up to 1%) [20], or by a complexometric method with the use of Trilon B (disodium EDTA) of various concentrations in the presence of xylenol orange as an indicator (under the bismuth content ranging from 1 to 90%). In both cases, certified reference materials (CRMs) for the composition of metallic bismuth or CRMs for the composition of solutions of bismuth (III) ions are required to establish the metrological traceability of the measurement results.

However, despite such a great practical significance of bismuth, the Federal Information Fund for Ensuring the Uniformity of Measurements[1] currently lacks CRMs for the composition of metallic bismuth, according to the data of the FIF EUM as of 1 January 2023. For example, the Vi 00 and Vi 02[2] CRMs for the composition of bismuth and the CRM for the composition of bismuth concentrates[3] have been past their shelf life, and the CRMs with metrological traceability are represented by two CRMs of bismuth (III)[4] solutions with the certified mass concentration value of 1 mg/cm^3 and the permissible relative error range of ± 1.0% (at $P = 0.95$).

The content of bismuth in medical preparations in terms of dry matter can reach 51% (bismuth subgallate) and 72% (bismuth subnitrate), or even 90% in bismuth oxide. Therefore, the development of an CRM for the composition of high-purity bismuth and its compounds certified by the mass fraction of the main component and having metrological traceability appears to be a relevant research task.

In this study, our aim was to develop a procedure for reproducing the units of bismuth mass fraction in metallic bismuth and the units of bismuth (III) mass concentration in bismuth nitrate solutions by controlled-potential coulometry using a reference installation (P-100 coulometric potentiostat-integrator) included in the GET

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Table 1 Composition of metallic bismuth, chemically pure

No.	Parameter	Result
1	Mass fraction of bismuth, (Bi), %, no less than	99.95
2	Mass fraction of lead, (Pb), %, no more than	0.01
3	Mass fraction of zinc, (Zn), %, no more than	0.0005
4	Mass fraction of iron (Fe), %, no more than	0.001
5	Mass fraction of antimony, (Sb), %, no more than	0.00005
6	Mass fraction of copper, (Cu), %, no more than	0.0001
7	Mass fraction of silver, (Ag), %, no more than	0.00002
8	Mass fraction of arsenic, (As), %, no more than	0.0001
9	Mass fraction of cadmium, (Cd), %, no more than	0.00005

176-2019 State primary standard of mass (molar, atomic) fraction and mass (molar) concentration of components in liquid and solid substances and materials on the basis of coulometry.[5]

In order to achieve the aim, the following objectives were formulated:

- to study the electrochemical oxidation–reduction reaction of the Bi^{3+}/Bi^0 system by obtaining voltammetric curves of cathodic and anodic polarization of a platinum electrode in bismuth nitrate solutions in the presence of nitric and hydrochloric acids;
- to determine optimal reaction conditions providing for a 100% current yield;
- to establish the uncertainty budget and to investigate the contribution of type A and type B uncertainties when determining bismuth by the method of controlled-potential coulometry.

Materials and Methods

Materials

Bismuth nitrate solutions were prepared using chemically pure metallic bismuth and analytical-grade pentahydrate bismuth nitrate (III). Tables 1 and 2 describe the characteristics of metallic bismuth and bismuth nitrate, respectively.

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[5] GET 176-2019 State primary standard of units of mass (molar, atomic) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on coulometry (includes 3 installations on the basis of constant current coulometry, controlled potential coulometry and inductively coupled plasma mass spectrometry): Custodian Institute UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1382712. Accessed 4 august 2022 (In Russ.).

Table 2 Composition of bismuth nitrate, analytical reagent grade

No.	Parameter	Result
1	Mass fraction of $Bi(NO_3)_3 \cdot 5H_2O$, %, no less than	99.5
2	Mass fraction of nonsolute in nitric acid, %, no more than	0.002
3	Mass fraction of sulfates (SO_4), %, no more than	0.005
4	Mass fraction of chlorides (Cl), %, no more than	0.001
5	Mass fraction of iron (Fe), %, no more than	0.001
6	Mass fraction of copper (Cu), %, no more than	0.0005
7	Mass fraction of lead (Pb), %, no more than	0.005
8	Mass fraction of magnesium (Mg), %, no more than	0.0005
9	Mass fraction of calcium (Ca), %, no more than	0.0005

Methods

Bismuth (III) solutions were prepared gravimetrically by dissolving a weighted portion of metallic bismuth or bismuth (III) nitrate in nitric acid of high purity according to GOST 11125-84 [21], further purified by distillation in a vacuum unit for low-boiling acid distillation. The content of impurity elements in nitric acid was measured by inductively coupled plasma mass spectrometry (ICP-MS). Solutions with concentrations of 10 g/dm^3 were prepared using weighted portions; solutions with concentrations of 4 and 1 g/dm^3 were prepared by multiple dilution with deionized water with addition of nitric acid. The concentration of HNO_3 in all prepared solutions was 0.25 M.

Measurements of the mass fraction of the main component in metallic bismuth and the mass concentration of bismuth in its solutions were carried out using the reference installation included in GET 176-2019, which implements the method of controlled-potential coulometry. The scheme and metrological characteristics of this installation can be found in [22].

The method is based on the reactions of electrochemical reduction of Bi^{3+} ions to Bi^0 on a platinum gauze at a potential of −150 mV and electrochemical oxidation of Bi^0 to Bi^{3+} at a potential of + 200 mV in an acid medium:

$Bi^{3+} + 3e \rightarrow Bi^0$ (reduction and separation of metal on a platinum gauze);
$Bi^0 - 3e \rightarrow Bi^{3+}$ (electro-dissolution of the metal from the electrode).

The electrochemical reaction is reversible; the formal potential of the Bi^{3+}/Bi^0 system comprises 0 mV relative to the ESr-10107-3.5 silver chloride reference electrode.

Titration was carried out using a three-electrode electrolytic cell consisting of a 50 cm^3 working chamber and two glass tubes ended with Vicor-glass frits used to separate the cathode and anode spaces. The generator and auxiliary electrodes were platinum gauzes by GOST 6563-75 [23]. The generator electrode was immersed into the working chamber; the auxiliary electrode and the silver chloride reference electrode were immersed into the corresponding glass tubes. The appearance of the electrolytic cell and the general procedure of measurements are given in [24].

Weighing of bismuth portions for dissolution, as well as aliquots of prepared solutions, were carried out taking into account atmospheric buoyancy using a CCE-66 mass comparator (Sartorius, Germany), accuracy class I (special), readability 0.000001 g. The density of bismuth nitrate solutions was measured using a DMA 4500 M density meter.

The mass fraction of bismuth A_m, (%) in metallic bismuth was calculated as follows:

$$A_m = \frac{M_{Bi} \cdot (Q_i - Q_f) \cdot m}{n \cdot F \cdot K_c \cdot m_1 \cdot m_2} \cdot 100\% = \frac{M_{Bi} \cdot \alpha \cdot (N_i - N_f) \cdot m}{n \cdot F \cdot K_c \cdot m_1 \cdot m_2} \cdot 100\%. \qquad (1)$$

The mass concentration C_{mass} (g/dm^3) in bismuth (III) solutions was calculated as follows:

$$C_{mass.} = \frac{M_{Bi} \cdot (Q_i - Q_f) \cdot \rho}{n \cdot F \cdot K_c \cdot m} = \frac{M_{Bi} \cdot \alpha \cdot (N_i - N_f) \cdot \rho}{n \cdot F \cdot K_c \cdot m}, \qquad (2)$$

where

M_{Bi}	is the molar mass of bismuth, g/mol;
Q_i	is the amount of electricity consumed during the oxidation (reduction) reaction of bismuth, C;
Q_f	is the electricity quantity consumed during the reaction of oxidation (reduction) of the background, C;
N_i	is the number of pulses recorded by an integrator pulse counter during the oxidation (reduction) time i of the sample, pulses;
N_f	is the number of pulses detected by an integrator pulse counter during the oxidation (reduction) of the background, pulses;
ά	is the calibration factor determining the quantity of electricity corresponding to 1 pulse at the voltage-to-frequency converter output, C/imp;
m	is the mass of the bismuth sample solution with allowance for atmospheric buoyancy, g;
m_1	is the mass of the bismuth portion with allowance for atmospheric buoyancy, g;
m_2	is the mass of the sample solution placed in the cell with allowance for atmospheric buoyancy, g;
ρ	is the density of the sample solution, g/dm^3;
n	is the number of electrons involved in the electrode reaction;
F	is the Faraday constant (96,485.33212 C/mol) [25];
K_c	is the coefficient of electrochemical reaction completion [26].

At the first stage of the work, in order to determine optimal experimental conditions, we analyzed the voltammetric curves of the cathodic and anodic polarization of a platinum electrode in a bismuth nitrate solution with a concentration of 4 g/

dm^3 in the presence of 0.25 M nitric and hydrochloric acids in the range from −250 to +500 mV with respect to a ESr 10107-3.5 silver chloride reference electrode. Voltammetric curves were recorded and processed using a software package included in the P-100 coulometric potentiostat-integrator. The acid concentration of 0.25 M was selected in order to avoid hydrolysis of bismuth nitrate solutions while shifting the reaction of hydrogen release from the solutions toward more negative potentials [27]. The study of the $Bi^{3+} + 3e^- \rightarrow Bi^0$ electrochemical reduction reaction of bismuth found that, in the presence of HCl, bismuth reduction occurs simultaneously with hydrogen release in the acid solution. In the presence of 0.25 M nitric acid, the reduction process of Bi^{3+} starts at a potential of −30 mV, with the maximum diffusion current reached at potentials from −80 to −100 mV. Due to the high overvoltage, the hydrogen release initiation potential shifts toward the negative region of up to −250 mV. This allows a dense precipitate completely covering the gauze to be obtained.

Under the selected conditions, cathodic and anodic polarization curves of the Pt electrode in solutions containing various amounts of Bi^{3+} were recorded (Fig. 1). It can be seen that, in the medium of 0.25 M nitric acid, the reduction process of Bi^{3+} in the range up to −200 mV proceeds without hydrogen release, thus being suitable for bismuth identification. However, an analysis of the $1'$-$3'$ anodic curves shows that the oxidation reaction of metallic bismuth released on the electrode is more preferrable for this purpose. The process of Bi^0 dissolution from the electrode is much faster than Bi^{3+} reduction from the solution. The limiting diffusive oxidation current occurring during Bi^0 dissolution is approximately twice greater than the cathodic reduction current in each polarization curve due to the higher concentration of bismuth on the electrode, which increases the sensitivity of analysis based on anodic oxidation curves. In addition, the oxidation curves ($1'$-$3'$) clearly show a proportional increase in the limiting diffusion current with increasing Bi^{3+} concentration in solution ($1'$—35 mA, $2'$—70 mA, $3'$—100 mA).

Results and Discussion

The mass concentrations of the prepared solutions were calculated according to the preparation procedure and then measured on the reference installation implementing the controlled-potential coulometry method as part of the GET 176-2019 State primary standard. Table 3 presents the measurement results of the bismuth mass fraction and concentration of the prepared solutions.

Figure 2 depicts factors affecting the accuracy of reproducing the unit of bismuth mass concentration in bismuth (III) nitrate solutions using the State primary standard in the form of a cause-and-effect diagram.

The relative standard type A uncertainty of reproducing the units of mass concentration was estimated as the relative standard deviation of the measurement result based on seven independent measurements. The relative standard uncertainty type B was estimated by combining uncertainty components caused by sample weighing,

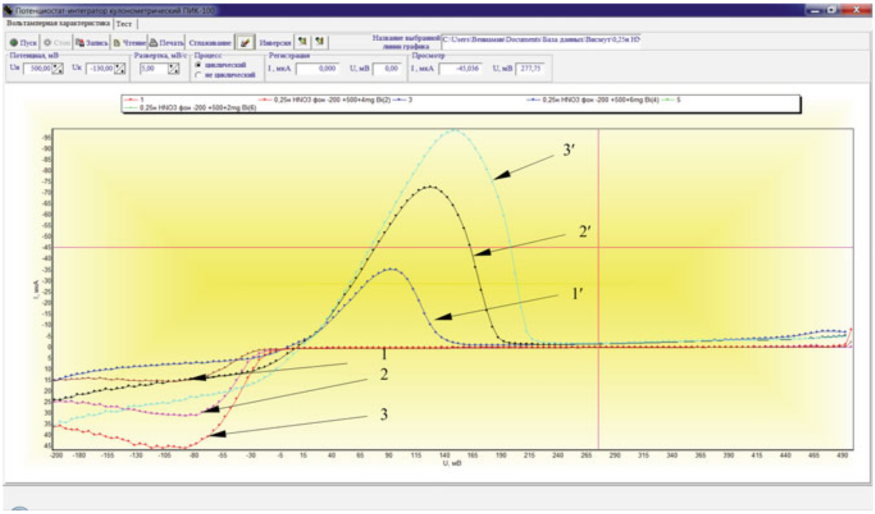

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Fig. 1 Cathodic and anodic curves for the Pt-electrode in $Bi(NO_3)_3$ solution
1-1′—2 mg of Bi^{3+} in the presence of 0.25M HNO_3 solution, scan frequency 5 mV/s
2-2′—4 mg of Bi^{3+} in the presence of 0.25M HNO_3 solution, scan frequency 5 mV/s
3-3′—6 mg of Bi^{3+} in the presence of 0.25M HNO_3 solution, scan frequency 5 mV/s

Table 3 Measurement results of the mass fraction of bismuth in metallic bismuth and the mass concentration of bismuth in bismuth (III) solutions with a nominal concentration of 10 and 1 g/dm^3, obtained using the controlled-potential coulometry installation as part of the GET 176-2019 State primary standard

Sample No.	Metallic bismuth, A = 99.95%	Solutions of bismuth (III) ions, C_{calc} = 10.002 g/dm^3	Solutions of bismuth (III) ions, C_{calc} = 1.0062 g/dm^3
	Mass fraction of bismuth, A, %	Mass concentration of bismuth, C, g/dm^3	
1	99.970	9.9907	1.0098
2	99.961	9.9963	1.0105
3	99.953	10.0088	1.0056
4	99.949	9.9944	1.0105
5	99.965	10.0036	1.0027
6	99.947	9.9880	1.0076
7	99.943	10.0072	1.0085
Mean value, $\overline{A}$, %	**99.955**	–	–
Mean value, $\overline{C}$, g/dm^3	–	**9.9984**	**1.0079**
Relative standard type A uncertainty, %	0.0038	0.031	0.108
Relative standard type B uncertainty, %	0.0063	0.006	0.006
Relative combined standard uncertainly, %	0.0073	0.032	0.108
Relative expanded uncertainty (k = 2), %	**0.015**	**0.06**	**0.22**

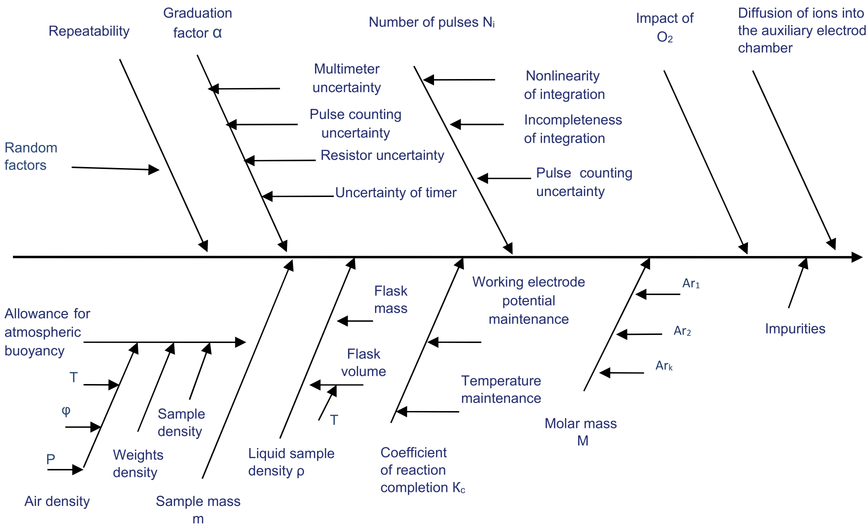

Fig. 2 Uncertainty sources in reproducing the unit of bismuth mass concentration in bismuth (III) nitrate solutions [22]

measuring the quantity of electricity consumed during electrolysis, as well as determination of the coefficient of electrochemical reaction completion, molecular weight, solution density, and other factors (Fig. 2) by the algorithms described in GOST R 54500.3-2011 [28], which is identic to GUM [29].

Tables 4 and 5 present uncertainty budgets when reproducing the units of bismuth mass concentration in solutions with mass concentrations of 10.0 g/dm^3 and 1.0 g/dm^3, respectively.

Table 4 Uncertainty budgets for the reproduction of the units of bismuth mass concentration (10.0 g/dm^3) in a bismuth (III) nitrate solution

Type	Input value	Estimate		Uncertainty		Probability distribution	Sensitivity factor		Contribution to u_c		ν_{eff}
		x_j	Units	u_j	Units		c_j	Units	$c_j u_j$	Units	
A	Repeatability	9.9984	g/dm^3	0.0031	g/dm^3	N	1	1	3.1×10^{-3}	g/dm^3	6
B	$\acute{\alpha}$	1.6×10^{-4}	C/puls	3.9×10^{-9}	C/puls	R	4.2×10^{4}	(g/dm^3)/ (C/puls)	1.7×10^{-4}	g/dm^3	∞
B	N_i	61,159	Pulses	9.2×10^{-1}	Pulses	R	1.2×10^{-4}	(g/dm^3)/puls	1.1×10^{-4}	g/dm^3	∞
B	N_f	2803	Pulses	4.2×10^{-2}	Pulses	R	-1.2×10^{-4}	(g/dm^3)/puls	-4.9×10^{6}	g/dm^3	∞
B	M	208.9804	g/mol	5.0×10^{-5}	g/mol	R	3.2×10^{-2}	mol/dm^3	1.6×10^{-6}	g/dm^3	∞
B	ρ	1015.65	g/dm^3	1.6×10^{-2}	g/dm^3	R	6.6×10^{-3}	–	1.1×10^{-4}	g/dm^3	∞
B	F	96,485.33	C/mol	0	C/mol	R	-1.0×10^{-4}	(g/dm^3)/ (C/mol)	0	g/dm^3	∞
B	K_c	1	–	2.0×10^{-6}	–	R	-10.0	g/dm^3	-2.0×10^{-5}	g/dm^3	∞
B	m	1.02112	g	6.3×10^{-6}	g	R	-9.8	(dm^3)$^{-1}$	-6.2×10^{-5}	g/dm^3	∞
B	Electrolyte impurities	–	–	1.7×10^{-3}	–	R	0.099984	–	1.7×10^{-4}	g/dm^3	∞
B	O_2 impact	–	–	5.5×10^{-3}	–	R	0.099984	–	5.5×10^{-4}	g/dm^3	∞
B	Diffusion	–	–	1.5×10^{-3}	–	R	0.099984	–	1.5×10^{-4}	g/dm^3	∞
Relative standard type A uncertainty, u_{A0}									0.031	%	6
Relative standard type B uncertainty, u_{B0}									0.006	%	∞

(continued)

Table 4 (continued)

Type	Input value	Estimate		Uncertainty		Probability distribution	Sensitivity factor		Contribution to u_c		ν_{eff}
		x_j	Units	u_j	Units		c_j	Units	$c_j u_j$	Units	
Relative combined standard uncertainty, u_{c0}									0.032	%	
Relative expanded uncertainty, U_0 ($k = 2$, $P = 0.95$)									0.06	%	

Input values

1. Graduation factor ά
2. Number of pulses at the voltage-to-frequency converter output N
3. Molar mass of the component M
4. Solution density ρ
5. Faraday constant F
6. Reaction completion coefficient K_c
7. Mass of the solution in the cell m

Table 5 Uncertainty budgets for the reproduction of the units of bismuth mass concentration (1 g/dm^3) in a bismuth (III) nitrate solution

Type	Input value	Estimate		Uncertainty		Probability distribution	Sensitivity factor		Contribution to u_c		ν_{eff}
		$\boldsymbol{x_j}$	Unit	u_j	Unit		c_j	Unit	$c_j u_j$	Unit	
A	Repeatability	1.0079	g/dm^3	0.0011	g/dm^3	N	1	1	1.0 × 10^{-3}	g/dm^3	6
B	$\acute{\alpha}$	1.6 × 10^{-4}	C/puls	3.9 × 10^{-9}	C/puls	R	4.0 × 10^3	(g/dm^3)/ (C/puls)	1.5 × 10^{-5}	g/dm^3	∞
B	N_i	31,591	Pulses	4.7 × 10^{-1}	Pulses	R	2.2 × 10^{-5}	(g/dm^3)/puls	1.0 × 10^{-5}	g/dm^3	∞
B	N_f	2803	Pulses	4.2 × 10^{-2}	Pulses	R	− 2.2 × 10^{-5}	(g/dm^3)/puls	− 9.4 × 10^{-7}	g/dm^3	∞
B	M	208.9804	g/mol	5.0 × 10^{-5}	g/mol	R	3.0 × 10^{-3}	mol/dm^3	1.5 × 10^{-7}	g/dm^3	∞
B	ρ	1003.35	g/dm^3	1.6 × 10^{-2}	g/dm^3	R	6.4 × 10^{-4}	–	1.0 × 10^{-5}	g/dm^3	∞
B	F	96,485.33	C/mol	0	C/mol	R	− 1.0 × 10^{-5}	(g/dm^3)/ (C/mol)	0	g/dm^3	∞
B	K_c	1	-	2.0 × 10^{-6}	–	R	− 1.0	g/dm^3	− 2.0 × 10^{-6}	g/dm^3	∞
B	m	5.18965	g	6.3 × 10^{-6}	g	R	− 1.9 × 10^{-1}	(dm^3)$^{-1}$	− 1.2 × 10^{-6}	g/dm^3	∞
B	Electrolyte impurities	–	–	1.7 × 10^{-3}	–	R	0.010079	–	1.7 × 10^{-5}	g/dm^3	∞
B	O_2 impact	–	–	5.5 × 10^{-3}	–	R	0.010079	–	5.5 × 10^{-5}	g/dm^3	∞
B	Diffusion	–	–	1.5 × 10^{-3}	–	R	0.010079	–	1.5 × 10^{-5}	g/dm^3	∞

(continued)

Table 5 (continued)

Type	Input value	Estimate		Uncertainty		Probability distribution	Sensitivity factor		Contribution to u_c		ν_{eff}
		$\boldsymbol{x_j}$	Unit	u_j	Unit		c_j	Unit	$c_j u_j$	Unit	
Relative standard type A uncertainty, u_{A0}									0.108	%	6
Relative standard type B uncertainty, u_{B0}									0.006	%	∞
Relative combined standard uncertainty, u_{c0}									0.108	%	
Relative expanded uncertainty, U_0 ($k = 2,\ P = 0.95$)									0.22	%	

Input values

1. Graduation factor ά
2. Number of pulses at the voltage-to-frequency converter output N
3. Molar mass of the component M
4. Solution density ρ
5. Faraday constant F
6. Reaction completion coefficient K_c
7. Mass of the solution in the cell m

Conclusion

The conducted studies have confirmed the applicability of the developed procedure based on the P-100 potentiostat-integrator included in GET 176-2019 for reproducing the units of bismuth mass fraction in metallic bismuth with a relative expanded uncertainty of 0.015% and bismuth mass concentration in bismuth (III) solutions in the range from 1.0 to 10.0 g/dm^3 with a relative expanded uncertainty of 0.06–0.22%. The procedure for reproducing the units of bismuth mass fraction in metallic bismuth and the mass (molar) concentration of bismuth in solutions of its salts by controlled-potential coulometry was developed and approved.

The results obtained can be used for the development and approval of CRMs for the composition of metallic bismuth with an expanded relative uncertainty of 0.015–0.03% and CRMs for the composition of solutions of bismuth (III) ions in the range from 1.00 to 10.00 g/dm^3 with an expanded relative uncertainty of 0.2–0.4%, having direct metrological traceability of the certified RM values to the GET 176-2019 primary standard.

The theoretical significance of the study consists in the development of methodological approaches to the certification procedure of an CRM for the composition of metallic bismuth and an CRM for the composition of bismuth solutions, allowing the accuracy of measurements in pharmacological, metallurgical, and nuclear industries to be improved.

The practical significance of the study consists in the possibility of providing metrological traceability of measurement results to the GET 176-2019 primary standard when controlling the release of pharmaceuticals based on bismuth compounds.

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Author Contribution Zyskin V. M.—research concept, measurement technique development, experimental data collection, literature data review, preparation of the original text of the article; Sobina A. V.—supervision, experimental data analysis, revision and editing the text of the article.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference materials in measurements and technologies" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized and the review procedure was carried out.

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Reference Materials of the Oil Refining, Gas, and Fuel Industry

Use of Reference Materials-Imitators for Metrological Support of Gas Analytical Measuring Instruments

Anna V. Kolobova, Leonid A. Konopelko, Timofei B. Sokolov, and Olga V. Fatina

Abstract Measurements in the field of gas analysis are one of the types of measurements of the physical and chemical composition and properties of substances, and cover a large amount of measurement tasks in various areas of human activity. The main means of calibration and verification of gas measuring instruments are 1st and 2nd category reference materials: gas mixtures, permeation tubes of gases and vapors, gas generators. The purpose of the research was to summarize the many years of experience of the specialists of the Research Department of State Standards in the field of Physical and Chemical Measurements, D.I. Mendeleyev Institute for Metrology, in terms of the use of reference materials—imitators for metrological support of the measuring instruments. The research considered the main features of the use of reference materials of gas mixtures as imitators of real environments. The main differences between imitators with a real environment were described. Approaches have been developed to assess the possibility of using imitators, the features of testing, calibration and verification of the measuring instruments. The practical significance is the use of the research results by metrologists in solving practical problems that arise during tests, verification and calibration of the measuring instruments.

Keywords Gas mixtures · Imitators · Concentration of components · Real environment · Gas analyzers · Ethanol vapor analyzers

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Abbreviations

FIF OEI	Federal Information Fund for Ensuring the Uniformity of Measurements
GET 154-2019	State Primary Standard of Units of Mole Faction, Mass Fraction and Mass Concentration of Components in Gas and Gas Condensate Media
MI	Measuring instrument
GM	Gas mixture
MT	Measuring transducer

Introduction

As a way of determining the physicochemical composition and properties of substances, gas analytical measurements cover a wide range of measurement problems in various spheres of human activity [1]:

- monitoring of atmospheric air pollutants (environmental monitoring);
- monitoring of hazardous substances in the workplace air;
- monitoring of industrial and automotive vehicle emissions;
- monitoring of explosive gases and vapors in the workplace air;
- monitoring of greenhouse gas emissions;
- monitoring of the component concentration during technological processes;
- composition control of hydrocarbon feedstock and derived products;
- monitoring of breath ethanol content, etc.

Over 5000 types of gas analytical measuring instruments (MIs) are registered in the Federal Information Fund for Ensuring the Uniformity of Measurements, which include gas analyzers, detectors, measuring systems, chromatographs, mass spectrometers, etc.

Gas analytical MIs are traceable to GET 154-2019 State Primary Standard of Units of Mole Faction, Mass Fraction and Mass Concentration of Components in Gas and Gas Condensate Media operating at the D.I. Mendeleyev Institute for Metrology (VNIIM) [2, 3].

The procedure for transferring the units of component concentration and content from GET 154-2019 to gas analytical MIs using 1st and 2nd class working standards is established in two state hierarchy schemes:

- state hierarchy scheme for instruments measuring the component concentration in gas and gas condensate environments, approved by the order of Rosstandart No. 2315 of Dec. 31, 2020 [4];

- state hierarchy scheme for instruments measuring ethanol concentration in gas environments, approved by the order of Rosstandart No. 3452 of Dec. 30, 2019 [5].

Gas analytical MIs are verified using primarily 1st and 2nd class working standards [1, 2, 6]:

- reference materials (RMs) for the composition of gas mixtures (GMs) in pressure cylinders;
- measures-sources of gas and vapor microflows—that are used together with thermal-diffusion generators of GMs;
- generators of various GMs.

One of the objectives set for the metrological support of gas analytical MIs consists in determining the metrological characteristics of MIs during verification and maintenance with the use of GMs corresponding to real analyzed environments in terms of their characteristics. Real analyzed environments can be both “simple” (binary and quasi-binary mixtures, e.g., methane-air, oxygen–nitrogen, etc.) and “complex.”

In the case of “simple” analyzed environments, gas analytical instruments can generally be verified and maintained using GMs that are most similar to real environments. As an example, we can consider gas analyzers of firedamp (methane), whose verification and maintenance are carried out according to the methane-air GM.

Examples of “complex” real environments include:

- flammable liquid vapors having a low saturation vapor pressure of the component to be determined mixed with air (saturated hydrocarbons in C_7H_{16}, aromatic hydrocarbons, etc.) and multicomponent mixtures (vapors of petroleum and petroleum products, etc.);
- explosive gas mixtures (hydrogen in oxygen, etc.) or non-explosive gas mixtures (hydrogen in carbon dioxide, etc.) that can become explosive when mixed with air;
- gas mixtures containing toxic or poisonous components (organophosphorus toxicants; rocket fuel components);
- gas mixtures in atmospheric air (containing moisture and undetectable components—argon, carbon dioxide, etc., corresponding to the atmospheric air composition);
- gas mixtures in industrial emissions (multi-component gas mixtures characterized by increased temperature and moisture, as well as the presence of chemically active components);
- exhaled air with ethanol vapor (humidity of $(95 \pm 5)\%$; sample temperature of about $+34$ °C; carbon dioxide content of about 5%).

A high degree of complexity is clearly involved in creating such “complex” gas environments for the metrological support of gas analytical MIs. In some cases, it is impossible or costly and labor-intensive to create GMs that correspond to real analyzed environments in terms of their characteristics. For example, in order to create air-vapor mixtures of flammable liquid vapors under laboratory conditions, working standards–dynamic systems (GGP-1,[1] DGK-NV,[2] etc.) are developed and successfully used; however, a certain level of qualification is required from the attending personnel to operate them, which does not solve the issue associated with the in-the-field verification and maintenance of a large number of in-service MIs.

In order to address these limitations, an approach has been developed to the use of imitators of real environments, which significantly simplifies the verification and maintenance of MIs, as well as greatly reducing user costs related to MI operation. For example, although RMs for the composition of gas mixtures in pressure cylinders do not fully correspond to real environments in terms of their composition or properties, they still provide a means to transfer the unit of component concentration with the required accuracy.

The study aims to identify and analyze the main factors affecting the metrological support of gas analytical MIs, taking into account the specifics of type approval testing and verification of MIs on the example of imitation RMs.

To this end, it is necessary to achieve the following objectives: to carry out a theoretical analysis of imitation GMs used for the metrological support of gas analytical MIs; to consider practical examples of static MI characteristics for a real environment and an imitator; to demonstrate algorithms for calculating MI correction factors and error when using imitators; to formulate recommendations for the use of imitation GMs for the metrological support of gas analytical MIs.

Theoretical Analysis

Widely used in operation and characterized by high stability, including in transportation and storage, GMs in pressure cylinders are generally used as imitators. The most commonly used GMs are methane-air, propane-air (and the same components in nitrogen), isobutylene-air, etc.

The feasibility of using imitators is due to the design and functional features of gas analytical MIs. In the general form, the conversion function of a gas analytical MI can be written as

$$I_{output} = K \cdot C_{input}, \tag{1}$$

where:

I_{output} output signal of the MI (indication);

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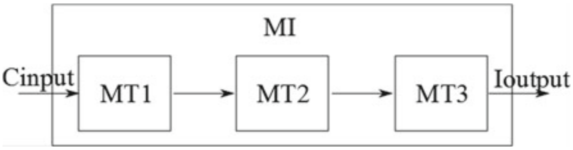
[1] Working standard of the 1st class—GGP-1 complex, reg. FIF OEI No 48775-11. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/360297. Accessed 15 July 2022 (In Russ.).

[2] Working standard of the 1st class—DGK-NV dynamic gas mixing complex, reg. FIF OEI number 47882-11. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/359260. Accessed 15 July 2022 (In Russ.).

Fig. 1 Functional flow block diagram of a gas analytical MI



C_{input}	concentration of the analyzed component at the MI input;
K	generalized conversion factor.

A general functional flow block diagram of a gas analytical MI can be represented by a set of three measuring transducers (MTs) [7], as shown in Fig. 1.

Let us consider the functional flow block diagram of a MI in more detail (Fig. 1) where the following notations are used:

C_{input}	input quantity (concentration of the component to be determined in the analyzed environment);
MT1	"composition–composition" measuring transducer ensuring scale transformation of the analyzed sample, e.g., preparation of the analyzed environment—temperature and flow rate stabilization, purification from mechanical impurities, etc.). Essentially, MT1 serves as a sampling and sample preparation device;
MT2	"composition–property" measuring transducer converting the measurand (mass concentration, volume fraction, etc.) into the physicochemical property of the sensing element (resistance, conductance, capacitance, electromotive force, etc.);
MT3	"property–output signal" measuring transducer converting the physicochemical property of the sensitive element into the output signal (indications) accessible to the operator;
I_{output}	output MI signal (displayed indications, analog or digital output signal, etc.)

Thus, according to the flow block, the generalized conversion factor of the gas analytical MI is functionally related to the conversion factors of individual MI blocks (measuring transducers)

$$K = K_1 \cdot K_2 \cdot K_3, \tag{2}$$

where: K_1, K_2, K_3—conversion factors of the corresponding blocks and modules MT1, MT2, and MT3, comprising the MI.

The conversion factors K_1 and K_3 are usually the characteristics of MIs that do not change when varying the composition of the analyzed environment.

The conversion coefficient K_2 determines the conversion from composition to property and, as a rule, is specific with respect to the composition determining MI selectivity.

As a rule, an imitation GM constitutes a GM which can be characterized as equivalent according to one of the following criteria:

- the main component is replaced, i.e., instead of the component to be determined in the real environment, the imitator uses an equivalent—a verification component having similar physicochemical properties;
- background component characterizing the real environment is replaced with an equivalent (e.g., the hydrogen–oxygen gas mixture is replaced with a hydrogen–nitrogen gas mixture);
- characteristics of the imitator do not fully reflect the real environment (e.g., moisture content, temperature, and the presence of unmeasurable components).

When using imitation GMs, it is necessary to ensure that the following inequality is satisfied

$$f(\delta_1, \delta_2, \delta_3) \leq \frac{\delta_{MI}}{K_{acc.m.}}, \tag{3}$$

where:

δ_1	error of the analyzed component concentration in a real environment;
δ_2	error of the imitation GM;
δ_3	error of the established factor for the correspondence between the concentration of the analyzed component (real environment) and the imitator;
δ_{MI}	MI error;
$K_{acc.m.}$	accuracy margin (as a rule, from 2 to 3).

In this connection, approaches have been developed to assess the feasibility of using imitation GMs for the verification and maintenance of gas analytical MIs:

- selection of an imitator, proceeding from the MI operating principle and design;
- experimental studies on determining the conversion factor (or conversion function) within the measurement range using GMs corresponding to the characteristics of the real environment and imitation GMs (taking into account data from the MI manufacturer);
- experimental studies on the conversion factor spread within a batch of single-type MIs;
- experimental studies on the stability of the conversion factor;
- establishment of special requirements for measurements and processing measurement results when using an imitator;
- estimation of the verification method error, taking into account the error of the imitator and the conversion factor.

Mathematical Approaches

The feasibility of using imitation GMs is generally assessed at the stage of MI development and/or type approval testing. In some cases, preliminary studies examining the metrological characteristics of MIs are recommended to select imitators.

Since 2000, the Research Department for State Standards in the Field of Physicochemical Measurements of VNIIM has conducted a series of studies and tests of several dozen MI types, whose verification and maintenance involves the use of imitation RMs for the composition of gas mixtures in pressure cylinders.

As an example, let us consider some types of such MIs and the results of experimental studies on the selection of imitators for individual measurement channels:

Example 1 DAF-M[3] gas analyzers are designed for continuous automatic mass concentration measurement of hydrocarbons, including petroleum and petroleum products in the air. Here, measurement relies on the photoionization principle. For the examined MI, acetone in air is the real environment, while hexane in air is used as the imitator. The general view of static MI characteristics for the real environment and the imitator is presented in Fig. 2. Hereinafter, the following notations are used in figures showing static MI characteristics:

- C_{input}—concentration of the component in the analyzed environment at the MI input, in measurand units;
- C_{meas}—result of measuring the component concentration in the analyzed environment, in measurand units.

Example 2 OGS-PGP/M-C_3H_8-A[4] gas analyzers are designed for the continuous automatic measurement of lower explosive limit or the volume fraction of flammable gases and liquid vapors in the ambient atmosphere. Here, measurement relies on the optical infrared absorption principle. For the examined MI, the real environment is diesel fuel vapor, while propane in air serves as the imitator. The general view of static MI characteristics for the real environment and the imitator is shown in Fig. 3.

Example 3 TP1142 gas analyzers[5] are designed for measuring the volume fraction of hydrogen, as well as detecting when it exceeds limits established for the gas

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[3] DAF-M gas analyzers manufactured by Analitpribor, Smolensk, Russia. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/530080. Accessed 15 July 2022 (In Russ.).

[4] OGS-PGP/M-C_3H_8-A gas analyzers manufactured by Pozhgazpribor LLC, St. Petersburg, Russia. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/536997. Accessed 15 July 2022 (In Russ.).

[5] TP1142 gas analyzers manufactured by ANAGAZ, St. Petersburg, Russia. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/1388854. Accessed: 15 July 2022 (In Russ.).

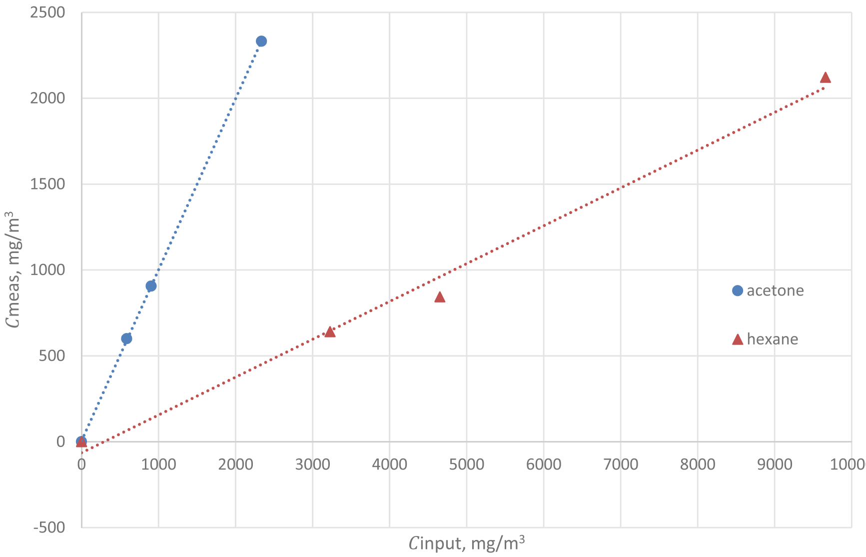

Fig. 2 General view of static characteristics (real environment and imitator) plotted for the DAF-M gas analyzer

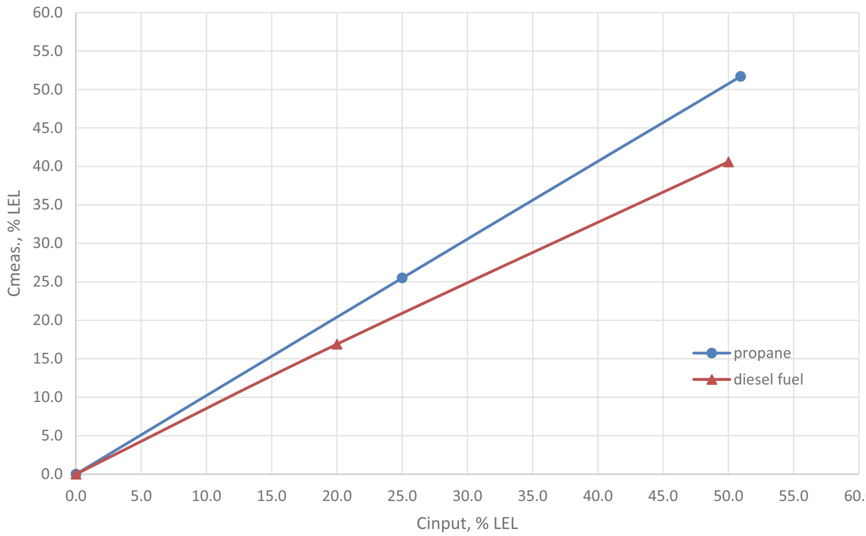

Fig. 3 General view of static characteristics (real environment and imitator) plotted for the OGS-PGP/M-C_3H_8-A gas analyzer

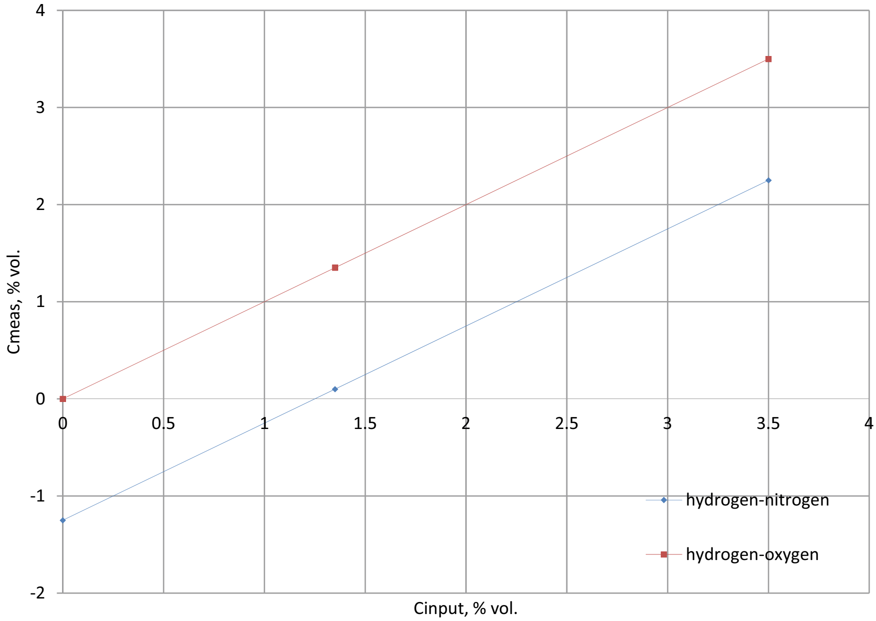

Fig. 4 General view of static characteristics (real environment and imitator) plotted for the TP1142 gas analyzer

environments of process lines in special systems. Here, measurement relies on the thermoconductometric principle. In this case, hydrogen in oxygen is the real environment, while hydrogen in nitrogen is used as the imitator. The general view of static MI characteristics for the real environment and the imitator is shown in Fig. 4.

Example 4 Alcotest 6820 breathalyzers[6] are designed for the rapid mass concentration measurement of ethanol vapor in an exhaled air sample. Here, measurement relies on the electrochemical principle. In this case, ethanol in exhaled air is the real environment (humidity—(95 ± 5)%; sample temperature— +34 °C), while ethanol in nitrogen serves as the imitator (humidity—about 0%; sample temperature— + 20 °C). The general view of static MI characteristics for the real environment and imitator is presented in Fig. 5.

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[6] Alcotest 6820 breathalyzers manufactured by Drager Safety AG & Co. KGaA, Germany. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/381645. Accessed 15 July 2022 (In Russ.).

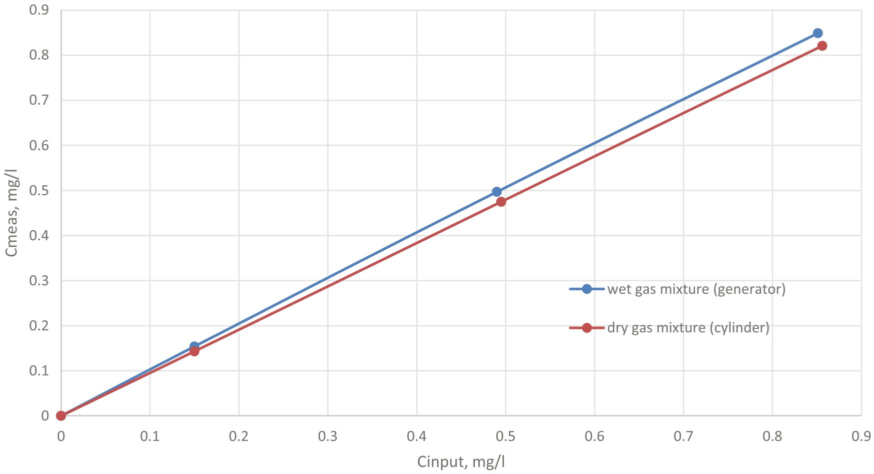

Fig. 5 General view of static characteristics (real environment and imitator) plotted for the Alcotest 6820 breathalyzer

According to the results of experimental studies, the correction factors for DAF-M and OGS-PGP gas analyzers (Examples 1 and 2) can be calculated as follows:

$$K_i = \frac{C_i^{(ver)}}{C_i^{conv.t.(ver)}} \cdot \frac{C_i^{conv.t.(det)}}{C_i^{(det)}}, \tag{4}$$

where:

$C_i^{(ver)}$	result of measuring the verification component concentration when feeding the *i*th GM containing the verification component (on the scale of the component to be determined);
$C_i^{conv.t.(ver)}$	conventional true concentration of the verification component in the *i*th GM containing the verification component;
$C_i^{(det)}$	result of measuring concentration obtained when feeding the *i*th GM containing the component to be determined;
$C_i^{conv.t.(det)}$	conventional true concentration of the component to be determined in the *i*th GM.

In the general case, the absolute MI error when using imitators is calculated according to the formula

$$\Delta = C_i^{(ver)} - K_i \cdot C_i^{conv.t.(ver)}, \tag{5}$$

where:

$C_i^{(ver.)}$ result of measuring the verification component concentration when feeding the *i*th GM containing the verification component (on the scale of the component to be determined);

$C_i^{conv.t.(ver)}$ conventional true concentration of the verification component in the *i*th GM containing the verification component;

K_i value of the correction factor for the *i*th verification point.

For the TP1142 (Example 3), the result of measuring the volume fraction of hydrogen in oxygen is calculated as follows according to the measured output signal (DC voltage from 0 to 10 V) when using imitation GMs (hydrogen in nitrogen):

$$C = K \cdot U_{output} + \Delta_{N2}, \tag{6}$$

where:

U_{output} value of the voltage output signal when feeding the hydrogen in nitrogen GM, V;

K conversion coefficient of 0.4%/V;

Δ_{N2} correction due to the replacement of hydrogen–oxygen GM with hydrogen–nitrogen GM, volume fraction of hydrogen, % ($\Delta_{\mathrm{N2}} = 1.25\%$).

The basic absolute error in measuring the volume fraction of hydrogen is calculated as follows:

$$\Delta C = C - C_{GMC}, \tag{7}$$

where:

C_{GMC} volume fraction of hydrogen specified in the GM certificate, %;

C volume fraction of hydrogen determined according to the output signal, taking the correction into account, %.

For the Alcotest 6820 (Example 4), the result of measuring ethanol mass concentration when feeding GM in a pressure cylinder C_i (mg/L) is calculated according to the following formula

$$C_i = I_i \cdot \frac{101.3}{P} \cdot K^{GM}, \tag{8}$$

where:

I_i analyzer indication when feeding the *i*th GM in a pressure cylinder, mg/L;

K^{GM} factor for converting indications, $K^{GM} = 1.05$;

P atmospheric pressure measured using a barometer, kPa.

The absolute error of the analyzer Δ_i (mg/L) when feeding the *i*th GM is calculated as follows

$$\Delta_i = C_i - C_i^{conv.t.}, \tag{9}$$

where:

C_i mass concentration of ethanol measured when feeding the *i*th GM, mg/L;
$C_i^{conv.t.}$ conventional true mass concentration of ethanol in the *i*th GM in a pressure cylinder, mg/L.

The relative error of the analyzer δ_i (%) when feeding the *i*th GM is calculated as follows

$$\delta_i = \frac{C_i - C_i^{conv.t.}}{C_i^{conv.t.}} \cdot 100, \tag{10}$$

The conventional true mass concentration of ethanol in the *i*th GM in a pressure cylinder $C_i^{conv.t.}$ (mg/L) is calculated according to the formula

$$C_i^{conv.t.} = C_i^{conv.t.}(\%) \cdot k \cdot 10, \tag{11}$$

where:

$C_i^{conv.t.}(\%)$ conventional true volume fraction of ethanol in the *i*th GM in a pressure cylinder, %;
k conversion factor for the following conditions: + 34 °C and 101.3 kPa, $k = 1.828$.

In some cases, the result of measuring the concentration of the component to be determined is calculated using a combination of the considered approaches: for example, for ARP1.0 acoustic resonance transducers[7] (INKRAM, Moscow), both the conversion and correction factors are used in the calculations [8].

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[7] ARP1.0 measuring acoustic-resonance transducers, manufactured by NPF INKRAM, Moscow, Russia. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/367156. Accessed 15 July 2022 (In Russ.).

ARP1.0 measuring acoustic-resonance transducers, manufactured by LLC NPF INKRAM, Moscow, Russia. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/1381989. Accessed 15 July 2022 (In Russ.).

Results and Discussion

The presented examples show that the application problem of imitation GMs is successfully solved for several MI types varying in terms of the measuring principle, purpose and design, by introducing the conversion factors and corrections determined as a result of MI studies or testing.

A special case of imitation GM application is a situation where the study results show that the use of imitation GMs does not require the use of a conversion factor due to the identity of MI indications in the analysis of a real environment and an imitator. For example, AGF-2[8] automatic stationary gas detectors for the detection of organophosphorus toxicants rely on the mass spectrometry operating principle.

Noteworthy is that in order to establish the identity of MI indications when analyzing the real environment and the imitator, virtually the same amount of experimental work is required as when establishing the conversion factor.

In special cases, it is established that the use of imitation GMs is impossible, for example, due to a significant spread of the factor within a MI batch or its instability over time; as a rule, such result is typical for measuring instruments relying on semiconductor or thermochemical measurement principles.

The imitation method requires careful development of a research (test) program, collection of a large experimental data array, processing of measurement results, as well as development of specific verification procedures.

In addition, the following factors should be taken into account when conducting studies:

- error of the verification method using imitators must provide the required accuracy margin;
- in some cases, special requirements exist with regard to the use of imitators: e.g., when testing breathalyzers, it is necessary to keep sessions conducted using dry and wet mixtures apart in time, etc.

The use of imitation GMs is specified in several national and interstate standards, for example, GOST R 8.838-2013, GOST R 8.922-2016, GOST 8.618-2013, GOST 8.629-2013, and GOST R 52350.29.1-2010 [9–13].

Conclusion

The study shows the relevance of using imitation GMs for the metrological support of gas analytical MIs. In the work, we performed a theoretical analysis of imitation GMs, defined the main stages involved in studies on the selection of imitation GMs,

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[8] Stationary automatic gas detectors for the detection of organophosphorus poisonous substances AGF-2, NPO “Pribor”, St. Petersburg, Russia. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/1389113. Accessed 15 July 2022 (In Russ.).

as well as considering practical examples of static MI characteristics for a real environment and an imitator. The algorithms for calculating the MI correction factors and error when using imitators are presented. The study results were used to develop approaches to the selection of imitation GMs and their use during type approval tests and their subsequent verification.

Contribution of the Authors Kolobova A. V.—setting the task and developing the concept of research, developing a methodology/method, analyzing research results, implementing the methodology, preparing abstracts, checking and editing the text of the article; Konopelko L. A.—setting the task and developing the concept of research, analyzing the results of research work, developing methodological approaches for implementing the methodology for using equivalents in gas analytical measurements, checking and editing the text of the article; Sokolov T. B.—carrying out research work on the use of equivalents for gas analytical measuring instruments, processing and analyzing the results of research work, implementing the methodology, writing a draft version of the article, preparing/creating visual materials; Fatina O. V.—carrying out research work on the use of equivalents for gas analytical measuring instruments, processing and analyzing the results of research work, implementing the methodology, checking and editing the text of the article.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Prospects for the Development of Reference Materials of the Wobbe Index

Natalia A. Malginova, Elena N. Korchagina, and Yaroslav V. Kazartsev

Abstract The thermophysical properties of gaseous fuel are essential for its industrial applications. The trueness of gas volumetric calorific values is currently ensured using certified reference materials for the net volumetric calorific value of pure gases and gas mixtures traceable to GET 16-2018. However, when determining the Wobbe index, a need arises to measure the relative gas density to air with traceability to GET 18-2014. This study aims to improve the accuracy of the pycnometer method for measuring gas density to establish metrological support for Wobbe index measurements. This method was used to estimate the uncertainty budget of gas density measurements. The mass determination of analyzed gas was found to make the greatest contribution to the combined uncertainty. To minimize the uncertainty, the pycnometer internal volume was increased and determined using the modified procedure. A pycnometer (imitator) having an increased volume and original design was produced. The modified method was tested to improve Wobbe index measurement accuracy. The study results provide a wider possibility of establishing metrological characteristics in the metrological support of Wobbe index analyzers, as well as enabling the application of the procedure for controlling the measurement accuracy of relative gas density to air.

Keywords Wobbe index · Volumetric calorific value · Relative gas density · Density determination methods · Pycnometer method · Reference material · Gaseous fuel

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Introduction

The use of alternative gaseous fuels is becoming increasingly popular in most industries today [1]. In 2019, as a result of the 21st Conference of the Framework Convention on Climate Change, Russia joined the Paris Agreement, a UN-ratified international treaty [2] aimed at slowing global climate change. Since then, the Russian strategy for socioeconomic development [3] has focused on reducing greenhouse gas emissions into the atmosphere, specifically those that have a negative impact on the environment and climate. Various types of renewable gaseous fuels are also essential as they can be used to produce thermal energy; they replace fossil fuels, thus reducing greenhouse gas emissions into the atmosphere.

One of the main thermophysical parameters characterizing gaseous fuels is the Wobbe index. The Wobbe index is defined as the ratio of the volumetric calorific value of gas to the square root of its relative density to air [4]. This parameter characterizes the interchangeability of combustible gases in technological processes. The Wobbe index of a gas mixture changes when different gases are mixed.

The Wobbe index is key in evaluating the interchangeability of gaseous fuels in the thermal power industry [5]. Therefore, Wobbe index measurements are actively used in many processes that require control over the calorific value of gaseous fuels. In order to control and regulate the operating modes of burner devices, it is common nowadays to use Wobbe index analyzers measuring the calorific value of gaseous fuels via direct calorimetric and some indirect methods, together with gas density measurement methods (optical or other [6]).

The development and establishment of metrological support in the field of Wobbe index measurements is currently a relevant issue. One of its objectives is to develop Wobbe index reference materials (RMs) for the verification, calibration, and type approval testing of gas calorimeters and Wobbe index analyzers.

In 2020–2022 in order to establish and control the metrological characteristics of instruments for measuring the volumetric calorific value of gases, the D. I. Mendeleyev Institute for Metrology (VNIIM) developed and certified GSO 11662-2020/GSO 11665-2020 and GSO 11904-2022/GSO 11907-2022—type-approved reference material sets for volumetric net calorific value of pure gases: hydrogen, methane, ethane, and propane (in the course of the study described in [7]).[1] The certified volumetric net calorific values for the certified reference materials (CRMs) of pure gases and gas mixtures are presented in Table 1.

The certified RM values are traceable to the unit of volumetric net calorific value reproduced by the GET 16-2018 State Primary Standard of the Units of calorific

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[1] GSO 11662-2020 ÷ GSO 11665-2020 Reference materials type-approved for the volumetric net calorific value of gases (NOES-VNIIM set). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1384812. Accessed: 1 October 2022 (In Russ.).

GSO 11904-2022 ÷ GSO 11907-2022 Reference materials type-approved for the volumetric net calorific value of gases (NOES-GS-VNIIM set). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1398624 [Accessed: 1 October 2022]. (In Russ.).

Table 1 Certified characteristic, permissible certified value range, and permissible expanded uncertainty of CRMs for the volumetric net calorific value of gases

GSO number in the set	CRM index in the set	Permissible certified value range of the volumetric net calorific value, $H_{inf}^{25/20}$, MJ/m^3*	Permissible relative expanded uncertainty (U)** at k = 2, %
GSO 11662-2020	NOES-VNIIM -H_2	10.03–10.26	0.3
GSO 11663-2020	NOES-VNIIM -CH_4	33.35–33.58	0.3
GSO 11664-2020	NOES-VNIIM -C_2H_6	59.74–59.98	0.3
GSO 11665-2020	NOES-VNIIM -C_3H_8	86.23–86.47	0.3
GSO 11904-2022	NOES-GS-1-VNIIM	3.00–10.00	0.40
GSO 11905-2022	NOES- GS-1-VNIIM	10.00–30.00	0.30
GSO 11906-2022	NOES- GS-1-VNIIM	30.00–36.50	0.20
GSO 11907-2022	NOES- GS-1-VNIIM	36.50–70.00	0.30

Note

*the superscript (25/20) indicates standard combustion conditions, i.e., a temperature of 25 °C (298.15 K) and a pressure of 101.325 kPa, as well as standard conditions for gas volume conversion, i.e., a temperature of 20 °C (293.15 K) and a pressure of 101.325 kPa

**corresponds to the permissible relative error at a confidence probability of $P = 0.95$

value, mass calorific value, and volumetric calorific value[2] [8]. Noteworthy is that the characterization results are obtained via direct calorimetric measurements using reference gas calorimeters KATET, USVG, and USNG (VNIIM, Russia) which are part of GET 16-2018.

However, for reliable measurements of the Wobbe index, it is necessary to determine the relative density of gases to air with established traceability to GET 18-2014 State Primary Density Standard [9].

The need to measure the absolute density of gases arose earlier during COOMET 780/RU-a/19[3] comparisons of national reference gas calorimeters using gas mixture RMs under the auspices of the International Bureau of Weights and Measures (BIPM) [10]. The metrological organizations of Russia, Turkey, and France took part in these comparisons. In European countries, reference gas calorimeters were developed as part of the GERG project aimed at solving the issues of metrological support in the gas industry.[4] The calorimeters used by PTB (Germany) and LNE (France) implement the principle of measuring the mass calorific value of gas, while in Russia volumetric calorific value is determined. This fact necessitated solving the problem of converting

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[2] GET 16-2018 State Primary Standard of Units of calorific value, mass calorific value, and volumetric calorific value: D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397842. Accessed 1 October 2022 (In Russ.).

[3] COOMET No. 780/Ru/2019 Comparisons of national reference gas calorimeters on samples of gas mixtures.

[4] The European gas research group. Available via GERG. https://www.gerg.eu/. Accessed 1 October 2022 (In Russ.).

the volumetric calorific value to a mass basis in order to compare results obtained in gas calorimetry. This problem can be solved by determining the analyzed gas density.

Therefore, this study aims to develop a modified pycnometer procedure for determining gas density.

To this end, the following study objectives were formulated: analysis of methods for determining gas density; selection of the optimal method for measuring gas density to determine the Wobbe index; development of a gas density measurement procedure; estimation of the uncertainty budget for density measurements within the developed procedure; testing of the developed procedure.

Materials and Methods

Selection of a Method for Determining Gas Density

The following measurement methods are currently used to determine gas density: calculation method, aerostatic method, vibration method, ultrasonic, and pycnometer method, among which calculation and pycnometer methods are the most widely used [10].

The calculation method is based on determining the physicochemical parameters of the gas mixture according to the known component composition and is described in ISO 6976 [11]. This method is designed to determine gross calorific value, net calorific value, density, relative density, and the Wobbe index for natural gas, natural gas imitators, and other gaseous fuels for which the component composition is known. In Russia, this method described in GOST 31369-2008 [12] is combined with the determination of component composition.

In the aerostatic method, the examined gas and air pass through vertical tubes under constant pressure. Gas and air columns of equal height are created in the internal cavities of the tubes. The pressure difference in the columns is determined using a differential manometer [13].

The vibration method for determining density relies on the dependence between the parameters of elastic vibrations transmitted to the gas-filled vessel and the analyzed gas density. In most cases, the natural frequency of the resonator, made in the form of a string, rod, tube, plate, or tuning fork, serves as a parameter of elastic vibrations [14].

Ultrasonic density meters are based on the use of ultrasound to determine the density of gaseous media. Since it is possible to perform measurements without direct contact of the sensing elements with the substance under study, this method can be used to study aggressive media [15, 16].

The pycnometer method for measuring gas density is based on the successive weighing of a glass pycnometer of known internal volume that is filled with dry gas, dry air, or nitrogen. This method is described in detail in GOST 17310-2002 and GOST 34721-2021 [17, 18]. In accordance with GOST 34721-2021, the analyzed gas density is determined by first filling the internal volume of the pycnometer with pure nitrogen and then by the analyzed gas. In the pycnometer method, the confidence limits of the absolute error amount to 0.004 kg/m^3 at a confidence probability of $P = 0.95$.

Given that the aerostatic, vibration, and ultrasonic methods are used primarily in the flow mode at high gas flow rates, and the calculation method is applicable only in the case when the component composition of the examined gas mixture is known, the analysis of specified methods led to the choice of the pycnometer method for determining gas density.

Practical Justification and Testing of the Pycnometer Method for Determining Gas Density

An uncertainty budget estimation for the pycnometer method shows that the largest contribution to the uncertainty of density measurements is made by the analyzed gas mass.

Thus, to test the pycnometer procedure under development, it was decided:

- to make a chamber (a pycnometer imitator) of increased volume having the original design (the internal volume of the chamber is increased from 200 cm^3 to 1 dm^3 in order to increase the mass of test gas inside the chamber);
- to evacuate the chamber instead of conducting a dry air blast;
- to use a liquid-comparator to determine the internal volume of the chamber.

The modified algorithm for determining the gas density is as follows:

- determine the internal volume of the chamber;
- evacuate the chamber;
- determine the mass of the evacuated chamber;
- fill the chamber with the analyzed gas;
- determine the mass of the chamber filled with the analyzed gas.

In order to test the modified pycnometer procedure, a spherical pycnometer chamber (imitator) having an internal volume of about 1 dm^3 and equipped with two shut-off valves was produced (Figs. 1 and 2). The chamber walls are made of aluminum. The design of shut-off valves allows the chamber to be filled with both gases and liquids.

The internal volume of the chamber was determined by filling it with a liquid-comparator (ethyl alcohol as per GOST R 55878-2013 [19]). The ethyl alcohol density was determined using a DMA 4200 M density meter (limit of the absolute error in measuring density—0.0002 g/cm^3) with traceability to GET 18-2014.[5] The

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[5] GET 18-2014 State Primary Density Standard: D. I. Mendeleyev Institute for Metrology. Available via FIF EUM https://fgis.gost.ru/fundmetrology/registry/12/items/397864. Accessed 1 October 2022 (In Russ.).

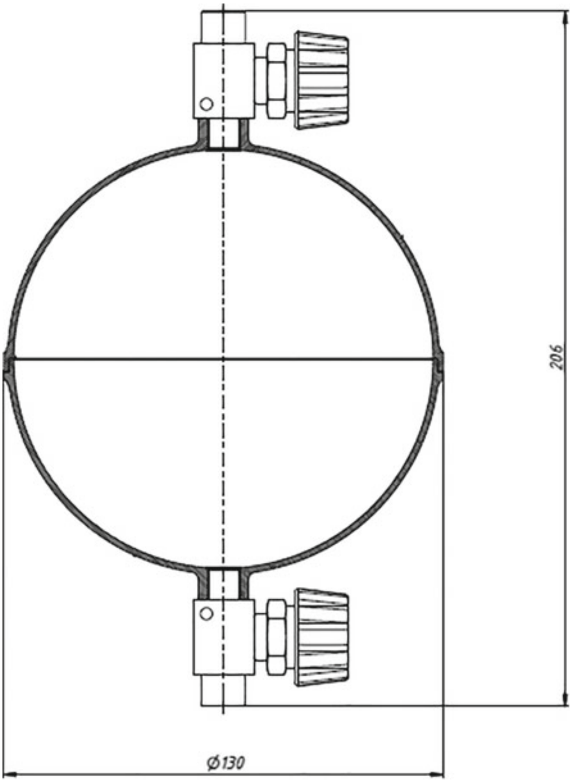

Fig. 1 A schematic of the pycnometer chamber (imitator)

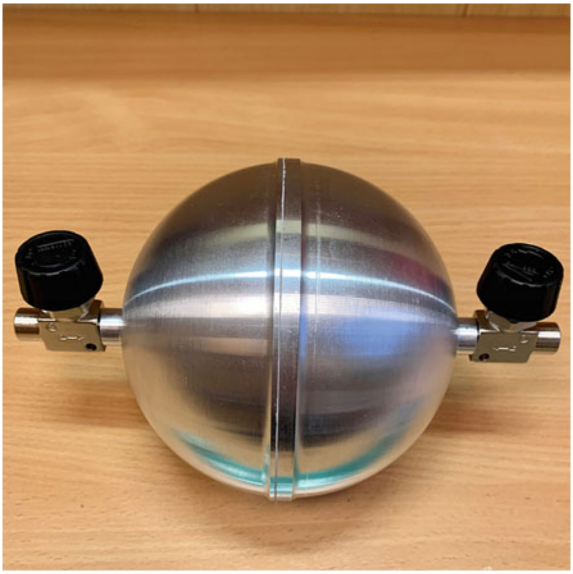

Fig. 2 A photograph of the manufactured pycnometer chamber (imitator)

filled chamber was kept in a circulation bath at (20.00 ± 0.02) °C for at least four hours.

The mass of the filled chamber was determined using Class I scales according to GOST OIML R 76-1-2011 [20]. The conventional true internal volume of the pycnometer chamber (imitator) was determined as follows:

$$V = \frac{m - m_{evac}}{\rho_{liq}}, \text{cm}^3, \tag{1}$$

where

m is the mass of the pycnometer chamber (imitator) filled with the liquid-comparator, g;
m_{evac} is the mass of the evacuated pycnometer chamber (imitator), g;
ρ_{liq} is the certified density of the liquid-comparator at the temperature t, g/cm^3.

The internal volume of the chamber was determined to be 963.9 ± 1.2 cm^3.

The density of the test gas was established by successively determining the mass of the evacuated and gas-filled pycnometer chamber (imitator). The environmental parameters were recorded (temperature, atmospheric pressure, and relative air humidity).

The density of gas analyzed at a temperature of 20 °C and a pressure of 101.325 kPa was obtained as follows:

$$\rho = \frac{M_1 - M_0}{K \cdot V}, \text{g/cm}^3, \tag{2}$$

where

M_1 is the result of measuring the mass of the pycnometer chamber (imitator) filled with the analyzed gas, g;
M_0 is the result of measuring the mass of the evacuated chamber, g;
V is the conventional true internal volume of the pycnometer chamber (imitator), cm^3;
K is the coefficient for converting the analyzed gas density under the experimental conditions (temperature t; atmospheric pressure P) to standard conditions (293.15 K; 101.325 kPa):

$$K = \frac{293.15 \cdot P}{(273.15 + t) \cdot 101.325}, \tag{3}$$

The relative density (to air) of the analyzed gas d was determined according to the formula:

$$d = \frac{\rho}{\rho_{air}}, \tag{4}$$

Table 2 Metrological characteristics of reference gas calorimeters KATET, USVG, and USNG (D. I. Mendeleyev Institute for Metrology, Russia)

Characteristic	KATET	USVG	USNG
Measurement range, MJ/m^3	10–66	25–90	3–35
Relative expanded uncertainty of unit transfer, $P = 0.95$	$16 \cdot 10^{-4}$	$30 \cdot 10^{-4}$	$(30 - 40) \cdot 10^{-4}$

where

ρ is the gas density, g/cm^3 at $t = 20$ °C (293.15 K) and $p = 101.325$ kPa;

ρ_{air} is the density of dry air having a standard composition at a temperature of $t = 20$ °C and a pressure of $p = 101.325$ kPa, g/cm^3 as per GSSSD 8-79 [21], amounting to 1.2044 kg/m^3.

The Wobbe index of the examined gas (W in MJ/m^3) was determined as follows:

$$W_{gross/net} = \frac{H_{gross/net}}{\sqrt{d}}, \tag{5}$$

where $H_{gross/net}$ is the gross or net volumetric calorific value of the analyzed gas, MJ/m^3.

According to Eq. 5, the Wobbe index can be determined by measuring the gross and net volumetric calorific value of the examined gas. To measure the volumetric calorific value, VNIIM can use (depending on the examined gas) the reference gas calorimeter KATET or gas calorimeter-comparators USVG or USNG realizing the relative expanded uncertainty in the unit transfer of volumetric calorific value which does not exceed 0.4%.

Table 2 provides the metrological characteristics of reference calorimeters.

Results and Discussion

In order to test the modified pycnometer procedure, we selected a gas mixture that was used in the COOMET 780/RU-a/19 gas calorimetry comparisons. The mixture was prepared gravimetrically at VNIIM. The certified mole fraction of the component to be determined is as follows: CO_2—40.22%; CH_4—rest. The relative expanded uncertainty of certification amounts to 0.20% at P = 0.95.

Table 3 provides gas mixture densities determined as per GOST 31369-2021 (ISO 6976:2016) and according to the modified pycnometer procedure under standard conditions.

The difference between results obtained using the modified pycnometer procedure ρ_{pyc} and calculated as per GOST 31369-2021 ρ_{calc} was determined as follows:

$$\Delta\rho = \frac{|\rho_{pyc} - \rho_{calc}|}{\rho_{calc}} \cdot 100\% = 0.3\%. \tag{6}$$

Table 3 Results of determining the gaseous mixture density (CO_2—40.22%, CH_4—rest)

Results of determining the gaseous mixture density (CO_2—40.22%, CH_4—rest) as per GOST 31369-2021 (ISO 6976:2016)	
Gaseous mixture density, kg/m^3	1.13797
Expanded uncertainty, $k = 2$, kg/m^3	0.00056
Results of determining the gaseous mixture density (CO_2—40.22%, CH_4—rest) according to the modified pycnometer procedure	
Gaseous mixture density, kg/m^3	1.1415
Expanded uncertainty, $k = 2$, kg/m^3	0.003

Table 4 presents the estimated uncertainty budget of gas mixture density measurements performed using the modified pycnometer procedure.

Table 4 Uncertainty budget of gas mixture density measurements (CO_2—40.22%, CH_4—rest)

Uncertainty budget of gas mixture density measurements (CO_2—40.22%; CH_4—rest) performed using the modified pycnometer procedure				
Source of uncertainty	Estimation of the relative uncertainty component, type	Probability distribution	Influence coefficient	Contribution to the relative combined standard uncertainty, %
Standard deviation of the arithmetic mean of measurement results (n = 5)	$7.4 \cdot 10^{-4}$ Type A	Normal	1	No more than $7.4 \cdot 10^{-2}$
Gas mass measurement	$4.0 \cdot 10^{-4}$ Type B	Uniform	1	$4.0 \cdot 10^{-2}$
Measurement of the internal chamber volume	$7.0 \cdot 10^{-4}$ Type B	Uniform	− 1	$7.0 \cdot 10^{-2}$
Coefficient for converting gas density to standard conditions	$1.0 \cdot 10^{-3}$ Type B	Uniform	− 1	$1.0 \cdot 10^{-1}$
Relative combined standard uncertainty, %				No more than 0.15
Relative expanded uncertainty (k = 2; P = 0.95), %				No more than 0.30

Conclusion

The study was aimed at developing a modified pycnometer procedure for determining gas density. The need for a new procedure primarily arises from the demand for developing Wobbe index RMs due to the increasing requirements of modern industry. The procedure can be used to convert the volumetric calorific value of gas to a mass basis during gas calorimetry comparisons.

The main changes introduced into the pycnometer procedure for determining gas density consist in increasing the internal volume of the pycnometer chamber (imitator) and implementing a different method of filling the chamber. In the course of the study, we developed a measurement algorithm and estimated the uncertainty budget.

For testing the pycnometer procedure under development, the following gas mixture was chosen: CO_2—40.22%; CH_4—rest. As a result of gas density measurements via the considered method and the calculation method as per GOST 31369-2021, a discrepancy not exceeding 0.3% was detected, which corresponds to the expanded measurement uncertainty as per the method under development. Of note is that a larger internal volume of the gas-filled chamber led to a decrease in the uncertainty of density measurements, as compared to the conventional pycnometer method, to the level of 0.003 kg/m^3.

Thus, the study led to an informed decision to use the modified pycnometer method for determining gas density.

The theoretical significance of obtained results consists in testing the modified pycnometer procedure to improve Wobbe index measurement accuracy for gaseous fuel.

The obtained study results provide a wider possibility of establishing the metrological characteristics of gas calorimeters and Wobbe index analyzers, including during type approval tests, as well as enabling the application of the procedure for controlling the measurement accuracy of relative gas density to air for other measurement methods.

The present study can be further developed in implementing the hydrostatic weighing of a gas-filled vessel to enhance the accuracy of gas density measurements. In order to implement the hydrostatic weighing method, VNIIM developed design documentation for a new spherical closed chamber having an internal volume of 5 dm^3 and produced it.

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Reference Materials for Physical Properties

Melting Point Certified Reference Materials for Organic Substances: Development Prospects

Yaroslav V. Kazartsev, Elena N. Korchagina, and Igor V. Solovev

Abstract Measurements of the melting point of high-purity organic substances are crucial in various fields, such as medicine, biology, and perfumery and cosmetic production. This research was aimed at developing melting point certified reference materials (CRMs) for organic substances, with metrological traceability to the SI base units for "temperature" (°C). The study analyzes the metrological assurance in the field of melting point measurements, including a reference complex used to measure the melting point and purity of organic substances in the range of + 40 to + 250 °C. The research also identifies the basic requirements for substances eligible as CRMs. The study presents the results of determining the melting point of benzophenone, benzoic acid, succinic acid, anthracene, and caffeine, along with interlaboratory comparisons. To harmonize the results, a method is suggested, which involves presenting the certified value of the melting point determined by direct measurements and certified values of the optical transparency temperature at various heating rates in the certificates of the developed CRMs. These results have theoretical significance in improving the accuracy of measurements in the field of thermal analysis to a higher quality level. The study also outlines research directions for future work.

Keywords Melting point · Melting point analyzers · Melting point certified reference materials · Thermal analysis · Heating rate · Temperature standard

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Abbreviations

ILC	Interlaboratory comparison
MI	Measuring instrument
CRM	Certified reference material
FIF EUM	Federal Information Fund for Ensuring the Uniformity of Measurements in Russia

Introduction

The melting point comprises one of the key physical and chemical properties of organic substances, since it determines other properties, including purity level, saturated vapor pressure, and solubility [1–4]. At present, the field of measuring the melting point of high-purity organic substances includes a large number of methods for thermal analysis of various types [3, 5, 6], used in medicine, biology, and perfumery and cosmetics production [7].

Leading international companies, such as Mettler Toledo, Buchi Labortechnic, Bibby Scientific, and others, are known for their high-quality measuring instruments (MI), including analyzers for the melting point of organic substances, which are characterized by high accuracy, efficiency, and automated measurement process. Over 10 types of such analyzers included in the Federal Information Fund for Ensuring the Uniformity of Measurements (FIF OEI)[1] undergo annual periodic verification. These analyzers are widely used in major medical and pharmaceutical companies, various scientific centers, and chemical laboratories.

In most melting point analyzers, an indirect method of measurement is used, which involves detecting the point at which the examined substance becomes optically transparent, while melting inside a glass capillary at various heating rates. The state of the samples is typically monitored using a digital camera. The point, at which optical transparency is observed, can be recorded manually or automatically by measuring the intensity of light passing through a sample. Since it is necessary to monitor the optical transparency of the melt during the verification and calibration of such MI, the temperature measures based on phase transitions of metals, whose metrological characteristics can be determined with high accuracy, are difficult to apply [8].

At present, the metrological supervision of this measurement area includes a number of CRMs for the melting point of pure organic substances of foreign origins, such as benzophenone, benzoic acid, and caffeine, as well as some CRMs for other physicochemical properties [9]. The CRMs, whose characteristics are listed in Table 1, are intended for use in the Russian Federation for the verification and calibration of analyzers.

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology. Accessed 7 October 2022.

Table 1 General information on melting point CRMs approved in the Russian Federation (based on information from FIF EUM)

GSO number	Substance	Producer	Certified value, °C	Expanded uncertainty, °C
GSO 7895-2001	Caffein	Sigma-Aldrich Chemie GmbH, Switzerland	235.0–237.0	± 0.3
GSO 11070-2018	Benzophenone	Mettler-Toledo Vostok JSC, Russia	47.6–48.6	± 0.2
GSO 11071-2018	Benzoic acid	Mettler-Toledo Vostok JSC, Russia	122.1–124.8	± 0.2

The certified value of the melting point of these CRMs indicates the temperature, at which the optical transparency of the melted substance occurs, while the certified range of values reflects the dependence of this temperature on the heating rate of a sample.

MI manufacturers or suppliers usually provide a set of imported CRMs for instrument calibration. The metrological characteristics of such CRMs established through traceability to foreign primary standards [10] can be determined using standard thermometry methods [11] or by measuring the heat of phase transitions with standard adiabatic calorimeters [12, 13].

Melting point analyzers are typically used in the temperature range from room temperature up to +350–400 °C, although the most practical range for industrial applications is from room temperature to +250 °C, with the measurement uncertainty of the instrument typically standardized by FIF EUM at ±0.3–(±0.5) °C. Therefore, the CRMs produced domestically and internationally, with an expanded uncertainty of the certified value in the range of 0.2–0.3 °C, fail to fully meet the demands of modern industry.

In this regard, this study focuses on identifying the characteristics and substantiating approaches to the development of certified reference materials for the melting point of organic substances, having metrological traceability to the basic SI units of the "temperature" (°C). The research objectives include the following: to justify the selection of substances for CRM; to establish the procedure for CRM certification; to assess the limitations that affect the certification process; to evaluate the uncertainty of certified melting point values in order to improve the accuracy of measurements in the field of thermal analysis.

Materials and Methods

The selection of substances for subsequent approval as melting point CRMs is a critical stage of research, since the properties of the examined substances significantly affect the results of the melting point measurements.

The following basic requirements were formulated for the organic substances selected as candidates for CRM:

- the melting point of the substance should be within the range from room temperature to +250 °C, taking into account the characteristics of already approved CRMs and the requirements of the approved methods of MI verification;
- the substance must be chemically stable during the entire melting process and melt without decomposition or danger of ignition;
- the substance must be non-toxic and non-hazardous to human health and analytical equipment in all aggregate states;
- the substance should be readily available for purchase, and samples of the initial substance of high purity (at least 99.0 mol.%) should be mass-produced by chemical industry enterprises;
- since the open capillary method is used in most ME, the substance should have a low evaporation capacity, which can be estimated from the saturated vapor pressure data.

Therefore, the following pure organic substances were selected for research: benzophenone, benzoic acid, succinic acid, anthracene, and caffeine. 5.0 ± 0.1 g of each candidate for CRM, comprising a finely dispersed polycrystalline substance with a molar fraction of the main component of at least 99.0%, was packed in screw-capped glass vials from the original package. The stability and homogeneity of the materials were examined in accordance with the requirements of GOST R 50779.60-2017 [14].

In order to compare the results obtained during the study, the melting point of benzophenone, benzoic acid, and caffeine (GSO 11070-2018, GSO 11071-2018, GSO 7895-2001)[2] manufactured by Fluka Chemie AG (Switzerland) and intended for use in optical analyzers were used.

It is recommended to define the melting point in order to avoid any ambiguity in its interpretation. Therefore, the melting point is defined as the temperature, at which thermodynamic equilibrium is achieved between the solid and liquid phases of the studied substance, with the liquid phase having the same initial composition as the substance and the number of solid phase crystals tending towards zero [1, 2].

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[2] GSO 7895-2001 Caffeine melting point reference material. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/392091. Accessed 7 October 2022.

GSO 11070-2018 Reference material for the melting point of benzophenone. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/593689. Accessed 7 October 2022.

GSO 11071-2018 Standard sample of the melting point of benzoic acid. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/394949. Accessed 7 October 2022.

With the introduction of the new state verification schedule (SVS) for temperature MI (instead of GOST 8.558–2009 [15]), new requirements for metrological characteristics of temperature measures based on pure organic substances (working standards of the 3rd grade), as well as the reference equipment for temperature units to transfer these measures (working standards of the 2nd grade), were introduced.

In order to comply with the requirements for the transfer of the temperature unit, a CryoTherm prototype standard complex for measuring the melting temperature and purity of organic substances was developed and investigated at D. I. Mendeleyev Institute for Metrology (VNIIIM).

The standard complex complies with the new GSO for the 2nd-grade operating temperature standard, with a range of confidence error of less than ±0.1 °C for temperature measurements at a confidence probability of 0.95 and the temperature range from +40 to +250 °C.

The standard complex described in [16, 17] allows the melting point of organic substances to be measured with traceability to the State Primary Standard of Temperature GET 34-2020.[3] The measurements are carried out by recording the temperature variations of the substance during the phase transition, in accordance with the accepted definition of the melting point. Temperature sensors in the standard complex comprise platinum resistance thermometers with nominal values of 50 and 100 ohms, connected to the measurement system based on the Keithley 2002 precision digital multimeter (Keithley Instruments, Inc., USA).

The functional diagram of the reference complex is shown in Fig. 1 [17].

The reference complex comprises two units:

1. A unit for measuring the melting point of organic substances in the temperature range of +40 to +250 °C is based on the conventional thermometry method of direct measuring of the phase transition temperature. The analyzed substance is placed in a molybdenum glass ampoule, which is located in a temperature calibrator ensuring the heating mode. The temperature sensor is in direct contact with the studied substance. The melting point is determined by analyzing the graph of the phase transition in the region where the sign of the first derivative changes (the inflection point in the transition to the heating of the liquid phase).
2. The cryometric analysis unit is designed for measuring the melting point and total molar fraction of impurities in organic substances in the range of +40 to +200 °C for substances that have a molar fraction of the main component ranging from 99.000 to 99.995%. Temperature measurements are carried out in the melting cell using a constant heat flux, ensuring that the thermodynamic equilibrium of the substance be reliably determined. The melting point is estimated by analyzing the temperature dependence graph on the reverse fraction of the liquid phase, in the area corresponding to the state of two-phase equilibrium. The inventor's certificate [18] describes the design of the melting cell developed at VNIIM, while the processing of measurement results is discussed in the works [16, 17].

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[3] GET 34-2020 State primary standard of temperature unit in the range from 0 to 3200 °C: D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1385580. Accessed 7 October 2022.

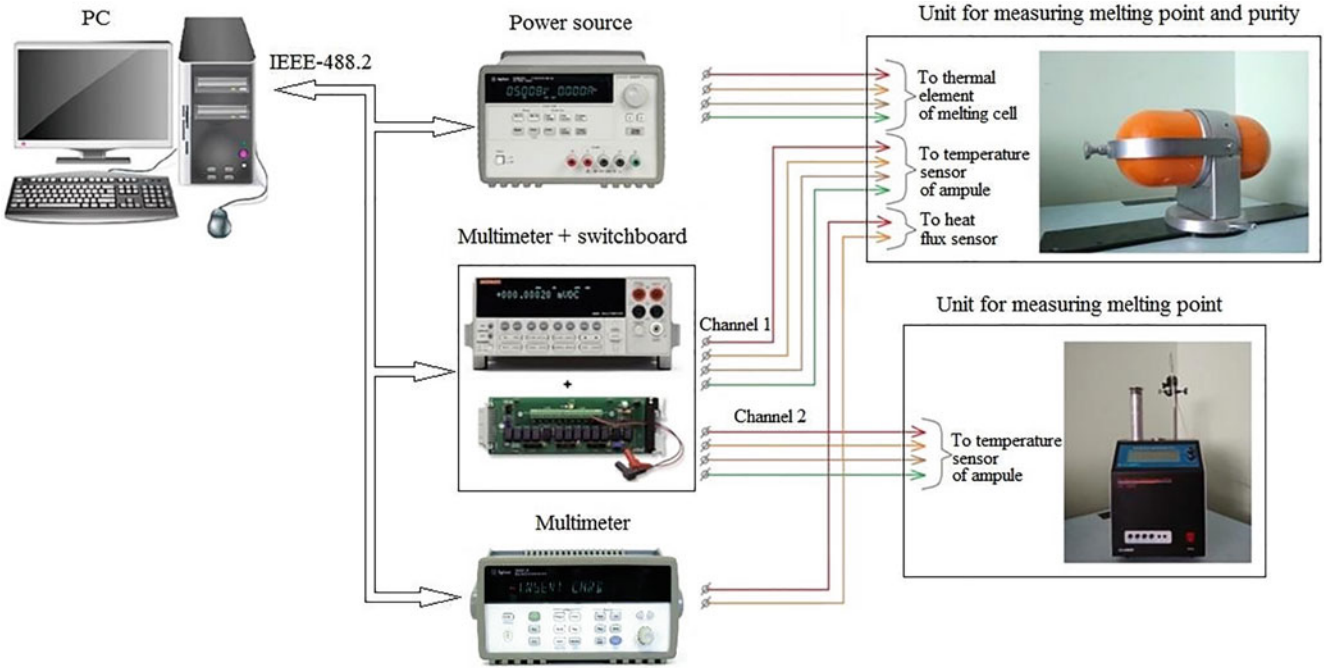


Fig. 1 Functional diagram of the CryoTherm reference complex designed to meet metrological characteristics of 2nd-grade operating temperature standard

In order to ensure the thermodynamic mode of experiments using the reference complex, a low heating rate of the substance (0.1–0.2 °C/min) is set, along with a prolonged melting process (a minimum of 15 min for 2 g of sample in the region where the two phases coexist). This process is primarily responsible for establishing conditions that lead to thermodynamic equilibrium.

Results and Discussion

When processing the measurement results obtained using the unit based on the temperature calibrator, the melting point on the T (temperature) against τ (time) diagram was determined at the inflection point where a sharp increase in temperature occurred, indicating the heating of the substance that transitioned to the liquid phase. The authors believe that this point characterizes the completion of the destruction of the crystalline lattice in the substance, thus allowing the melting point to be determined according to the previously adopted definition. Summary data of the experimental results are presented in Table 2.

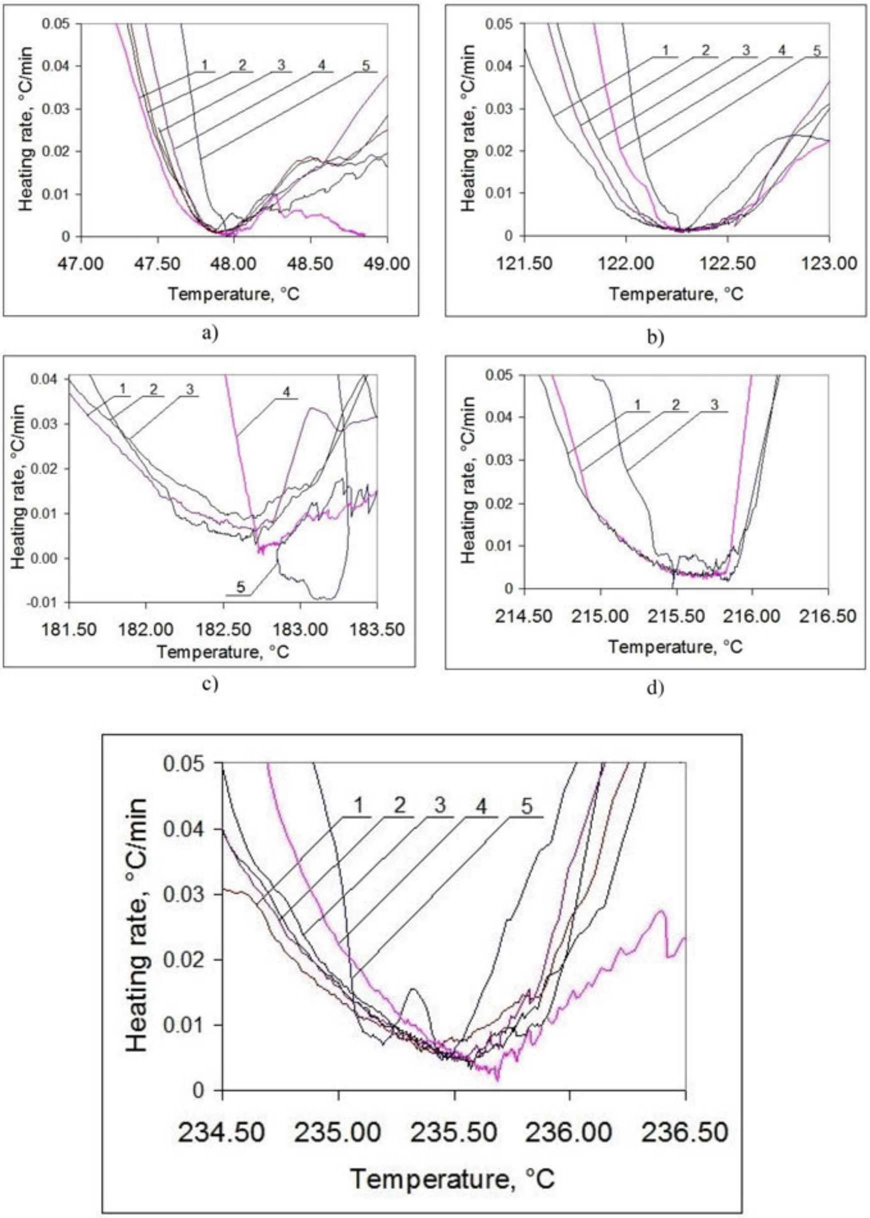
The melting temperature can also be determined by locating the inflection point on the X-axis from the plot of the heating rate U as a function of temperature T. Moreover, the U–T plot provides insights into the melting process dynamics, along with the thermal stability of a substance under temperature repeatability at the inflection point during multiple "melting-crystallization" cycles (Fig. 2).

Here, for succinic acid, much lower reproducibility of the melting point was observed, as well as a different heating rate profile (Fig. 2c), compared to the other studied substances. This finding suggests that the substance may be thermally unstable and unsuitable for use as a CRM.

It should be noted that the melting point obtained by classical thermometry (Table 2) often fails to comply with the certified values of the melting point of the approved CRMs (Table 1).

Table 2 Melting point of studied substances measured using the CryoTherm reference complex

Experiment	Melting point, °C				
	Benzophenone	Benzoic acid	Succinic acid	Anthracene	Caffein
1	47.95	122.27	182.86	216.02	235.45
2	48.00	122.30	182.82	215.88	235.69
3	47.89	122.27	182.81	215.84	235.59
4	47.90	122.28	182.80	–	235.58
5	47.91	122.28	–	–	235.56
6	47.91	–	–	–	235.42
Average value	47.93	122.28	182.82	215.91	235.55



a)

b)

c)

d)

e)

Fig. 2 Temperature variations during melting of benzophenone (**a**), benzoic acid (**b**), succinic acid (**c**), anthracene (**d**), and caffeine (**e**) at a heating rate of the solid phase within 0.2 °C/min obtained under repeatability conditions (footnotes "1–5" denote diagrams of successive melting cycles of a substance)

This was confirmed by a comparative analysis of results obtained for substances from the same batches using different methods: classical thermometry and optical transparency. The research focused on CRM samples of benzophenone, benzoic acid, and caffeine, with melting point specifications as per GSO 11070-2018, GSO 11071-2018, and GSO 7895-2001. As presented in Table 3, a difference was observed between the temperature corresponding to the time of complete destruction of the crystal lattice in the substance and the temperature corresponding to the time of occurrence of optical transparency of the melt (the certified value) for benzoic acid and caffeine samples.

In order to ensure a reliable comparison between the results obtained using the reference complex and those obtained using instruments based on the optical principle of registration, it is essential to establish accurate data on the occurrence of optical transparency in the melt.

In light of the inconsistent measurement results obtained by different methods, an Interlaboratory comparison (ILC) was organized in order to increase the reliability of the data intended for inclusion in the certificates of the CRMs under approval. The ILC was carried out in compliance with the guidance of GOST 17043-2013 [19] using samples of benzophenone, benzoic acid, succinic acid, and anthracene, tested for stability and homogeneity in accordance with the requirements of GOST R 50779.60-2017. During the ILC, seven chemical, pharmaceutical, and medical laboratories in the Russian Federation, used melting temperature analyzers based on the optical principle of registration, produced by different manufacturers. Measurements were carried out at a sample heating rate in a capillary of 0.2 and 1 °C/min in accordance with the requirements of GOST 18995.4-73, GOST 21553-76, and OFS.1.2.1.0011.15 [20–22].

The ILC requires that three melting point determinations be made for each substance at the specified heating rates. Tests were carried out under conditions of method repeatability to establish the degree of consistency of independent measurement results. The statistical processing of the ILC results was carried out in accordance with the requirements of GOST R 50779.60-2017, GOST R ISO 5725-5-2002, GOST R ISO 5725-6-2002, and RMG 103-2010 [15, 23–25].

Table 3 Comparative analysis of melting point values of organic substances obtained by various methods

Benzophenone, GSO 11070-2018 (Lot BCBZ6573)		Benzoic acid, GSO 11071-2018 (Lot BCBZ7431)		Caffeine, GSO 7895v2001 (Lot BCBB1813)	
CryoTherm[a]	CRM certificate[b]	CryoTherm[a]	CRM certificate[b]	CryoTherm[a]	CRM certificate[b]
47.9	47.9	122.3	122.7	235.6	236.3

[a] Thermometric method analysis: The temperature corresponding to the inflection point in the phase transition diagram is recorded, with a heating rate of less than 0.2 °C/min

[b] Optical method analysis: The temperature is recorded, which corresponds to the occurrence of optical transparency when heated in a capillary in thermodynamic mode, with a heating rate of 0.2 °C/min

Table 4 Assigned values of melting point of substances and their expanded uncertainty

Studied substance	Heating rate, °C/min	Assigned value of melting point of substances and its expanded uncertainty, °C
Benzophenone	0.2	48.76 ± 0.18
	1	49.60 ± 0.53
Benzoic acid	0.2	123.07 ± 0.06
	1	124.37 ± 0.11
Succinic acid	0.2	184.57 ± 0.06
	1	187.21 ± 0.22
Anthracene	0.2	216.99 ± 0.30
	1	217.84 ± 0.04

The melting point values of the studied substances, corresponding to the occurrence of their optical transparency, obtained as a result of the ILC, are listed in Table 4.

As observed, the obtained results also exhibit significant discrepancies from the results obtained by the classical thermometry method (Table 2). Furthermore, the data obtained is dependent on the sample heating rate, indicating the method-dependence of the measurement results.

Conclusion

The results of the research validate the type of CRMs for melting point based on pure organic substances. Developing such CRMs is a challenging scientific and technical task, since it requires the initial substance, available in sufficient quantities to produce the required CRMs, to have stable characteristics and a high degree of purity.

The technical challenges in developing CRMs for melting points are primarily due to limitations in the equipment used for registration and control of the phase transition. The method used to obtain the results is a major factor that affects the results. The authors suggest that it is essential to differentiate between melting temperature as a characteristic of phase transition and the temperature associated with a specific physical effect, such as transparency. The findings assume that the moment of transparency in the melt may not necessarily correspond to the moment of complete destruction of the crystal lattice.

The non-uniformity of the temperature field in the heating unit of the analyzer is an additional source of error when measuring the temperature of optical transparency, particularly when the heating element, temperature sensor, and capillary containing the substance are distant from each other. This temperature imbalance of the heated elements becomes particularly noticeable at higher heating rates.

In order to address the issue of method-dependence of the certified melting point value, it is recommended to include the certified melting point value determined by the classical thermometry method under conditions of approaching thermodynamic equilibrium, as the primary characteristic of the substance in the certificates of the developed CRMs. The temperature of optical transparency at different heating rates (0.2 and 1 °C/min) should be included as an additional characteristic of the substance.

Here, the concept of measurement uniformity adopted in the Russian Federation is adhered to, which ensures that the certified CRM value is traceable to the primary standard of the temperature unit. Including additional characteristics specific to the phase transition of a particular substance in the certificates of developed CRMs follows the requirements for CRM development.

In order to further enhance the capabilities of the CryoTherm reference complex, it is recommended to integrate a melting point analyzer based on the optical principle of registration. The added functionality will offer the expansion of the range of substances investigated for potential use as CRMs. Following the example of foreign calibration standards, compounds such as vanillin, phenacetin, and saccharin in a wide melting point range may be considered potential CRMs. Broadening the range of CRMs will allow the analyzers to be calibrated for narrower measurement ranges specific to certain tasks, thereby increasing the level of metrological support in this field.

The obtained results have both theoretical and practical significance. Theoretical significance is attributed to the development of theoretical and methodological approaches for the certification of CRMs for melting point based on pure organic substances. This improvement can lead to increased accuracy in thermal analysis measurements. The practical significance of the results is related to the expansion of possibilities for establishing and controlling the accuracy and stability of calibration characteristics of melting point analyzers. It can also contribute to the certification of measurement techniques (methods) and accuracy control of measurement results for the temperature of phase transitions of organic substances.

The development of multi-parameter CRMs based on pure organic substances, including certified values of melting point and total molar fraction of impurities determined by cryometric analysis, is a promising area of research in this field. The authors suggest that such CRMs have the potential to find wide-ranging applications in scientific research, as well as in various industries.

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Contribution of the Authors Kazartsev Ya. V.—development of measurement methodology, programming, conducting research, carrying out a formal analysis of the data obtained, writing a draft of the article, preparation and creation of visual materials; Korchagina E. N.—development of the idea (concept) of the study, management of research and development work, obtaining funding, revision of the article; Solovev I. V.—conducting research, carrying out a formal analysis of the data obtained, revision of the article.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Application of the Traceability Concept in Determining the Mechanical Properties of Metals Under Static Tension Using a GSO 11854-2021 Reference Material

Ilona N. Matveeva, Vladimir V. Tolmachev, and Anastasia A. Zabelina

Abstract This paper deals with the specific features of applying approved reference materials as a tool for ensuring traceability and controlling the accuracy of mechanical property measurements. The authors analyzed the theoretical approach based on ISO/IEC Guide 98-3:2008 and calculation algorithms provided by ISO 21748:2017 for evaluating measurement uncertainty. The methodology of using a GSO 11854-2021 certified reference material of St20 steel mechanical properties for evaluating the uncertainty of static tensile test measurements is considered. In order to ensure the traceability of measurement results, two options for accounting the laboratory systematic component are proposed: as a correction or a contribution to the standard combined uncertainty. According to the conducted study, the modeling approach of theoretical concepts based on ISO/IEC Guide 98-3:2008 and calculation algorithms provided by ISO 21748:2017 (Eq. 1) can be applied by accredited laboratories when assessing uncertainty in accordance with clause 7.6 of GOST ISO/IEC 17025-2019.

Keywords Mechanical properties · Static tension · Metrological traceability · Primary reference measurement technique · Reference material

Introduction

Determination of the mechanical characteristics of metals under static tension is a key method for identifying their strength and ductility indicators. Static tensile tests represent indirect measurements, where strength (tensile strength, proof strength) and ductility (percentage elongation after fracture, percentage total extension at fracture) are determined using corresponding measurement equations under specified test conditions. In this regard, although the results of determining mechanical properties cannot be correlated with a comparison base in the form of a physical quantity

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reference, either a primary reference measurement procedure or a reference material (RM) can be applied. However, the use of RMs or primary reference measurement procedures as a comparison base is associated with a number of specific features, which are not immanent to quantitative chemical analysis. These features arise due to the heterogeneity of either RMs or the material used for comparing the results of a laboratory procedure according to GOST 1497-84 [1] with those obtained by a primary reference measurement procedure.

Bahng and Roebben [2–4] classify mechanical properties as characteristics that depend on test procedures. A confusion in establishing the traceability of mechanical properties is caused by expressing the results of testing the mechanical properties of metals in SI basic units. Thus, strength in static tensile tests is measured in MPa, which are the units of pressure (force, divided by the area). However, the assumption that the properties of metals in static tensile tests can be traced to force 01basic units, seems to be incorrect, since these properties are measured prior to sample destruction. In order to obtain a response from a sample, it is necessary to use an external action in the form of tension, e.g., under a certain deformation rate. This means that any variations in the method or procedure of external action will affect the measurement results. Therefore, an RM is needed to ensure the traceability of properties and to control the accuracy of applying a particular test method.

Adamczak et al. [5–7] discussed various approaches to calculating the uncertainty of strength and ductility characteristics. An essential drawback of these studies involves uncertainty budgeting only on the basis of data on the traceability to the units of force and length, i.e., without using an RM. Tolmachev and Matveeva [8] considered an approach to ensuring the metrological traceability of the results of measuring mechanical properties during static tensile tests to a primary reference measurement technique using RMs. When evaluating the uncertainty of static tensile test results, a contribution of traceability, assessed using RMs, requires accounting for the laboratory systematic component either as a correction or as a contribution to the standard combined uncertainty.

In this paper, we aim to study and verify a methodology of using RMs to establish traceability in determining the mechanical properties of metals under static tension. To this end, the following research objectives were formulated: to analyze the theoretical principles specified in ISO/IEC Guide 98-3:2008 [9] and calculation algorithms proposed by ISO 21748:2017 [10] for evaluating measurement uncertainty; to evaluate the uncertainty of static tensile test results using the example of a GSO 11854-2021[1] reference material of the mechanical properties of St20 steel; to develop an approach based on uncertainty budgeting taking into account the combined effect from all sources of uncertainty, including bias, caused by traceability.

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[1] GSO 11854-2021 Reference materials of the approved type of mechanical properties of steel grade 20. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1395637. Accessed 15 October 2022 (In Russ.).

Table 1 Metrological characteristics of GSO 11854-2021 CRM of the mechanical properties of grade 20 steel

Certified characteristic	RM certified value	Absolute expanded uncertainty of certified values ($P = 0.95$, $k = 2$)
Tensile strength R_m, MPa (N/mm^2)*	446	± 6
Lower yield strength R_{eL}, MPa (N/mm^2)*	250	± 10

* For proportional cylindrical samples according to GOST 1497-84 with the original gauge length $l_0 = 5d_0$, where d_0 is the original diameter of the parallel length of a circular test piece

Materials and Methods

According to JCGM 200:2012 [11] (clause 2.41), metrological traceability is the property of a measurement result whereby the result can be correlated with a reference system through a documented continuous chain of calibrations, each contributing to the measurement uncertainty. In this research, we study the mandatory characteristic of the result of measuring the strength properties of a metal, i.e., the uncertainty of tensile strength, and carry out an analysis of traceability contribution to uncertainty budgeting. The GSO 11854-2021 certified reference material (CRM) of the mechanical properties of St20 steel was selected as a comparison base.

A GSO 11854-2021 approved type CRM was obtained as a result of the study and characterization of steel hot-rolled circular products according to GOST 2590-2006 [12] made of St20 steel according to GOST 1050-2013 [13]. The characterization of rolled samples in terms of their tensile strength and lower yield strength was carried out on the State standard of the unit of force of the first category[2] provided by GOST 1497-84. The standard uncertainty of the characterization was 0.9 N/mm^2. In order to evaluate the expanded uncertainty of certified values, during the type approval tests, the standard uncertainty from the heterogeneity of the reference material equal to 2 N/mm^2 was established.

Table 1 provides the metrological characteristics of the CRM of the mechanical properties of St20 steel, established as a result of testing batch No. 1.

Various approaches can be used to evaluate measurement uncertainties. All of them include determination of the measurand and careful identification of all possible contributions to an increase in the uncertainty of measurements.

Figure 1 illustrates the classification of approaches to the evaluation of uncertainty proposed in [14]. The classification is based on the difference between the evaluation of uncertainty, conducted by the laboratory itself (intralaboratory approach) and uncertainty, based on combined studies in various laboratories (interlaboratory approach).

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[2] GET 32-2011 State working standard of the unit of force of the first category in the range of values from 1 to 50 kN. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/11/items/415290. Accessed 15 October 2022 (In Russ.).

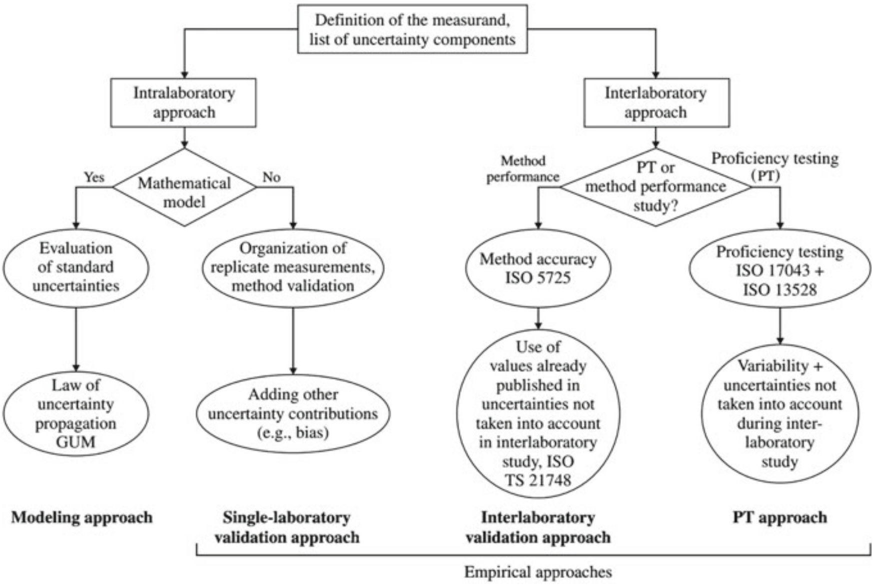

Fig. 1 Classification of approaches to the evaluation of uncertainty according to [9]

In this study, we used a modeling approach based on ISO/IEC Guide 98-3:2008. A mathematical model was created, representing an equation that determines the quantitative relationship between the measurand and all dependent quantities, including all components that contribute to the uncertainty of measurements. An evaluation of the standard uncertainties of all individual components of uncertainty was made. Standard deviations of repeated measurements are directly the standard uncertainties for the corresponding components (given the normal distribution). The combined standard uncertainty is calculated by applying the uncertainty propagation law, which depends on the partial derivatives for each input value. The expanded uncertainty U (providing the interval from $(y - U)$ to $(y + U)$ for the measurand y is calculated. For a normal distribution, the coverage factor $k = 2$ is typically selected. The measurement result, together with its uncertainty, is represented in accordance with the rules of ISO/IEC Guide 98-3:2008.

It should be noted that empirical approaches are just as valid as the modeling approach, and sometimes can lead to a more realistic evaluation of uncertainty. In fact, empirical approaches are substantially based on experimental data and long-term experience, thus reflecting the conventional practice.

Results

The statistical model, forming the basis of uncertainty evaluation methods, can be written in the form of Eq. (1) of ISO 21748:2017:

$$R_m = R + \xi_{R_m} + \sum c_i x_i' + g_{R_m} + e_{R_m}, \tag{1}$$

where

R_m is the measurement result, for which it is assumed that it can be calculated by the corresponding function ($R_m = \frac{P_{\max}}{F_0}$, $P_{\max}$ is the maximum force preceding the rupture of the sample, F_0 is the initial cross-sectional area);
R is the (unknown) expected value of ideal results;
ξ_{R_m} is the bias due to traceability;
x_i' is excursion x_i;
c_i is the sensitivity factor equal to $\frac{\partial R_m}{\partial x_i}$;
g_{R_m} is rounding of the measurement result according to GOST 1497-84;
e_{R_m} is a random component of measurement uncertainty under repeatability conditions.

It was assumed that x_i' are normally distributed with a zero expected value and variance $u^2(x_i)$.

Given the model described by Eq. (1), the uncertainty of measuring R_m was evaluated using Eq. (2):

$$\begin{aligned} u^2(R_m) &= u^2(\xi_{R_m}) + \mathrm{c}^2(\mathrm{P}_{\max})u^2(\mathrm{P}_{\max}) + \mathrm{c}^2(d_0)u^2(d_0) + u^2(g_{R_m}) + u^2(e_{R_m}) \\ &= u^2(\xi_{R_m}) + \left(\frac{4}{\pi d_0^2}\right)^2 u^2(\mathrm{P}_{\max}) + \left(-\frac{8\mathrm{P}_{\max}}{\pi d_0^3}\right)^2 u^2(d_0) + u^2(g_{R_m}) + u^2(e_{R_m}), \end{aligned} \tag{2}$$

where

$u^2(\xi_{R_m})$ is the uncertainty caused by the uncertainty of the estimate obtained based on the measurements of the reference material with the certified value;
$u^2(x_i')$ is the uncertainty corresponding to x_i';
$u^2(g_{R_m})$ is the uncertainty due to rounding of the measurement result;
$u^2(e_{R_m})$ is the random component of the uncertainty of measuring the reference material under repeatability conditions.

The uncertainty, corresponding to the bias due to traceability, can be given by Eq. (3):

$$u^2(\xi_{R_m}) = u^2(R_{mGSO}) + \frac{(R_{mGSO} - \underline{R}_m)^2}{3}, \tag{3}$$

where $u^2(R_{mGSO})$ is the uncertainty corresponding to the certified value R_{mGSO} used to assess the correctness in a joint study.

If a reference material was tested n times (at least 3 times), the recommended procedure for evaluating the limits of $u^2\left(e_{R_m}\right)$ is as follows:

(a) determination of the mean $\underline{R}_m$ and the standard deviation $s_{\underline{R}_m}$;
(b) determination of the confidence interval of the mean according to formula (4):

$$u\left(e_{R_m}\right) = \frac{s_{\underline{R}_m} t(P, f)}{\sqrt{n}}, \tag{4}$$

where

t is Student's coefficient; P is the confidence level; $f = (n - 1)$ is the number of degrees of freedom;
n is the number of measurements. For $P = 0.7$ and $n = 3$, $t = 1.386$.

Table 2 represents an example of calculating the uncertainty budget of measuring the "ultimate stress" characteristic.

Discussion

An analysis of the uncertainty budget presented in Table 2 distinguishes four equivalent contributions to the combined standard uncertainty: the RM original diameter of the parallel length of a circular test piece, rounding of the result, traceability to an approved type RM, and the random component of measurements under repeatability conditions. Let us consider each of the components individually.

The contribution from the initial RM diameter is caused by the tolerance for the diameter of the test piece, equal to 0.10 mm by GOST 1497-84. This contribution, in accordance with JCGM 106:2012 [15], describes the global risk for an object (sample) selected randomly from the production process. The decision about accounting for this contribution should be taken depending on the object under assessment: an individual sample or a test procedure implemented using certain equipment in a laboratory.

The requirement of GOST 1497-84 to account for the contribution from the rounding procedure, equal to 10 $\mathrm{N/mm^2}$, is apparently related to the past practice of applying tensile testing machines based on analog signals, which exhibit insufficient sensitivity to dynamic force variations during testing. When contemporary tensile testing machines based on digital signals are used, this contribution can be neglected.

The contribution from the random component of RM measurements under the repeatability conditions typically describes the quality of the operator's work and random effects, occurring during the test procedure in a laboratory using certain equipment, since the material heterogeneity is already taken into account in RM

Table 2 Uncertainty budget of measuring the "ultimate strength" characteristic

Source of uncertainty	Designation	Formula	Applied variables, parameters	Example
Original diameter of the parallel length of a circular test piece	$u(d_0)$ $c(d_0)$	$u(d_0) = \frac{\Delta_{d_0}}{\sqrt{3}}$ $\mathrm{c}(d_0) = -2\frac{R_m}{d_0}$ $\mathrm{c}(d_0)u(d_0)$	Table 8 GOST 1497-87 $\Delta_{d_0} = \pm 0.10$ mm $R_m =$ 570 N/mm^2 $d_0 = 10.06$ mm	$u(d_0) = \frac{0.10}{\sqrt{3}} = 0.0577$ $\mathrm{c}(d_0) = -2\frac{570}{10.06} = -113$ $\mathrm{c}(d_0)u(d_0) = 113{\cdot}0.0577 = 6.52$ N/mm^2
Maximum force	$u(\mathrm{P}_{\max})$ $c(\mathrm{P}_{\max})$	$u(\mathrm{P}_{\max}) = \frac{\Delta_{R_m}}{\sqrt{3}}$ $\mathrm{c}(\mathrm{P}_{\max}) = \frac{R_m}{\mathrm{P}_{\max}}$ $\mathrm{c}(\mathrm{P}_{\max})u(\mathrm{P}_{\max})$	Limit of the permissible relative error of the force measurement $\frac{\Delta \mathrm{P}}{\mathrm{P}} = \pm 0.5\%$ $R_m =$ 570 N/mm^2 $\mathrm{P}_{\max} = 45120$ N	$\mathrm{P}_{\max} = 45120$ N $u(\mathrm{P}_{\max}) = \frac{45120{\cdot}0.005}{\sqrt{3}} = 130$ $\mathrm{c}(\mathrm{P}_{\max}) = \frac{570}{45120} = 0.0126$ $\mathrm{c}(\mathrm{P}_{\max})u(\mathrm{P}_{\max}) = 130 \cdot 0.0126 = 1.64\frac{\mathrm{N}}{\mathrm{mm}^2}$
Result rounding	$u\left(g_{R_m}\right)$	$u\left(g_{R_m}\right) = \frac{g_{R_m}}{\sqrt{12}}$	Table 2 GOST 1497-87 $g_{R_m} =$ 10 N/mm^2 for $\sigma > 500$ N/mm^2	$u\left(g_{R_m}\right) = \frac{10}{\sqrt{12}} = 2.88$ N/mm^2

(continued)

Table 2 (continued)

Source of uncertainty	Designation	Formula	Applied variables, parameters	Example
Mean and MSE of CRM measurements	$\underline{R}_m, s_{\underline{R}_m}$	$\underline{R}_m = \frac{\sum_{i=1}^{n} \underline{R}_{mi}}{n} = \frac{1}{3}\sum_{i=1}^{3} \underline{R}_{mi}$ $s_{\underline{R}_m} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}\left(\underline{R}_{mi} - \underline{R}_m\right)^2} = \sqrt{\frac{1}{2}\sum_{i=1}^{3}\left(\underline{R}_{mi} - \underline{R}_m\right)^2}$	$\underline{R}_{mi}$—single measurement of a GSO n—number of GSO measurements ($n = 3$)	Measurement results (**465; 470; 475**) N/mm^2 $\underline{R}_m = \mathbf{470}$ N/mm^2 $s_{\underline{R}_m} = \sqrt{\frac{1}{2}\left(5^2 + 0 + 5^2\right)} = 5\ \text{N/mm}^2$
Random component of the uncertainty of measuring a GSO under repeatability conditions	$u\left(e_{R_m}\right)$	$u\left(e_{R_m}\right) = \frac{s_{\underline{R}_m} t(P,f)}{\sqrt{n}} = \frac{s_{\underline{R}_m} t_{0.7,2}}{\sqrt{3}}$	$t_{0.7,2} = 1.386$ for $n = 3$	$u\left(e_{R_m}\right) = \frac{5 \cdot 1.386}{\sqrt{3}} = 4\ \text{N/mm}^2$

(continued)

Table 2 (continued)

Source of uncertainty	Designation	Formula	Applied variables, parameters	Example
Certified GSO value and its uncertainty (Based on the GSO certificate)	R_{mGSO} $u(R_{mGSO})$	$u(R_{mGSO}) = \frac{U(R_{mGSO})}{2}$	$R_{mGSO} =$ **446** N/mm^2 $U(R_{mGSO}) =$ 6 N/mm^2 is the value of the expanded uncertainty of the GSO according to the certificate	$u(R_{mGSO}) = \frac{6}{2} = 3$ N/mm^2
Traceability to GSO	$u(\xi_{R_m})$	$u(\xi_{\sigma_{inst}}) = \sqrt{u^2(R_{mGSO}) + \frac{(R_{mGSO} - \underline{R}_m)^2}{3}}$		$u(\xi_{R_m}) = \sqrt{3^2 + \frac{\mathbf{(446-470)}^2}{3}} =$ **14.2** N/mm^2
Combined standard uncertainty	$u(R_m)$	$u(R_m) =$ $\sqrt{\mathrm{c}^2(\mathrm{P}_{\max})u^2(\mathrm{P}_{\max}) + \mathrm{c}^2(d_0)u^2(d_0) + u^2(g_{R_m}) + u^2(\xi_{R_m}) + u^2(e_{R_m})}$		$u(R_m) =$ $\sqrt{6.52^2 + 1.64^2 + 2.88^2 + 4^2 + \mathbf{14.2}^2} =$ 16.0 N/mm^2
Effective number of the degrees of freedom	ν_{eff}	$\nu_{eff} = \frac{u^4(R_m)}{\frac{u^4(\mathrm{P}_{\max})}{\nu(\mathrm{P}_{\max})} + \frac{u^4(d_0)}{\nu(d_0)} + \frac{u^4(g_{R_m})}{\nu(g_{R_m})} + \frac{u^4(\xi_{R_m})}{\nu(\xi_{R_m})} + \frac{u^4(e_{R_m})}{\nu(e_{R_m})}}$	$\nu(\mathrm{P}_{\max}) = \infty$ $\nu(d_0) = \infty$ $\nu(g_{R_m}) = \infty$ $\nu(\xi_{R_m}) = 2 - 1 = 1$ $\nu(e_{R_m}) = 3 - 1 = 2$	$\nu_{eff} = \frac{\mathbf{16.0}^4}{0+0+0+\frac{\mathbf{14.2}^4}{1}+\frac{4^4}{2}} = \frac{\mathbf{65536}}{\mathbf{40787}} = \mathbf{1.6}$
Expanded uncertainty	$U(R_m)$	$U(R_m) = t_{0.95,\nu_{eff}} \cdot u(R_m)$	$t_{0.95,\nu_{eff}} = 4.303$	$U(R_m) = 4.303 \cdot 16.0 = 69$ N/mm^2

metrological characteristics. In the considered example, the estimate of the contribution is equal to 4 N/mm^2. It is assumed that it is necessary to evaluate the random component for each “operator-tensile machine” system.

The contribution from traceability to the RM includes a systematic component, connected with the implementation of the test procedure in a laboratory, including due to the algorithms of tensile testing machine software, and RM heterogeneity. The main affecting factors can be preparatory operations prior to testing (resetting to zero of force and strain sensors, sample fastening type, preliminary loading value), assigned test conditions (deformation rate or loading rate), calculation errors, including in the algorithms of built-in software [8]. The testing rate used in practice is typically close to the maximum values, permitted by the test procedure, since it is necessary to conduct the maximum number of tests per one operator’s work shift. However, the following implicit assumptions are ignored. On the one hand, GOST 1497-84 is a static tensile test method, i.e., reliable results are obtained at a minimum testing rate. On the other, the calibration of tensile testing machines is performed in the static mode, while tests are conducted in the dynamic mode. Therefore, the recorded values of force and elongation may have a systematic error associated with the testing rate. In the analyzed example, the contribution due to traceability is equal to **14.2** N/mm^2. It is assumed that the systematic error should be evaluated for each existing tensile testing machine.

The expanded uncertainty in the example has a value of 69 N/mm^2, which is due to the effective number of the degrees of freedom $\nu_{eff} = 1.6$. A reduction in the effective number of the degrees of freedom can be achieved using two approaches. The first is to reduce the systematic component by varying the testing rate. The second can be implemented given that the random component is not less than the contribution from traceability to a GSO.

Conclusion

The use of an approved RM as a comparison base represents one of the basic tools for ensuring traceability and controlling the accuracy of mechanical property measurements. The approach based on uncertainty budgeting for the measurement Eq. (1) can be used by laboratories to properly assess the uncertainty of the results of measuring mechanical property characteristics, taking into account the combined effect of all uncertainty sources, as well as the bias due to traceability.

The practical significance of the study lies in the possibility of applying the modeling approach of theoretical concepts based on ISO/IEC Guide 98-3:2008 and calculation algorithms provided in ISO 21748:2017 (Eq. 1) by accredited laboratories when evaluating uncertainty according to clause 7.6 of GOST ISO/IEC 17025-2019 [16].

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Metrological Support for Titration Isothermal Calorimetry: Prospects for the Development of Certified Reference Materials

Karina A. Mishina

Abstract Isothermal titration calorimetry is used to study the thermal effects of chemical and physicochemical processes. The wide application of this method has revealed a number of problems. Among them is insufficient metrological support for isothermal titration calorimeters, which leads to inconsistency of the results obtained using different models of calorimeters. The electrical and chemical procedures currently used for calibrating calorimeters have several limitations. This work aims to develop an approach to creating certified reference materials (CRMs) for the unit of heat quantity of physical and chemical interactions having metrological traceability to the main SI units. An analysis of the state of metrological support in isothermal titration calorimetry measurements was carried out. The basic requirements for CRM candidate substances were formulated. The target uncertainty of the integral heat of dilution of propan-1-ol solutions was estimated by analyzing the characteristics of measuring instruments, which allowed the requirements for the measurement method used to characterize CRMs to be specified. A method for determining the certified value of integral heat of dilution traceable to the GET 133 State Primary Special Standard of the unit of heat quantity in the field of solution and reaction calorimetry was proposed.

Keywords Isothermal titration calorimetry · Measurement uncertainty · Chemical calibration · Certified reference materials · Primary standard

Abbreviations

GET 133-2012	State Primary Special Standard of the unit of heat quantity in the field of solution and reaction calorimetry
CRM	Certified reference material

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MI	Measuring instrument
ITC	Isothermal titration calorimetry
SD	Standard deviation

Introduction

Isothermal titration calorimetry (ITC) is a relatively new method for determining the heat of chemical and physicochemical interactions, which has become commercially available since the 1990s. In recent years, the ITC method has been gaining popularity in chemical, medical, and biological research [1–5]. An essential advantage of ITC consists in its ability to study the entire thermodynamic profile of the process during one experiment, including the binding affinity and changes in the enthalpy, entropy, and Gibbs free energy [2].

Every year, more than 500 publications present the results of scientific research obtained using ITC. About 80% of these publications relate to studies of biomolecular interactions: proteins with other proteins, metal ions, fats, nucleic acids, and carbohydrates [2–4]. At the same time, the ITC application is constantly expanding to cover, e.g., enzyme kinetics and medicine characterizations. In [5], Prozeller et al. reported studies of protein and nanoparticle interactions.

In light of the growing spectrum of ITC application, as well as tightening requirements for the accuracy and reliability of ITC measurements, the issues of metrological support for isothermal titration calorimeters are attracting increased attention. At present, the accuracy of calorimeters is assured mostly by electrical calibration with subsequent verification using the chemical and physical interactions with known values of thermal effects. Such interactions include chemical reactions, processes of dissolving pure substances or diluting their solutions, and are generally referred to as calibration, test [6], or standard reactions [7]. In this paper, the term “standard reactions” is used.

Mention should be made that the current calibration practice is associated with serious metrological problems. Thus, the authors in [8, 9] observed significant differences in the thermal effect of the reaction obtained using different models of calorimeters. The researchers paid attention to discrepancies between the obtained values and those reported in literature, exceeding the claimed uncertainty estimates in some cases. In [12], Myszka et al. presented the results of ABRF-MIRG interlaboratory comparisons, which showed the standard deviation of the reaction thermal effect obtained by the participants to reach about 20%. This value significantly exceeded the accuracy levels stated by laboratories.

Some researchers [13, 14] explain such discrepancies with differences in starting materials and procedures for their preparation, emphasizing the need for a primary standard with sufficient stability and accessibility for users, i.e., meeting the requirements for certified reference materials (CRMs). In addition, comparative studies of electrical and chemical calibration procedures [13, 14], as well as recommendations of the International Union of Pure and Applied Chemistry (IUPAC) [6], show that the use of standard reactions in chemical calibration enables a more accurate determination of the effective cell volume and the energy equivalent of a calorimeter. The IUPAC report [6] suggested a number of reactions that could be used as the standard reaction. At the same time, the possibility of using many of the standard reactions given in literature is limited due to the discrepancy between the thermal effect of the reaction and the operating range and application area of the calorimeter, the risk of contamination of the sample syringe and measuring cell, the lack of commercially available materials, etc.

The use of standard reactions is also recommended by manufacturers of measuring instruments (MIs). Thus, the MicroCal and TA Instruments propose sets of reagents (test kits) of their own production in the microcalorimeter operation guidelines. It should be mentioned that these sets are not reference materials per se, as their homogeneity and stability are not guaranteed, nor is the direct traceability to the reference value taken from literature sources. Another question is how the reference measurand value and its uncertainty are determined, since the information on sample preparation and measurement procedures is lacking in the majority of cases.

It is noteworthy that the data presented in [7, 8, 13, 16, 17] should be used as reference values with some caution, since previously obtained results may have been subject to systematic effects and presented with a significantly underestimated uncertainty. For example, this may result from the negligence of such important components, as the titrant concentration.

Therefore, assessment of the uncertainty of isothermal titration calorimeter measurements represents a relevant research problem attracting the attention of both MI manufacturers [13] and scientific organizations [8, 17–20]. An analysis of published literature and FIF EUM[1] data reveals the lack of metrological instruments for ensuring the uniformity of titration calorimetry measurements. This requires CRMs with the certified value of the heat of dissolution, dilution, and reactions capable of ensuring the traceability of measurements to SI units and intended for validating and calibrating MI, as well as for assuring the accuracy of certified and standardized procedures.

The present research is aimed at developing a general approach to creating CRMs for the unit of heat of dilution with the established metrological traceability to the main SI units. To this end, the following research objectives were formulated: to review available publications; to justify the selection of candidate substances for CRMs; to determine the procedure for CRM certification; to conduct type-approval tests; and to establish the metrological characteristics of CRMs.

In the course of the work, candidate substances were selected based on the conducted literature review; the requirements for CRMs and their target uncertainty were determined; and a measurement method to assess the CRM uniformity and

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology. Accessed 4 August 2022 (In Russ.).

stability was proposed. As a result of improving GET 133-2012,[2] its functional and measuring capabilities were extended to microcalorimetric measurements. This allowed the use of direct measurements based GET 133-2012 equipment for CRM characterization. The proposed approach was tested on selected candidate CRM substances.

Materials and Methods

Project Design

The research project was designed taking into account the recommendations of ISO Guide 35 [21]. The developed plan included the following stages: determination of candidate materials for CRM; determination of CRM target uncertainty; planning of experiments, selection and testing of characterization methods and instruments; planning of experiments and selection of methods and instruments for CRM homogeneity and stability studies; homogeneity and stability studies; CRM characterization; uncertainty estimation of the certified value.

The CRM material was selected based on a review of publications reporting the use of standard reactions. The advantages and disadvantages of candidate substances, as well as the experience of their practical application, were considered.

The evaluation of the target uncertainty of the CRM was carried out based on a study of the application area and characteristics of microcalorimeters stated by their manufacturers, an analysis of interlaboratory experiments, as well as the information on the metrological characteristics of MIs presented in publications.

The experimental design, along with the selection and testing of characterization procedures, were developed based on the target uncertainty of CRM. The selection of methods and instruments for studying the CRM uniformity and stability was carried out taking into account the repeatability standard deviation when estimating the thermal effect of the reaction.

Future research stages will focus on the CRM uniformity and stability, its characterization, and the assessment of the CRM certified value uncertainty.

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[2] GET 133-2012 State Primary Special Standard of the unit of heat quantity in the field of solution and reaction calorimetry: Custodian Institute D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397821 2. Accessed 4 August 2022 (In Russ.).

Equipment

Preliminary experimental studies were carried out using an MKT reference titration microcalorimeter (VNIIM, Russia) and a NanoITC SV microcalorimeter-comparator (TA Instruments, USA) from the GET 133-2012 composition.

The MKT microcalorimeter is a heat flow differential titration microcalorimeter. This installation consists of a two-cell measuring calorimeter unit (sample and reference cells), a dosing system, a temperature monitoring system, a measurement process monitoring system, and an information collection and processing system. The OriginPro 2022B software package is used for processing experimental data. The appearance of the assembled MKT microcalorimeter is shown in Fig. 1.

The MKT metrological characteristics include the following values (Table 1): the range of heat quantity, across which the unit is reproduced under special conditions; standard deviation (SD) of estimating the measured value S_0 under the number of independent measurements n, non-excluded systematic error Θ_0, the total standard deviation of the measured value $S_{0\Sigma}$, tolerance limits of measurement error $\Delta_{0\Sigma}$, type A standard uncertainty u_{0A}, type B standard uncertainty u_{0B}, combined standard uncertainty u_{0C}, and expanded uncertainty U_0 with the coverage factor $k = 2$.

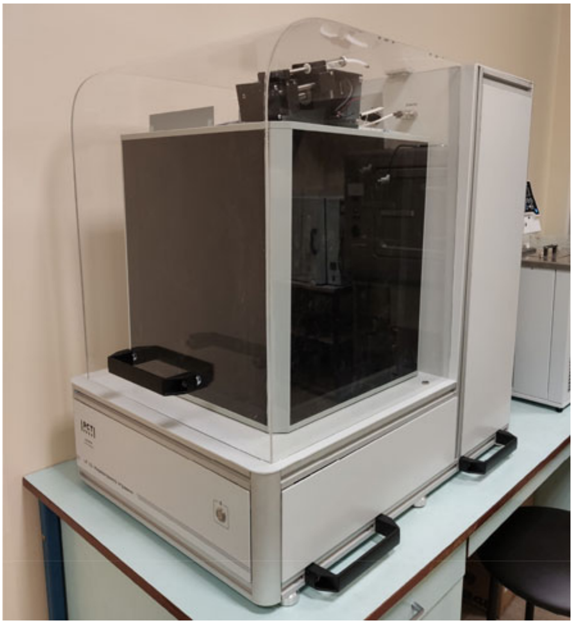

Fig. 1 MKT reference titration microcalorimeter from GET 133-2012

Table 1 Metrological characteristics of the MKT microcalorimeter from GET 133-2012

Measurement error					
Physical quantity	Range of values, μJ	S_0, %	Θ_0, % at P = 0.95	$S_{0\Sigma}$, %	$\Delta_{0\Sigma}$, % at P = 0.95
Heat quantity	100–5000	0.2 ÷ 4.0 (n = 10)	0.8 ÷ 2.9	0.5 ÷ 4.3	1.0 ÷ 8.6
Measurement uncertainty					
Physical quantity	Range of values, μJ	u_{0A}, %	u_{0B}, %	u_{0C}, %	U_0, % at k = 2
Heat quantity	100–5000	0.2 ÷ 4.0 (n = 10)	0.4 ÷ 1.5	0.5 ÷ 4.3	1.0 ÷ 8.6

CRM Candidate Materials

When selecting candidate substances for CRM development, the following basic requirements were formulated:

- candidate materials must ensure the possibility of the standard reaction under a given heat release across the respective range;
- candidate materials must be chemically stable during storage and sample preparation;
- candidate materials should not cause side chemical reactions with the solvent and calorimeter materials;
- candidate materials of the required purity must be commercially available.

The problem of selecting suitable CRM materials was studied in a number of works, e.g., in [6, 22]. The reported results of theoretical and practical studies of standard reactions were taken into accound when selecting a CRM candidate substance.

Since the aim of the present study was to develop CRMs for assessing the metrological characteristics of MIs, standard reactions requiring complicated sample preparation techniques and intended for a comprehensive quality assurance of microcalorimetric measurement procedures were not considered. For this research, the reactions of barium salts with dibenzo-18-crown-6 and calcium chloride with ethylenediaminetetraacetic acid were identified as promising. Neutralization reactions were also excluded from consideration, as they may be affected by dissolved CO_2. Moreover, substances for such reactions may be corrosively active against some calorimeter materials.

In this work, for the development of CRMs for the unit of heat, the dilution reaction of propan-1-ol solutions in water was selected as the standard reaction due to the availability of domestically produced high-purity materials and the simplicity of data preparation and processing techniques. Solutions of different concentrations can be used to evaluate the metrological characteristics of MIs across the entire measurement range.

Table 2 Characteristics of candidate substances

Reference material (RM)	Certified characteristic	Value of the certified characteristic
RM for the composition of propan-1-ol solution, GSO 11383-2019[3]	Mass concentration of propan-1-ol	(4.00 ± 0.04) g/dm^3
RM for the composition of propan-1-ol, SOP 0023-03	Mass fraction of propan-1-ol	(99.9 ± 0.1) %

Table 2 presents commercial products made from propan-1-ol selected as CRM candidate substances.

In order to prepare samples of a given concentration from starting solutions, a procedure that included estimation of uncertainty when determining the concentration of the resultant solutions was developed in accordance with the EUROCHEM/CITAK CG 4 Guideline [23].

Solutions for preliminary experimental studies were prepared by the gravimetric method without subsequent control by other methods. Pure propanol (RM for the composition of propan-1-ol, SOP 0023-03) was prepared according to the instructions for handling and use given in the RM passport and cooled for at least 1 h at a temperature of 2–6 °C. A solution with a 2% mass fraction was prepared using 2 g of propanol and 98 g of deionized water, which was obtained using a UPVA-5-1 bidistillator (Livam, Russia) as part of GET 133-2012. Sartorius ME 235s scales were used for weighting sample portions.

Results and Discussion

Defining Target Uncertainty

The operation principle of ITC is described in detail in [2]. The general calorimeter scheme is outlined in Fig. 2.

A typical isothermal titration calorimeter consists of two—sample and reference—cells. The control system monitors the cell temperature such that the temperature difference between the cells was close to zero. When a sample is fed into the sample cell, the resulting chemical process is accompanied by a release or absorption of heat, which alters the temperature in the sample cell. The operation of such calorimeters is based on the measurement of the thermoelectric force arising in the thermopile under the passage of a thermal flow from the sample cell to the large metal calorimeter block of the calorimeter.

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[3] GSO 11383-2019 materials of the composition of propanol solution 1. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/812255. Accessed 4 August 2022 (In Russ.).

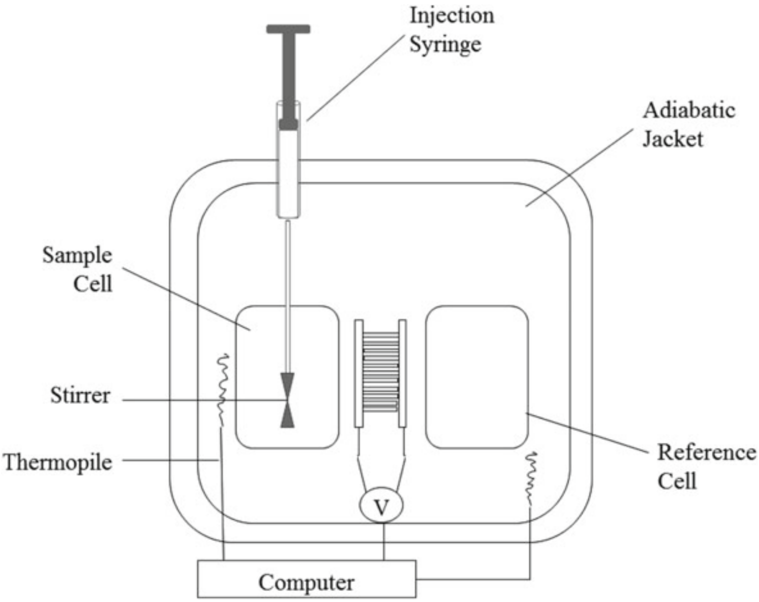

Fig. 2 General scheme of an isothermal titration microcalorimeter

Despite the similar principle of operation, the design of various working microcalorimeters is characterized by the following differences: the principle of power compensation; the material, shape, and volume of the calorimeter cells; the design of the sample feeding equipment and stirrer. These differences significantly affect the technical and metrological characteristics of the microcalorimeter. At the same time, manufacturers do not specify the measurement accuracy of the thermal effects of reactions in their official documentation.

Therefore, the CRM target uncertainty was estimated based on a review of literature data on the measurement results obtained by modern microcalorimeters, on the estimated standard uncertainty (Table 3), interlaboratory standard deviation from the results of interlaboratory experiments (Table 4), and the standard deviation of repeated observations (Table 5).

The researchers in [16] assumed that an uncertainty of about 2% could be achieved when measuring the thermal effect of a reaction. At the same time, a significant dispersion of uncertainty estimates of up to 20% was observed depending on the experimental conditions and data processing methods.

Taking into account the more common uncertainty estimates in other publications [17, 24–27] and the typical intralaboratory standard deviation of 1–2%, it was decided to take an approximate relative standard uncertainty of the measurements of the reaction heat effects equal to 4.5%. The target uncertainty of the developed CRM was set at 1.5%, taking into account the required 1:3 ratio of the verification means to the verifiable MI.

Table 3 Measurement uncertainty of the thermal effect of a reaction using microcalorimeters, according to the published data

Thermal effect of reaction, ΔH, kJ/mol	Standard uncertainty of measurements of the thermal effect of reaction, u (ΔH), kJ/mol*	Relative standard uncertainty of measurements of the thermal effect of reaction, u_0(ΔH), %*	Source
– 26.6	1.1	4.1	[24]
8.8 ÷ 43.0	0.9**	4.9**	[25]
0.5 ÷ 46.6	0.3**	4.4**	[26]
– 8.8***	1.3	14.3	[17]
46.5***	2.3	5.0	[27]
27.2***	0.5	2.0	[16]

Notes

*—the calculation of the uncertainty value given in the source to another form of expression was carried out in accordance with the equations:

$u_0(X) = \frac{u(X)}{\overline{X}}$,

$U = k \cdot u_C$,

**—the value was obtained as the average of the given results.

***—the value was recalculated from kcal/mol to kJ/mol by multiplying by a coefficient of 4.1868.

Table 4 Interlaboratory standard deviation of measurements of the thermal effect of a reaction using microcalorimeters, according to the published data

Thermal effect of reaction, ΔH, kJ/mol	Number of participants	SD, kJ/mol	Relative SD, %	Source
– 43.5	14	10.5	24	[10]
– 17.5 15.0	12 12	0.4 0.4	2.3 2.6	[14]
– 9.26 – 8.53	7 6	0.7 0.35	7.5 4.1	[13]

The established metrological and technical requirements for the developed CRMs are shown in Tables 6 and 7.

Methods and Instruments for Studying Uniformity and Stability

In accordance with clause 7.5.1 of ISO Guide 35 [21], the method used in the study of uniformity must have good repeatability, i.e., the following equation must be fulfilled:

Table 5 Standard deviation of measurements of the thermal effect of a reaction using microcalorimeters, according to the published data

Thermal effect of reaction, ΔH, kJ/mol	SD, kJ/mol	Relative SD, %	Source
– 28.8 ÷ – 32.9*	0.3	0.8	[28]
– 27.5 – 20.4	1.1 0.7	4.0 3.4	[29]
55 – 9.07 – 17.4	1 0.05 0.2	2 0.6 1	[18]
– 50.7*	1.7	3.5	[9]
– 46.0	1.2	2.6	[8]
– 1.540 – 0.604 – 0.196	0.01 0.01 0.01	0.7 1.8 2.8	[15]
– 59.5	3.3	5.5	[30]
– 17.5* 15.0*	0.4 0.2	2.1 1.1	[14]

*—the value was recalculated from kcal/mol to kJ/mol by multiplying by a coefficient of 4.1868

Table 6 CRM certified characteristic, certified value range, and expanded uncertainty range

Index of the CRM in the set	Certified value range of the integral heat of dilution, $\Delta H^0{}_m$, kJ/mol	Relative expanded uncertainty (U) with the coverage factor k = 2, %
ITR-VNIIM-2	– 0.190 ÷ – 0.210	3.0
ITR-VNIIM-5	– 0.600 ÷ – 0.640	3.0
ITR-VNIIM-10	– 1.530 ÷ – 1.560	3.0

Table 7 Technical characteristics of initial materials

RS index in the set	Initial substance	Documents regulating the initial substance
ITR-VNIIM-2 ITR-VNIIM-5 ITR-VNIIM-10	CRM for the composition of propan-1-ol, SOP 0023-03	TU 2631-031-56278322-2008

$$\frac{s_r}{\sqrt{n}} = \frac{u_{trg}}{3}, \tag{1}$$

where

s_r is the standard deviation of method repeatability, %;
n is the number of experiments;
u_{trg} is the CRM target uncertainty, %.

An analysis of standard deviations of repeatability shows that $s_r = 1\%$ can be achieved under optimal conditions. Therefore, Eq. (1) is fulfilled when n ≥ 4.

This assumption was experimentally tested using a NanoITC SV comparator microcalorimeter in a series of experiments to measure the thermal effect of the reaction of calcium chloride and ethylenediaminetetraacetic acid. The obtained average value was −17.58 kJ/mol with an SD of 0.05 kJ/mol (0.3%).

When selecting a method for studying stability, its repeatability according to (1) was taken into account. The stability of the measuring system was also considered, due to the long-term nature of stability studies. The stability of the measuring system was considered sufficient when fulfilling the equation:

$$\frac{|X_{t1} - X_{t0}|}{\overline{X}} \cdot 100\% \leq \frac{u_{trg}}{3}, \tag{2}$$

where

X_{t1}, X_{t0} are measurement results at points in time t_1, t_0, accordingly;
$\overline{X}$ is the average of measurement results.

The stability study is planned to last for 6 months, according to the conventional approach. The same period was set for assessing the stability of the measuring system.

The stability of the measuring system was experimentally tested for an NanoITC SV comparator microcalorimeter in an experiment to measure the thermal effect of the reaction of calcium chloride and ethylenediaminetetraacetic acid. The received deviation $|X_{t1} - X_{t0}|$ was 0.07 kJ/mol (0.4%).

The conducted studies confirmed the applicability of the NanoITC SV comparator microcalorimeter when studying the uniformity and stability of CRMs.

Testing the Method of CRM Characterization

The CRM characterization was carried out by the method of direct measurements using the MKT microcalorimeter from GET 133. The method was tested by measuring the integral heat of dilution of a 2% propan-1-ol solution (n = 2). The obtained value of the integrated heat of dilution was −0.200 kJ/mol. The expanded uncertainty estimated based on the characteristics of the MKT microcalorimeter was 1.4%, which meets the requirements for the CRM target uncertainty under the condition of confirmed uniformity and stability.

The obtained results were validated by comparing the measurement results obtained by the MKT calorimeter from GET 133-2012 and literature data [6, 15] obtained by precision calorimetric installations.

The results obtained at CIQ-UP (Portugal) are given in accordance with [15]. The uncertainty shown in Fig. 3 is represented by two standard deviations. The results obtained at the Lund University (Sweden) are calculated in accordance with the

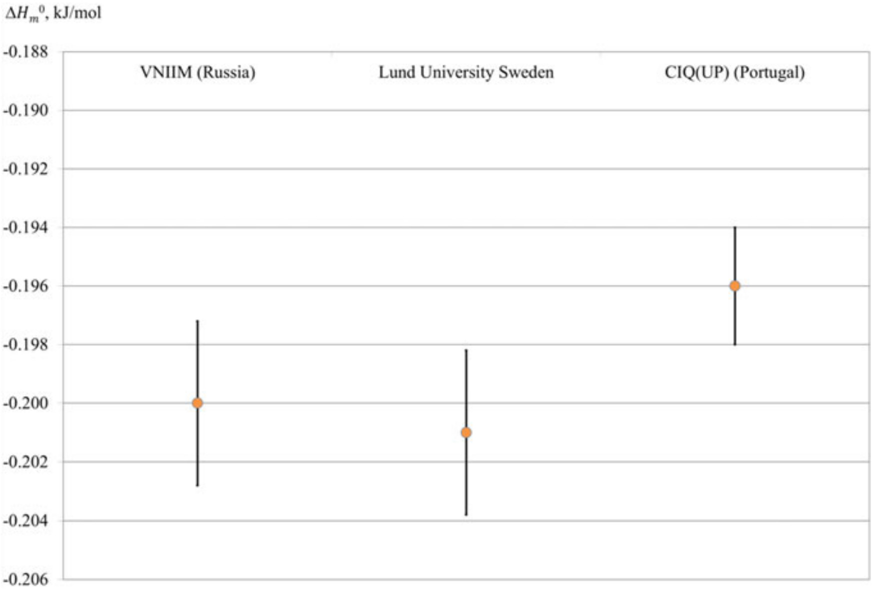

Fig. 3 Comparative analysis of the integral heat of dilution of a 2% propan-1-ol solution

equation given in [6]. The uncertainty shown in Fig. 3 is represented by two standard deviations of the mean value.

The comparison of the integral heat of dilution determined in this study with literature data shows their good agreement. The method of direct measurements using an MKT microcalorimeter can be considered applicable for CRM characterization.

Conclusion

When measuring the thermal effects of reactions using isothermal titration microcalorimeters, the results obtained by different MI models frequently disagree with those reported in literature. The present work aimed to contribute to metrological support in this field by launching research into the development of CRMs with the certified value of the integral heat of dilution having established metrological traceability.

The research design included selection of initial materials, assessment of the target uncertainty of the heat of dilution, and establishment of requirements for CRMs. To meet these requirements, the methods for homogeneity and stability studies were selected, and a direct method of CRM characterization was proposed and tested, providing traceability to the GET 133 State Primary Special Standard of the unit of heat quantity in the field of solution and reaction calorimetry. In order to complete the

CRM development, future works will include experimental studies into characterization and evaluation of the CRM homogeneity and stability, as well as estimation of the uncertainly of the certified value.

The theoretical significance of the research consists in the development of a basic approach to ensuring the traceability and uniformity of ITC measurements using CRMs as a mean of unit transfer and a reference microcalorimeter for their characterization. The practical significance is directly related to the development and application of CRMs intended for the verification and calibration of MIs and accuracy assurance of certified and standardized techniques. Their implementation is expected to increase the accuracy and reliability of measurement results obtained by isothermal titration calorimeters.

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Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Study of Certified Reference Materials for Temperature and Specific Enthalpy of Phase Transitions of Metals and Metal Salts

Andrei M. Nepomiluev, Artyom P. Shipitsyn, and Anastasiya E. Tyurnina

Abstract The development of calibration procedures, as well as the certification of readily available reference materials (RMs) and the improvement of their metrological characteristics, are topical issues for thermal analysis methods. In the course of the work, the necessity of conducting a study in order to expand the range of CRMs for temperature and enthalpy of phase transitions is substantiated. The substantiation of the choice of the measurement procedure and starting materials is given. The conditions for conducting experiments are described in detail, and the choice of these conditions is justified. The developed CRMs passed the metrological examination and were included in the Register of approved types of reference materials FIF EUM as a set of certified reference materials for temperature and specific enthalpy of phase transitions (set SOTSF-2) GSO 11890-2022/GSO 11896-2022. The practical significance of the obtained results is as follows: certified reference materials allow expanding the possibility of establishing and monitoring the stability of the calibration characteristics of thermal analysis installations and measuring instruments; certification of measurement procedures (methods) and accuracy control of the measurement results of the phase transition temperature of metals, metal salts, metal oxides, and polymeric materials, organic and inorganic substances.

Keywords Reference material · Thermal analysis · Phase transition temperature · Specific enthalpy of phase transition · State primary standard

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Abbreviations

TA	Thermal analysis
DTA	Differential thermal analysis
TGA	Thermal gravimetric analysis
DSC	Differential scanning calorimetry
STA	Simultaneous thermal analysis
MP	Measurement procedure
MI	Measuring instrument
RMS	Root mean square
CRM	Certified reference material

Introduction

Currently, thermal analysis (TA) refers to a group of methods in which a physical property of a substance is measured as a function of temperature or time while the substance is subjected to a temperature controlled program [1]. The temperature program can include heating, cooling at a constant rate, exposure at a constant temperature (isotherm), and a combination of these modes or a temperature modulation mode with amplitude and frequency setting.

One of the most advanced, highly sensitive and, therefore, the most commonly used methods for phase analysis of heterogeneous systems are differential thermal analysis (DTA) and differential scanning calorimetry (DSC). They make it possible to determine the temperature and enthalpy of transitions, the heat capacity and its dependence on thermodynamic parameters, as well as the kinetic characteristics of physical and chemical processes under conditions of a linear temperature variation. Instruments that implement these methods are widely used, quite simple in design, and easy to operate. However, DSC/DTA instruments, unlike adiabatic calorimeters, are not absolute measuring instruments. The values of the amount of released or absorbed heat and heat flow rates are measured under dynamic conditions, and not under heat equilibrium conditions. Therefore, the measurement results of DSC/DTA instruments are influenced by numerous parameters associated with the measured sample, instrument, and operator. The main influencing parameters include: the mass and geometry of the sample, its state of aggregation and thermophysical properties, the type of the measured effect, the magnitude of the measured effect, the dimensions of the crucible and the material from which it is made, the thermal resistance between the calorimeter, the crucible, and the sample, the response time of the heating/cooling device, the design and material of the measuring cell, the position of the sample in the crucible and the crucible in the calorimeter, the type of purge gas, its pressure, and purge rate, the procedure for collecting and processing experimental data. Nevertheless, errors in measuring the phase transition temperature from 0.01 to 0.5 °C, the enthalpy changes of phase transitions from 0.5 to 1.0%, and heat capacity from

1 to 2% [2] can be achieved in the temperature range from 200 to 800 K subject to careful calibration of DSC/DTA instruments and accurate measurements. In addition, the measurement accuracy of thermophysical properties performed using DSC/DTA devices can be affected by changes occurring in the device—the drift of electronic parameters and degradation of thermally sensitive elements (thermocouples and thermal resistances). The dynamics of these changes primarily depends on the intensity of MI operation and measurement conditions: heating rate, atmosphere, and sample holder material. Therefore, a fundamental requirement for every thermoanalytical study is the pre-calibration of the respective instrument, which should be performed using uniform and reliable procedures. Calibration is understood as the procedure for establishing the relationship between the value of a quantity determined by the measuring instrument and its true value. The developments of calibration procedures, as well as the certification of readily available reference materials (RMs) and the improvement of their metrological characteristics, are topical issues for thermal analysis methods.

Theoretical Background

The range of reference materials used should correspond to the operating ranges of measuring instruments, as well as the materials used for the manufacture of measuring cells and crucibles. Since the development and implementation of the ISO-9000 standard [3], ensuring the comparability and traceability of thermal analytical data has become a fundamental requirement.

One of the first calibration procedures for DSC/DTA instruments were those described in [4–6]. But none of them acted as an instrument-independent procedure. In order to unify the calibration methods that existed at that time and reduce the magnitude of systematic measurement errors, the German Society for Thermal Analysis[1] formed a working group "Calibration of scanning calorimeters" (1987), whose goal was to develop scientifically based, instrument-independent, and generally accepted calibration procedures, the application of which would significantly reduce the systematic errors of the values measured by DSC/DTA. The results of the working group were published in [7–13] and were adopted by the International Confederation for Thermal Analysis and Calorimetry (ICTAC)[2] as a recommendation to its members (2000).

Since DSC/DTA instruments are used to determine the temperature and heat of phase transitions, as well as the rate of heat flow (when determining the temperature dependence of the heat capacity of a substance or studying the kinetics of a reaction),

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[1] Gesellschaft fur Thermische Analyse (GEFTA). Available via GEFTA. https://www.gefta.org/index.php. Accessed 10 October 2022.

[2] International Confederation for Thermal Analysis and Calorimetry (ICTAC). Available via ICTAC. http://www.ictac.org/index.html. Accessed 10 October 2022.

it becomes necessary to calibrate MI using three scales: temperature, enthalpy, and heat flow.

The temperature scale is calibrated by comparing the known phase transition (melting/crystallization) temperatures of RMs with the corresponding temperatures measured at the appropriate heating rate. Then a correction curve is constructed in the given temperature range. Depending on the range to be covered and the shape of the calibration curve, at least three RMs are required to be measured—even if the calibration is linear, a third point is needed to confirm this. Since temperature sensors are most often located inside the device and not in the sample, the need for extrapolation to a zero heating rate requires measuring RMs at three or more heating rates [2].

Melting (crystallization) or a phase transition in a solid appears on the DSC curve as an endo- or exothermic peak; therefore, the enthalpy calibration procedure makes it possible to relate the peak area obtained for the RM to the enthalpy change known for it and obtain peak area to enthalpy conversion factor. Since experience has shown that this factor is a function of temperature and temperature scan rate (or any other of the experimental parameters that should be kept constant throughout the measurement series), the measurements of several enthalpy reference materials are required to cover the entire temperature range of the DSC/DTA instrument.

The heat flow rate in the DSC experiment is related to the heat capacity of the sample; and if any reaction occurs in the sample, it is related to the "apparent" heat capacity. The ideal RM for heat flow calibration should be thermodynamically inert (have no phase transformations) over a wide temperature range. From this point of view, α-alumina (polycrystalline corundum or monocrystalline leucosapphire) is a universal RM. With its use, experimental data on heat capacity for other well-studied substances can be reproduced with an accuracy of $\pm$ 1%, and in some cases even better [14].

The recommendations of the GEFTA working group for calibrating DSC/DTA instruments and performing subsequent measurements are formulated as follows [15]:

- Experimental conditions for calibration and sample measurements should differ as little as possible. Therefore, a valid calibration procedure should specify in detail how to establish reproducible environmental conditions and how to check for non-linearities;
- Any asymmetry in heat flows, temperature gradients, and measurement effects should be avoided. It is necessary to create quasi-steady-state conditions. However, a detailed study of the dependence of the measured values on the parameters of the sample and instrument is mandatory to eliminate systematic errors;
- Calibrations already performed by the manufacturer of DSC/DTA instruments should be carefully checked;
- Calibrations should be checked at regular intervals according to the required accuracy. It also provides information on any long-term systematic changes to the measuring system.

The recommended substances by GEFTA and ICTAC for use as RMs cover the temperature range from 120 to 1350 °C and mainly refer to materials that determine the fixed points of the International Temperature Scale 1990 (ITS-90) [15] (Table 1).

The GEFTA and ICTAC recommendations also describe the procedure for processing experimental data obtained from measurements of RMs on a calibrated DSC/DTA instrument, including a method for determining the characteristic temperatures of endothermic (exothermic) peaks of phase transformations. Figure 1 shows the determination of the characteristic temperatures in the case of an endothermic peak (for example, metal melting) [2].

It is more convenient to determine the values of temperatures T_i, T_f from the first derivative of the DSC/DTA curve, where the moment of deviation of the DSC/DTA signal from the interpolated baseline is determined much better.

Using the example of UNIIM, a competent manufacturer of CRMs for thermophysical properties (SOTS), 6 types of CRMs for temperature and heat of phase transitions were developed based on potassium chloride, as well as gallium, indium, tin, zinc, antimony (set SOTSF, Table 2). The development and certification of CRMs were carried out in accordance with the State verification schedule for means of measuring specific enthalpy and specific heat-conguction of solid frames in the range of temperature from 700 to 1800 K [16] using the National primary special standard GET 67-2013 for the units of specific enthalpy and specific heat of solids at temperatures in the 700–1800 K range [17].

As shown in Table 2, the temperature range provided by the available GSOs is from ~ 300 to ~ 1050 K, and the range of specific heat of fusion is from ~ 28 to ~ 59 kJ/kg.

At the same time, TA instruments supplied to the Russian market by leading manufacturers of thermoanalytical equipment, such as “Netzsch-Geratebau GmbH” (Germany), “TA Instruments” and “PerkinElmer, Inc” (USA), “Setaram Instrumentation” (France), and “Mettler-Toledo AG” (Switzerland) cover a much wider range of temperatures and heats of phase transitions: from ~ 90 to ~ 1920 K and from 10 to 1000 kJ/kg, respectively. Moreover, recently a number of high temperature instruments have appeared on the market, the upper limits of the operating range of which are 2273 K (DSC 404 F1/F3 Pegasus, STA 449 F1 Jupiter, Netzsch, Germany), 2673 K (STA 449 F3 Jupiter, Netzsch, Germany, SETSYS Evolution TGA-DTA/DSC, Setaram Instrumentation, France), and even 3073 K (DIL 402 Expedis HT, Netzsch, Germany).

The analysis showed that the available list of GSOs for temperatures and heats of phase transitions do not fully provide the measurement ranges of the corresponding thermophysical quantities. In addition, Table 3 indicates the existing limitations on the compatibility of GSO materials with the materials of crucibles used in measurements [8].

Table 3 shows that, for example, GSO 2312-82/2316-82 can be used for graduation, calibration, and temperature verification of thermal analyzers in the case of measurements in non-metallic (ceramic, glass, etc.) crucibles. At the same time, only GSO 2313-82 (In) and GSO 2314-82 (Sn) can be used for graduation, calibration, and

Table 1 Substances recommended for calibration by temperature and specific enthalpy of phase trasformation

Substance	Type of transformation	Transformation temperature		Uncertainty, u^a, μm	Specific enthalpy[b]		Uncertainty, u, %
		K	°C		ΔH, J/g	ΔH, kJ/mol	
C_5H_{10} (Cyclopentane)	s.–s.	122.38	− 150.77	50	69.60	4.881	0.5
C_5H_{10} (Cyclopentane)	s.–s.	138.06	− 135.09	50	4.91	0.345	1.1
C_5H_{10} (Cyclopentane)	s.–l.	179.72	− 93.43	50	8.63	0.605	1.1
Water	s.–l.	273.15	0.00	10			
Gallium	s.–l.	302.914	29.764	0	79.88	5.569	0.9
Indium	s.–l.	429.748	156.598	0	28.62	3.286	0.4
Tin	s.–l.	505.078	231.928	0	60.40	7.170	0.6
Bismuth	s.–l.	(544.55)	(271.40)	–	53.83	11.25	3.9
Lead	s.–l.	600.61	327.46	10			
Zinc	s.–l.	692.677	419.527	0			
Lithium sulfate	s.–l.	851.43	578.28	250	228.1	25.07	4.6
Aluminium	s.–l.	933.473	660.323	0	398.1	10.74	2.3
Silver	s.–l.	1234.93	961.78	0			
Gold	s.–l.	1337.33	1064.18	0			

Note

a—uncertainty *u* is an estimate of the range of values within which the true value lies. The uncertainty of phase transformation temperatures for materials corresponding to the ITS-90 fixed points is zero

b—the indicated transition temperatures are average values calculated as a weighted average of the most reliable measurements given in the relevant literature. The indicated uncertainty describes the double experimental standard deviation

s.–s.—polymorphic transformation

s.–l.—solid–liquid transition (melting)

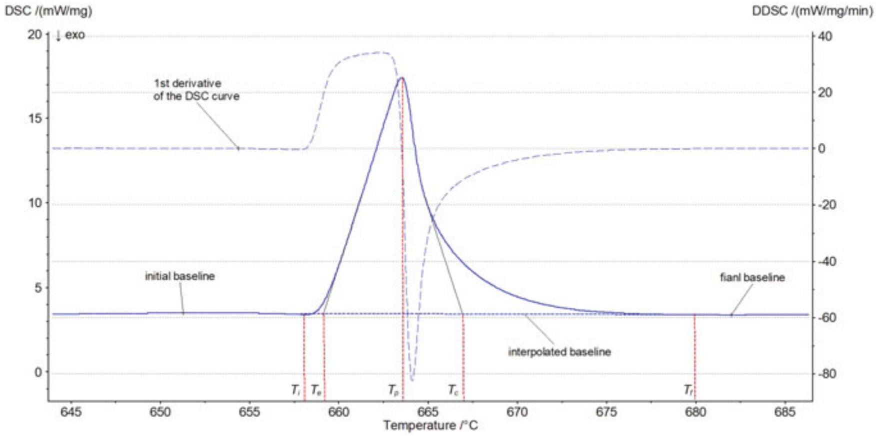

Fig. 1 Determination of characteristic DSC/DTA peak temperatures, where: T_i is the initial temperature: the first visible deviation of the DSC/DTA curve from the interpolated baseline; T_e is the extrapolated peak start temperature: the temperature at which the tangent at the inflection point of the rising slope of the peak crosses the interpolated baseline. The temperature T_e is taken as the melting or phase transition temperature; T_p is the peak temperature: the temperature corresponding to the largest distance between the DSC/DTA curve and the interpolated baseline; T_c is the extrapolated peak end temperature: the temperature at which the tangent at the inflection point of the downward slope of the peak crosses the interpolated baseline; T_f is the final temperature: the last visible deviation of the DSC/DTA curve from the interpolated baseline

verification by specific heat of fusion. There is only GSO 1363-78 based on potassium chloride for use with metal crucibles (platinum, steel, etc.), which is clearly not enough for graduation, calibration, and verification of thermal analysis MI.

Thus, the study of determining the temperature and heat of phase transitions for the metrological support of MI of higher temperature and traceability to the basic units of physical quantities is of particular relevance.

Therefore, the purpose of this work is the development and certification of new RMs for temperature and heat of phase transitions for thermal analysis instruments, primarily for DSC/DTA instruments.

Materials and Methods

Reagents

The commercially available Netzsch RMs used for calibration of thermal analysis instruments were selected as candidate RMs (Table 4).

This list of 24 substances can be divided into three groups: the first is for the low temperature range (from − 87 to 122 °C) and consists mainly of organic compounds;

Table 2 Certified reference materials for thermophysical properties developed by UNIIM[3]

State register number, the material of CRM	Certified characteristic	Unit designation	Certified value	Error (±)
GSO 2312, gallium	Melting point	K	303.04	0.06
GSO 2313, indium	Melting point, crystallization point, specific heat of fusion	K K kJ/kg	429.85 429.79 28.58	0.06 0.08 0.12
GSO 2314, tin	Melting point, crystallization point, specific heat of fusion	K K kJ/kg	505.20 505.12 59.92	0.12 0.01 0.25
GSO 2315, zinc	Melting point, crystallization point	K K	692.71 692.67	0.26 0.03
GSO 2316, antimony	Crystallization point	K	903.76	0.03
GSO 1363, potassium chloride	Melting point, specific heat of fusion	K kJ/kg	1044.75 357.29	0.6 1.79

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[3] GSO 2312-82/2316-82 Reference materials of the approved type of temperatures and heats of phase transitions (set SOTSF). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/393963. Accessed 10 October 2022 (In Russ.).

GSO 1363-78 Reference materials of thermodynamic properties (potassium chloride) (SOTS-5). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/394230. Accessed 10 October 2022 (In Russ.).

Table 3 Interaction of reference materials with crucible materials

Crucible material	Reference material								
	Cyclopentane, C_5H_{10}	Water, H_2O	Gallium, Ga	Indium, In	Tin, Sn	Lead, Pb	Zinc, Zn	Lithium sulfate, Li_2SO_4	Aluminum, Al
Corundum, $A1_2O_3$	o	o	+	+	+	+	+	+	+
Boron nitride, BN	o	o	+	+	+	+	+	+	+
Graphite, C	o	o	+	+	+	+	+	+	+
Silica glass	+	+	+	+	+	+	?	+	–
Silicon dioxide, SiO_2	+	+	+	+	+	+	+	+	–
Aluminum, Al	+	•	–	+	–	+	–	+	×
Aluminum oxidized	+	+	+	+	+	+	+	+	×
Silver, Ag	+	+	–	–	–	–	–	?	–
Gold, Au	+	+	•	•	–	–	–	+	–
Nickel, Ni	+	+	•	•	•	•	•	?	–
Iron, Fe	+	•	•	+	•	+	–	?	–

(continued)

Table 3 (continued)

Crucible material	Reference material								
	Cyclopentane, C_5H_{10}	Water, H_2O	Gallium, Ga	Indium, In	Tin, Sn	Lead, Pb	Zinc, Zn	Lithium sulfate, Li_2SO_4	Aluminum, Al
Stainless steel	+	+	•	+	•	+	–	?	–
Platinum, Pt	+	+	•	•	–	–	–	+	–
Molybdenum, Mo	+	+	•	?	•	?	•	?	?
Tantalum, Ta	+	+	?	+	?	?	?	+	–
Tungsten, W	o	o	•	?	?	•	+	?	•

Note

+—solubility and effect on melting point is not expected

−—the melt dissolves the crucible material, which leads to a significant change in the melting point

•—partial dissolution and slight effect on the melting point are possible

×—the crucible melts

?—this interaction is unknown

o—sealing the crucible can be difficult

Table 4 List of reference materials supplied by Netzsch for calibration of thermal analysis instruments

Substance		Melting point (phase transition)		Specific enthalpy, J/g	Note
Formula	Designation	°C	K		
C_6H_{12}	Hexene	− 87.0	186.0	− 79.4	
$C_{10}H_{16}$	Monoterpene	− 64.7	208.3	− 22.3	
Hg	Mercury	− 38.8	234.2	− 11.4	
H_2O	Water	0.0	273.0	− 333.4	
Ga	Gallium	29.8	302.8	− 80.0	
$C_{12}H_{10}$	Biphenyl	69.2	342.2	− 120.5	
C_6H_5COOH	Benzoic acid	122.4	395.4	− 147.4	
KNO_3	Potassium nitrate	128.7	401.7	− 50.0	For metal crucibles
In	Indium	156.6	429.6	− 28.6	
$RbNO_3$	Rubidium nitrate	164.2	437.2	− 26.6	For metal crucibles
Sn	Tin	231.9	504.9	− 60.5	For ceramic crucibles
Bi	Bismuth	271.4	544.4	− 53.1	For ceramic crucibles
$KClO_4$	Potassium perchlorate	300.8	573.8	− 104.9	For metal crucibles
Pb	Lead	327.5	600.5	− 23.0	For ceramic crucibles
Zn	Zinc	419.5	692.5	− 107.5	For ceramic crucibles
Ag_2SO_4	Silver sulfate	426.4	699.4	− 51.0	For metal crucibles
CsCl	Caesium chloride	476.0	749.0	− 17.2	For metal crucibles
Al	Aluminum	660.3	933.3	− 397.0	For ceramic crucibles
K_2CrO_4	Potassium chromate	668.0	941.0	− 37.0	For metal crucibles
$BaCO_3$	Barium carbonate	808.0	1081.0	− 94.9	For metal crucibles
Ag	Silver	961.8	1234.8	− 104.6	For ceramic crucibles
Au	Gold	1064.2	1337.2	− 63.7	For ceramic crucibles

(continued)

Table 4 (continued)

Substance		Melting point (phase transition)		Specific enthalpy, J/g	Note
Formula	Designation	°C	K		
Ni	Nickel	1455.0	1728.0	− 290.4	For ceramic crucibles
Pd	Palladium	1554.8	1827.8	− 157.3	For ceramic crucibles

the second and third are for the high temperature range and consist of salts (for metal crucibles) and metals (for ceramic crucibles).

The main objective in selecting a candidate RM for the development and certification of new GSO for temperature and heat of phase transitions from those given in Table 4 was to expand the temperature ranges provided by new RMs for both metal and non-metal crucibles. Therefore, the GSO set, later called SOTSF-2, included samples of four high-purity metals (wt%): Bi (99.999%), Al (99.999%), Ag (99.99%), Au (99.999%), as well as three samples of high-purity metal salts: Ag_2SO_4 (99.999%), CsCl (99.999%), $BaCO_3$ (99.98%).

Thus, the temperature range provided by the GSO was supposed to be expanded to 1337.2 K with a total amount of RMs of 10 pieces (6—SOTSF, 4—SOTSF-2) for non-metal crucibles. According to the enthalpy of phase transitions, the upper limit of the range will be 397 J/g with a total number of RMs of 6 pieces (2—SOTSF, 4—SOTSF-2). Similarly, the lower and upper limits of the temperature range provided by the GSO should be 699.4 K and 1081 K with a total number of reference materials of 4 pieces (GSO 1363-78, 3—SOTSF-2) for metal crucibles. According to the enthalpy of phase transitions, the range is from 17.2 to 357.29 J/g with a total amount of RMs of 4 pieces (GSO 1363-78, 3—SOTSF-2).

Equipment

All phase transformation temperature studies were performed on a thermal analyzer STA 449 F5 Jupiter (NETZSCH, Germany), which implements the method of simultaneous thermal analysis (STA), which combines the method of differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA), from the State Primary Standard of units of mass fraction, mass (molar) concentration of water in solid and liquid substances and materials GET 173-2017.[4]

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[4] GET 173-2017 State primary standard of units of mass fraction, mass (molar) concentration of water in solid and liquid substances and materials: UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397857. Accessed 10 October 2022 (In Russ.).

The mass of the starting material (candidate RM) was measured on a laboratory balance of special accuracy class I (Sartorius, Germany) with a weighing resolution of 10 μg and an expanded uncertainty of 80 μg. The mass of the crucible and the weight of the crucible with a sample were obtained in ten parallel measurements. The sample mass was determined by the mass differences. The establishment of certified values of the specific enthalpy of phase transformations of new GSO was carried out by direct measurements on the National primary special standard GET 67-2013 for the units of specific enthalpy and specific heat of solids at temperatures in the 700–1800 K range.[5] GET 67-2013 is an isothermal mixing calorimeter designed to measure specific enthalpy and specific heat, as well as to study phase transformations in solid and liquid materials in a wide temperature range (from room temperature to 1800 K).

A description of the GET 67-2013 design and methods for measuring the specific enthalpy and specific heat capacity are given in [17]. The theory of calorimetric measurements by the mixing method is considered in detail in [18]. The derivation of the equation for measuring the specific enthalpy of phase transformations using an isothermal mixing calorimeter is described in [19].

Research Methods and Procedure

The establishment of certified values of the temperature of phase transformations of new GSO was performed using a certified measurement method FR.1.31.2021.40481.[6] The indicators of the measurement method are presented in Table 5.

The establishment of certified values of the specific enthalpy of phase transformations of new GSO was performed in accordance with the operating manual and technical documentation for GET 67-2013.

To reduce the impact of factors affecting the accuracy, the measurements were carried out in accordance with the following procedure:

- in accordance with the requirements of ASTM E 967, high-purity Al_2O_3 crucibles were used for metal measurements in order to avoid interaction of the test material with the crucible material. High-purity metal salts were measured in platinum crucibles. Closed crucibles were used to reduce the influence of sample color on its heating rate, as well as a more uniform heat distribution;

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[5] GET 67-2013 State primary special standard of units of specific enthalpy and specific heat capacity of solids in the temperature range from 700 to 1800 K: UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397945. Accessed 10 October 2022 (In Russ.).

[6] FR.1.31.2021.40481 Method for Measuring Phase Transition Temperatures of High-Purity Metals and Inorganic Compounds by Differential Scanning Calorimetry:: UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/1391509. Accessed 10 October 2022 (In Russ.).

Table 5 Measurement ranges, values for accuracy, correctness, repeatability, intralaboratory precision of the combined standard and expanded uncertainty

Measurement range, °C	Repeatability, °C	Intralaboratory precision, °C	Correctness, °C	Accuracy, °C	Combined standard uncertainty, °C	Absolute expanded uncertainty, °C $P = 0.95$ and $k = 2$
From 28 to 200 incl	0.01	0.05	0.06	0.11	0.055	0.11
From 200 to 400 incl	0.01	0.05	0.12	0.17	0.085	0.17
From 400 to 700 incl	0.02	0.07	0.26	0.33	0.165	0.33
From 700 to 1600 incl	0.02	0.07	0.60	0.67	0.335	0.67

- it is recommended to use a gas with low thermal conductivity, therefore, the measurements were carried out in N_2 atmosphere with a purity of 99.99% at a flow rate of 40 ml/min;
- the measurements were carried out using a DSC/TG sample holder with an S-type thermocouple and a sensitivity of 1.2 μV/mW;
- good contact between the material and the bottom of the crucible is essential to increase the heat transfer and, therefore, the measurement accuracy. In this regard, the starting material was placed in the crucible so as to completely cover the bottom for the case of measuring samples of metal salts; then the compaction was carried out using special equipment, which is a common technique in sample preparation [20].
- samples weighing no more than 20 mg were used to reduce the effect of sample weight on the measurement result;
- the thermal analyzer was calibrated using GSO 2312-82/2316-82 and GSO 1363-78 (set SOTSF), the accuracy indicators of which correspond to the working standards of the 1st category according to the state temperature verification scheme (GOST 8.558-2009 [21]);
- a heating rate of 10 °C/min was used during the study of RMs, since high heating rates lead to a shift in heat effects to higher temperatures. This heating rate value is recommended for use in verification methods for TA MI.

Results and Discussion

The DSC curves for some materials obtained in determining the certified values of the phase transition temperature of CRMs are shown in Figs. 2, 3, 4 and 5.

The DSC curves presented in Figs. 2, 3, 4 and 5 were processed in accordance with the requirements of the MP; two or three sample weights were used for each material.

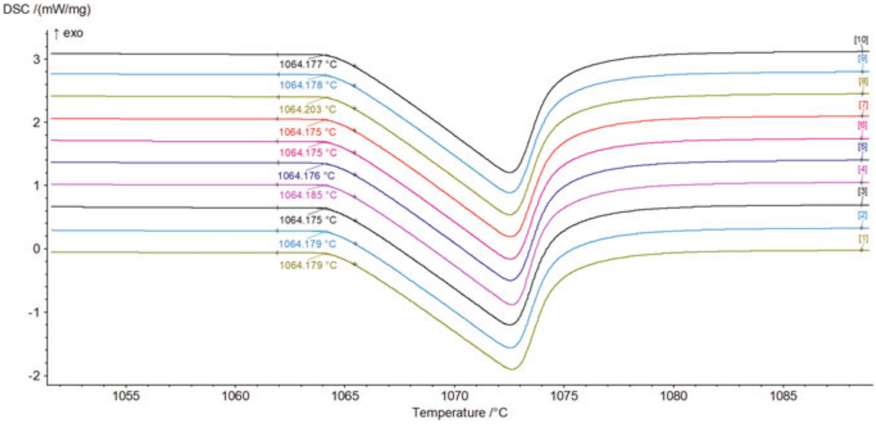

Fig. 2 DSC curves of gold samples (Au)

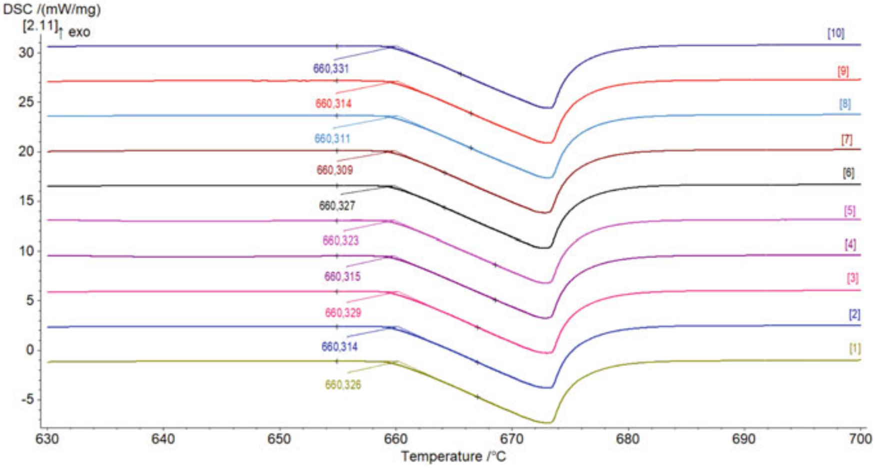

Fig. 3 DSC curves of aluminum samples (Al)

It follows from the obtained data that the standard deviation of the measurement results does not exceed 0.01 °C, which meets the requirements of the MP in the entire temperature range.

When determining the certified value of RMs in accordance with GOST ISO Guide 35-2015 [22], studies were carried out, and the contributions to the uncertainty due to heterogeneity of the starting materials, short- and long-term stabilities of materials were estimated.

The obtained values of the metrological characteristics are as follows: the phase transition temperature (T_{PhT}) and specific enthalpy of phase transition (H_{PhT}) of the investigated batch of CRMs are presented in Table 6.

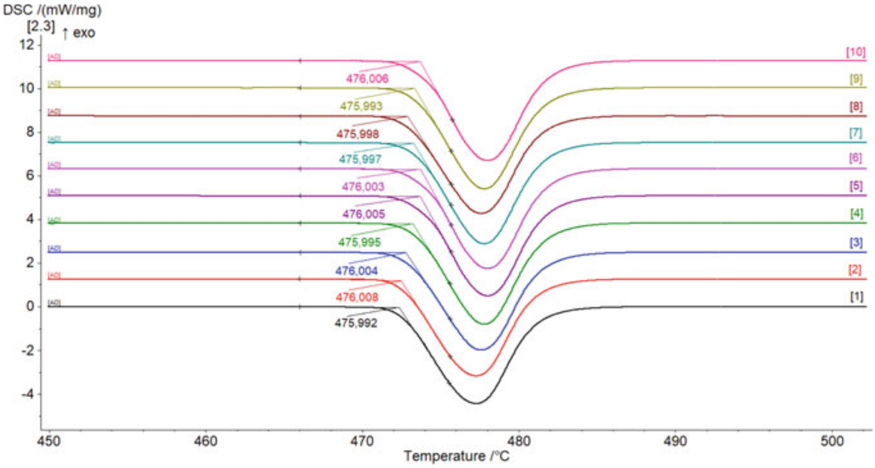

Fig. 4 DSC curves of caesium chloride samples (CsCl)

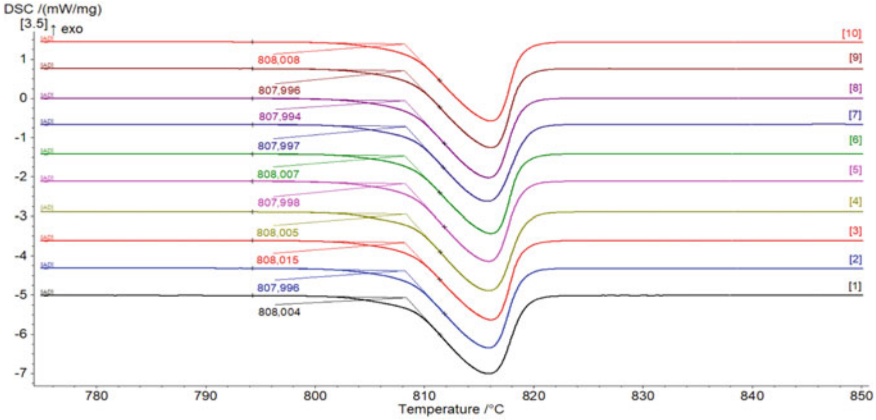

Fig. 5 DSC curves of barium carbonate samples ($BaCO_3$)

It should be noted that the melting temperatures of metals coincide within the error of the measurement method used by us with fixed points of the ITS-90 [23]. The H_{PhT} values for metals are close to the data given in [24], as well as in a number of other sources [2, 8, 12, 15]. Similarly the values of the phase transition temperature are consistent with our results better than the values of the enthalpy of phase transformations [25] for metal salts.

The existing differences can be primarily due to differences in the chemical composition of micro-impurities in the composition of the substances researched in different studies, as well as some differences in sample preparation and

Table 6 Metrological characteristics of the certified reference material[7]

GSO number in the set	Index	Certified characteristic	Certified value	Limits of the absolute error at $P = 0.95$	Absolute expanded uncertainty at $P = 0.95$ and $k = 2$
GSO 11890-2022	SOTSF-2-Ag_2SO_4	T_{PhT}, K	699.55	± 0.4	± 0.4
		H_{PhT}, J/g	51.9	± 0.24	± 0.24
GSO 11891-2022	SOTSF-2-CsCl	T_{PhT}, K	749.15	± 0.4	± 0.4
		H_{PhT}, J/g	17.2	± 0.12	± 0.12
GSO 11892-2022	SOTSF-2-$BaCO_3$	T_{PhT}, K	1081.15	± 1.2	± 1.2
		H_{PhT}, J/g	94.9	± 0.45	± 0.45
GSO 11893-2022	SOTSF-2-Bi	T_{PhT}, K	544.55	± 0.2	± 0.2
		H_{PhT}, J/g	53.1	± 0.2	± 0.2
GSO 11894-2022	SOTSF-2-Al	T_{PhT}, K	933.47	± 1.0	± 1.0
		H_{PhT}, J/g	397.0	± 2.0	± 2.0
GSO 11895-2022	SOTSF-2-Ag	T_{PhT}, K	1234.93	± 1.3	± 1.3
		H_{PhT}, J/g	104.6	± 0.5	± 0.5
GSO 11896-2022	SOTSF-2-Au	T_{PhT}, K	1337.33	± 1.4	± 1.4
		H_{PhT}, J/g	63.7	± 0.25	± 0.25

measurements, which is completely difficult to avoid even with the most careful implementation of the GTEFTA and ICTAC recommendations [15].

Taking into account the obtained results for other materials, the correctness of the chosen method and experimental conditions, it can be concluded that the obtained values are representative.

The traceability of the certified values of the phase transition temperature is ensured to the temperature unit (°C) reproduced by the State Primary Standard of temperature unit in the range from 0 to 3200 °C GET 34-2020,[8] and provided through the use of reference materials of In, Sn, Zn, Al, Ag, and Au, which are the fixed points of the International Temperature Scale (ITS–90). The procedure described above can be recommended for determining the characteristics of other high-purity metals and their salts, as well as for certification of RMs based on them.

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[7] GSO 11890-2022/11896-2022 Reference materials of the temperature and specific enthalpy of phase transitions of metals and metal salts (SET RM SOTSF-2). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1398408. Accessed 10 October 2022 (In Russ.).

[8] GET 34-2020 State primary standard of temperature unit in the range from 0 to 3200 °C: D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1385580. Accessed 10 October 2022 (In Russ.).

Conclusion

The purpose of this work was the development and certification of new certified reference materials for phase transition temperature based on high-purity metals and metal salts traceable to SI unit “temperature” °C for thermal analysis measuring instruments, primarily for DSC/DTA instruments.

In the course of experimental studies by differential scanning calorimetry using the thermal analyzer STA 449 F5 Jupiter from GET 173-2017, CRM certified values were determined. The procedure was carried out in accordance with GOST ISO Guide 35-2015; the contributions to the uncertainty due to heterogeneity of the starting materials were evaluated; the short- and long-term stabilities of the materials were studied.

The obtained metrological characteristics of the investigated batch of CRMs are as follows: the phase transition temperature (T_{PhT}) and specific enthalpy of phase transition (H_{PhT}) of the investigated batch of CRMs. The certified T_{PhT} values for the developed CRMs based on high-purity metals (Bi, Al, Ag, Au) are consistent with the temperatures of the ITS-90 fixed points with an accuracy of no worse than ± 0.01 °C. The certified H_{PhT} values for CRMs based on high-purity Bi, Al, Ag, Au are consistent with the results given in [2, 10, 12, 13, 15, 25] in the range from 0.3 to 1.3%.

Similarly, there is also a fairly good agreement between the T_{PhT} and H_{PhT} certified values and the reference data for CRMs based on high-purity metal salts (Ag_2SO_4, CsCl, $BaCO_3$). For example, the obtained T_{PhT} value for $BaCO_3$ practically coincides, and the H_{PhT} value differs by 2.7% from the results obtained in [25].

The developed CRMs passed the metrological examination and were included in the Register of approved types of reference materials FIF EUM[9] as a set of certified reference materials for temperature and specific enthalpy of phase transitions (set SOTSF-2) GSO 11890-2022/GSO 11896-2022.

The practical significance of the obtained results is as follows: certified reference materials allow expanding the possibility of establishing and monitoring the stability of the calibration characteristics of thermal analysis installations and measuring instruments; certification of measurement procedures (methods) and accuracy control of the measurement results of the phase transition temperature of metals, metal salts, metal oxides, and polymeric materials, organic and inorganic substances.

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Contribution of the Authors Nepomiluev A. M.—development of the research concept, conducting research work;

Shipitsyn A. P.—development of a methodology/procedure, implementation of a formal analysis, writing a draft version of the article, conducting research work;

Tyurnina A. E.—manuscript revision and editing.

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[9] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19. Accessed 10 October 2022 (In Russ.).

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Development of Reference Materials for AC Magnetic Properties of Cold-Rolled Non-oriented Electrical Steel

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Abstract Electrical steel is a soft magnetic material used in the manufacture of various electrical equipment intended for the transmission and conversion of electricity and used mainly in alternating magnetic fields of industrial frequencies. The key properties of such a material are AC magnetic properties, which are standardized when assessing the compliance of the material with the requirements of both Russian and foreign standards. The article presents information about the aspects of the development of reference materials for AC magnetic properties of electrical cold-rolled non-oriented steel made in the form of strips for the Epstein frame. The main feature of these reference materials is that the value of one of the two certified characteristics (specific power of magnetic losses) is transmitted directly from the State primary standard, and the other (magnetic flux density amplitude) is determined in accordance with a certified measurement procedure. Their use will provide the possibility of a comprehensive and reliable determination of the magnetic characteristics of samples of non-oriented electrical steel normalized in accordance with the requirements of current standards.

Keywords Soft magnetic materials · Non-oriented electrical steel · Epstein frame · AC magnetic properties · Specific power of magnetic losses · Magnetic flux density · Reference materials

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Abbreviations

GOST	National standard
GSO	Formal name of certified reference material in Russia
GET	State primary standard
SMM	Soft magnetic material
RM	Reference material

Introduction

Electrical steel is a soft magnetic material (hereinafter referred to as SMM) widely used in industry for the manufacture of generators, transformers, various electric motors, and other electrical equipment intended for the transmission and conversion of electricity. The most important properties of SMMs that determine their consumer appeal are AC magnetic properties—the specific power of magnetic losses for given values of the magnetic flux density amplitude and magnetization reversal frequency, as well as the magnetic flux density amplitude for given values of the amplitude of the magnetic field strength and magnetization reversal frequency [1–7]. Both of these quantities determine the quality of the material and are standardized in the standards regulating the technical requirements for materials. Specifications for rolled products made from electrical steel in the Russian Federation are presented in the standards of technical requirements GOST 21427.2-83, GOST 32482-2013, GOST 33212-2014 [8–10].

The volume of generation and, accordingly, consumption of electricity was 1.131 trillion kWh in Russia in 2021. Reducing energy losses in the production, transmission, and consumption of electrical energy, at least in the amount of fractions of a percent, can save billions of kWh of electricity. This can be achieved by improving the quality of manufactured SMMs through the use of new technological processes (for example, a new complex for laser machining of transformer electrical steel with a capacity of 54 thousand tons per year is being prepared for launch at the Novolipetsk Iron and Steel Works). Another way to reduce losses is to increase the accuracy of quality control of the AC magnetic characteristics of SMMs.

Traditionally, measurements of AC magnetic characteristics are carried out on samples made in the form of rings, sheets, or sets of strips for the Epstein frame. The general principle is to pass an alternating current of a given value through the primary (magnetizing) winding and measure the voltage from the secondary (measuring) winding. The sample material serves as a magneto conductor. In this case, the given value of the magnetizing current determines the magnetic field strength in the sample, and the measured voltage is proportional to the magnetic flux density that occurs in the material under study during its magnetization reversal. Windings are applied directly to the sample when measuring ring-shaped samples, and special magnetizing devices are used to measure the characteristics of strip and sheet samples. The method of measuring stripe-shaped samples in the Epstein frame has received the widest application for determining the properties of SMMs and, as a rule, is indicated in regulatory documents as a referee method when establishing the characteristics of the material under test.

The FIF EUM[1] analysis revealed that at the moment in Russia there are several certified reference materials (hereinafter referred to as CRMs) for AC magnetic properties made of anisotropic electrical steel (for example, made in the form of rings (GSO 10270-2013[2]), sheets (GSO 2129-89[3]), and strips for the Epstein frame (GSO 10271-2013[4])). The main certifiable characteristic for these CRMs is the value of the specific power of magnetic losses reproduced by the State primary standard of units of power of magnetic losses, magnetic flux density of a constant magnetic field in the range from 0.1 to 2.5 T, and magnetic flux in the range from $1 \cdot 10^{-5}$ to $3 \cdot 10^{-2}$ Wb GET 198-2017 [11]. In accordance with the State verification scheme for instruments for measuring the power of magnetic losses of magnetically soft materials and the magnetic characteristics of magnetically hard materials,[5] the value of specific magnetic losses is transmitted directly from GET 198-2017 to the CRM.

The relevance of the development of a new type of RMs for AC magnetic properties of electrical cold-rolled non-oriented steel made in the form of strips for the Epstein frame is due to the fact that they can be used for metrological support of instruments for measuring the magnetic properties of SMMs simultaneously for both AC magnetic characteristics (specific power of magnetic losses and amplitude of magnetic flux density).

Thus, the aim of this study was to develop RMs that would be able to provide a complete and reliable determination of the magnetic characteristics of samples of non-oriented electrical steel in accordance with the requirements for these characteristics, while ensuring sufficient stability of the magnetic properties over time.

The objectives of the study were analysis and consideration of the characteristic features of the material, determination of the requirements for the preparation of

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via. https://fgis.gost.ru/fundmetrology. Accessed 4 August 2022 (In Russ.).

[2] GSO 10270-2013 Reference materials of dynamic magnetic properties of electrical cold-rolled anisotropic steel. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/389912. Accessed 4 August 2022 (In Russ.).

[3] GSO 2129-89 Reference materials of specific magnetic losses (electrical steel, cold-rolled anisotropic) set SOTESL. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/393998. Accessed 4 August 2022 (In Russ.).

[4] GSO 10271-2013 Reference materials of dynamic magnetic properties of electrical cold-rolled anisotropic steel (sotes). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/389911. Accessed 4 August 2022 (In Russ.).

[5] The state verification scheme for instruments for measuring the power of magnetic losses of magnetically soft materials and the magnetic characteristics of magnetically hard materials was approved by order of Rosstandart dated December 29, 2018 No. 2816 [12]

the material and the geometric dimensions of the samples, the production of an experimental batch of RMs, and the establishment of metrological characteristics of RMs.

Materials and Methods

One of the main criteria in the development of new types of RMs is to ensure the traceability of certified values to the standards of measurement units. Therefore, an important feature of the material for the type of RMs being developed is the ability to characterize non-oriented electrical steel by two magnetic quantities at once during certification of RMs.

It was decided that the transfer of the unit size of the specific power of magnetic losses will be carried out by direct measurements on GET 198-2017.[6] Samples of electrical cold-rolled non-oriented steel made in the form of strips for the Epstein frame were selected as candidate RMs. The steel is made according to GOST 21427.2-83 [8].

A method for measuring magnetic flux density in alternating magnetic fields of SMM samples by the induction method M.261.0059/RA.RU.311866/2022[7] was developed and certified to determine certified RM values by the magnetic flux density amplitude value. The measurement procedure is based on the induction method with analog-to-digital conversion of instantaneous voltage values on the measuring winding of the sample (magnetizing device) and voltage on the secondary winding of the mutual inductance coil, the primary winding of which is connected in series with the primary winding of the sample (magnetizing device), into digital codes with subsequent calculation of the magnetic characteristics.

Determination of Metrological Characteristics

The approaches outlined in GOST 34100.3-2017/ISO/IEC Guide 98-3:2008 [13] were used to assess the uncertainty of the measurement results of the magnetic flux density amplitude. The standard uncertainty of type A, u_A, T, of the magnetic flux density amplitude measurements was calculated as the standard deviation of the mean value:

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[6] GET 198-2017 State primary standard of units of power of magnetic losses, magnetic induction of a constant magnetic field in the range from 0.1 to 2.5 T and magnetic flux in the range from 1 10^{-5} to 3 10^{-2} Wb. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397884. Accessed 4 August 2022 (In Russ.)].

[7] M.261.0059/RA.RU.311866/2022 Method for measuring magnetic induction in alternating magnetic fields of samples of magnetically soft materials by the induction method. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/1400432. Accessed 4 August 2022 (In Russ.).

$$u_{Aj} = \sqrt{\frac{1}{n(n-1)} \sum_{i=1}^{n} \left(X_{ij} - \overline{X}_j\right)^2}, \tag{1}$$

where

X_{ij} is the result of determining the *j*-th magnetic characteristic, T;
$\overline{X}_j$ is the arithmetic mean of the measurement results of the magnetic flux density amplitude under the *j*-th measurement conditions (set value of the magnetization reversal frequency and amplitude of the magnetic field strength), T;
n is the number of measurements on the sample of the magnetic flux density amplitude under the *j*-th measurement conditions (set value of the magnetization reversal frequency and the amplitude of the magnetic field strength).

The standard uncertainty of type B, u_B, T, was estimated as a composition of uncertainty components due to the uncertainty of the results of the measuring instruments used and the influence of the measurement method used.

The standard uncertainty of type B of the measurement result of the magnetic flux density amplitude was calculated by the formula:

$$u_B = \sqrt{\sum_{z=1}^{m} \left(\frac{\partial f}{\partial x_z}\right)^2 \cdot u^2(x_z)} = \sqrt{\sum_{z=1}^{m} c^2(x_z) \cdot u^2(x_z)}, \tag{2}$$

where

x_z is an estimate of the *z*-th input quantity contributing to the type B standard uncertainty;
$u(x_z)$ is the standard uncertainty of estimating the *z*-th input quantity;
$c(x_z)$ is a sensitivity coefficient describing the change in the output quantity when the *z*-th input quantity changes.

The sensitivity coefficients $c(x_z)$ for each input quantity x_z contributing to u_B were determined as partial derivatives of the measurement model. Experimental measurement data of SMM samples were used to calculate the numerical values of the sensitivity coefficients.

The total standard uncertainty of the certified RM values was calculated using the formula:

$$u_C = \sqrt{u_{char}^2 + u_{stab}^2}, \tag{3}$$

where

u_{char} is the relative standard uncertainty from characterization, %;
u_{stab} is the relative standard uncertainty due to instability, %.

The expanded uncertainty was calculated using the formula:

$$U = k \cdot u_C, \tag{4}$$

where k is the coverage factor, $k = 2$ for $P = 0.95$.

Within- and between-bottle heterogeneity of RMs was not studied, since RM certification is carried out individually, while the certified value is assigned to the entire sample.

The equation for measuring the magnetic flux density amplitude B_m, T, for a given value of the magnetization reversal frequency and the magnetic field strength amplitude:

$$B_m = \frac{U_{avg}}{4fSW_2} + \Delta(B_m)\Delta H_m, \tag{5}$$

where

$\Delta(B_m)\Delta H_m$ is the uncertainty component of the variation of the magnetic flux density amplitude due to the uncertainty in establishing the amplitude of the magnetic field strength, T;

U_{avg} is the average value of the voltage on the secondary winding of the sample or magnetizing device, V;

f is the magnetization reversal frequency, Hz;

S is the cross-sectional area of the sample determined by the shape and geometric dimensions of the sample, m^2;

W_2 is the number of turns of the secondary (measuring) winding of the sample or magnetizing device.

Type B standard uncertainty was calculated using the formula:

$$u_B = \sqrt{\left(\frac{u\left(U_{avg}\right)}{4fSW_2}\right)^2 + \left(\frac{u(f)U_{avg}}{4f^2SW_2}\right)^2 + \left(\frac{u(S)U_{avg}}{4fS^2W_2}\right)^2 + \left(\frac{\partial B_m}{\partial H_m}u(H_m)\right)^2}, \tag{6}$$

where

$u\left(U_{avg}\right)$ is the standard measurement uncertainty of the sinusoidal alternating current voltage on the secondary winding, V;

$u(f)$ is the standard uncertainty of establishing the magnetization reversal frequency, Hz;

$u(S)$ is the standard uncertainty in determining the sample cross-sectional area, m^2;

$u(H_m)$ is the uncertainty of establishing the set value H_m determined by the formula:

$$u(H_m) = \sqrt{\left(\frac{u(U_{avgM})W_1}{4fMl_{avg}}\right)^2 + \left(\frac{U_{avgM}W_1u(f)}{4f^2Ml_{avg}}\right)^2 + \left(\frac{U_{avgM}W_1u(M)}{4fM^2l_{avg}}\right)^2}, \quad (7)$$

where

$u(U_{avg\ M})$ is the standard uncertainty of measuring the AC voltage on the primary winding of the mutual inductance coil corresponding to the given value H_m, V;
$u(M)$ is the uncertainty in determining mutual inductance of the mutual inductance coil determined during calibration, H;
$u(f)$ is the standard uncertainty of establishing the magnetization reversal frequency, Hz;
l_{avg} is the average magnetic path length determined by the shape and dimensions of the sample, m;
$\frac{\partial B_m}{\partial H_m}$ is the variation of the magnetic flux density amplitude with a variation in the magnetic field strength amplitude of the sample near the measured value B_m, T A^{-1} m, determined from the experimentally determined dynamic curves of magnetization of samples of various types of SMMs.

In this case, the uncertainties of the input values (frequency, voltage on the primary winding, voltage on the secondary winding, mutual inductance coefficient of the mutual inductance coils, geometric dimensions of the sample) were determined from the calibration results of the measuring instruments used. An example of the uncertainty budget of the measurement result of the magnetic flux density amplitude of a strip sample at a set magnetization reversal frequency $f = 400$ Hz and a magnetic field strength amplitude $H_m = 40$ A/m is given in Table 1.

When assessing the uncertainty of the measurement results of the specific power of magnetic losses obtained by direct measurements on GET 198-2017, we were guided by the method for calculating the uncertainty in reproducing units of magnetic loss power MRN 02-GET 198-2016. The method establishes the procedure for calculating the uncertainty of reproduction of power units and specific power of magnetic losses on the State primary standard. The standard implements an induction measurement method with analog-to-digital conversion of instantaneous voltage values on the measuring winding of the sample and voltage on the standard reference resistor in the magnetizing circuit into digital codes stored in memory and used to calculate the magnetic characteristics.

The stability study of the properties of the candidate RMs was carried out according to the algorithms given in GOST ISO Guide 35-2015 [14] using the measurement results obtained in the period from 2018 to 2022. Five samples of different strip thicknesses with values of magnetic characteristics in all ranges of permissible values of the certified characteristics were selected for study.

Table 1 Uncertainty budget of the measurement result of the magnetic flux density amplitude of a strip sample at $f = 400$ Hz, $H_m = 40$ A/m

Type	Source	Value estimation	Estimation of the standard uncertainty		Sensitivity coefficient		Probability distribution	Degrees of freedom, v_j	Contribution to uncertainty	
			u_j	Unit	$c_j = \frac{\partial f}{\partial x_j}$	Unit			$c_j u_j$	Unit
A	u_A	0.8052	$0.63{\cdot}10^{-3}$	T	1	-	N	9	$0.63{\cdot}10^{-3}$	T
B	$U_{avg\,M}$	0.8594	$0.43{\cdot}10^{-3}$	V	46.542	$\frac{\mathrm{A/m}}{\mathrm{V}}$	R	∞	$0.20{\cdot}10^{-2}$	A/m
B	f	400	$1{\cdot}10^{-6}$	Hz	0.750	$\frac{\mathrm{A/m}}{\mathrm{Hz}}$	R	∞	$0.75{\cdot}10^{-6}$	A/m
B	M	0.01	$2.5{\cdot}10^{-6}$	H	4131.7	$\frac{\mathrm{A/m}}{\mathrm{H}}$	R	∞	$1.03{\cdot}10^{-2}$	A/m
B	H_m	40	$1.05{\cdot}10^{-2}$	A/m	0.01525	$\frac{\mathrm{T}}{\mathrm{A/m}}$	R	∞	$0.16{\cdot}10^{-3}$	T
B	S	$0.593{\cdot}10^{-4}$	$0.12{\cdot}10^{-7}$	m^2	$12.94{\cdot}10^{4}$	$\frac{\mathrm{T}}{\mathrm{m}^2}$	R	∞	$1.54{\cdot}10^{-3}$	T
B	U_{avg}	53.487	$0.449{\cdot}10^{-2}$	V	0.1230	$\frac{\mathrm{T}}{\mathrm{V}}$	R	∞	$0.55{\cdot}10^{-3}$	T
Type A standard uncertainty, u_A									$0.63{\cdot}10^{-3}$	T
Type B standard uncertainty, u_B									$1.64{\cdot}10^{-3}$	T
Total standard uncertainty, u_C									$1.76{\cdot}10^{-3}$	T
Expanded uncertainty, U ($k = 2$, $P = 0.95$)									$3.52{\cdot}10^{-3}$	T
Relative value of expanded uncertainty, $\tilde{U}$									0.44	%

Results and Discussion

The metrological characteristics given in Table 2 were obtained based on the results of the studies carried out for the developed type of RMs.

The magnitude of the error (uncertainty) depends on the magnetic field strength and magnetization reversal frequency. The values of the certified characteristics can be determined in the magnetization reversal frequency range from 50 to 5000 Hz. The expected frequency of re-determination of metrological characteristics is two years.

In addition, a number of requirements are established for the geometric dimensions of the strips: length from 280 to 350 mm, width (30 ± 0.2) mm, thickness from 0.1 to 0.5 mm. The strips should not differ in length by more than ± 0.5 mm. The cross-sectional area of the sample should be in the range from $0.5 \bullet 10^{-4}$ to $1.5 \bullet 10^{-4}$ m^2.

Strips after cutting should be annealed to relieve mechanical stress. Strips after annealing should be subjected to aging to stabilize the magnetic properties. The number of strips in the sample should be a multiple of four; one half of the strips is cut along the rolling direction (the limiting angle between the cutting and rolling direction is no more than ± 5°), the other half is cut across the rolling direction.

The obtained results make it possible to include in the purpose of this type of RMs their use for certification of measurement procedures for calibration and verification of measuring instruments, as well as for monitoring the metrological characteristics of measuring instruments during type approval tests.

Table 2 Metrological characteristics of the developed RMs

Certified characteristics		Result
Specific power of magnetic losses	Range of permissible values of the certified characteristics	From 0.1 to 100.0 W/kg
	Permissible values of relative expanded uncertainty	From 0.3 to 1.5%
	Limits of permissible values of relative error	From ± 0.3 to ± 1.5%
Magnetic flux density amplitude	Range of permissible values of the certified characteristics	From 0.01 to 2.0 T
	Permissible values of relative expanded uncertainty	From 0.5 to 1.5%
	Limits of permissible values of relative error	From ± 0.5 to ± 1.5%

Conclusion

In the course of the study, an approach to the creation of RMs for the metrological support of AC magnetic properties of electrical cold-rolled non-oriented steel made in the form of strips for the Epstein frame was proposed and tested. Based on the results of the study, the authors developed practical recommendations for the geometric dimensions of the samples and the process of preparing the RM material. It was noted that the magnitude of the error (uncertainty) directly depends on the magnitude of the magnetic field strength and magnetization reversal frequency.

The metrological characteristics of the investigated RM type were established:

- the specific power of magnetic losses in the range from 0.1 to 100.0 W/kg with permissible values of relative expanded uncertainty from 0.3 to 1.5% and limits of permissible values of relative error from ± 0.3 to ± 1.5%;
- the magnetic flux density amplitude in the range from 0.01 to 2.0 T with the permissible values of the relative expanded uncertainty from 0.5 to 1.5% and the limits of the permissible values of the relative error from ± 0.5 to ± 1.5%.

A pilot batch of RMs was manufactured. The main feature of RMs is that the value of one of the two certified characteristics (specific power of magnetic losses) is transmitted directly from GET 198-2017, and the other (magnetic flux density amplitude) is determined in accordance with the certified measurement procedure.

The achieved results are of practical importance, because the developed RMs can provide the possibility of a complete and reliable determination of the magnetic characteristics of samples of non-oriented electrical steel in accordance with the requirements of the current standards.

Further research will focus on the development of RMs for AC magnetic properties made in the form of rings and sheets and manufactured for the needs of a wider range of consumers and manufacturers of various SMMs.

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Certified Reference Materials for the Phase Transition Temperature of Organic Substances Based on Anhydrous Sodium Acetate and Sodium Methansulfonate

Artyom P. Shipitsyn, Andrei M. Nepomiluev, and Anastasiya E. Tyurnina

Abstract The requirements for thermal analysis instruments are increasing in terms of extending the measurement ranges and improving their accuracy with scientific and technological progress in the field of metrological supervision, chemical, and pharmaceutical industries. The study of determining the melting point of organic substances is of particular relevance for the metrological support of the applied thermal analysis instruments and traceability to the base physical units. The purpose of the research was to test the possibility of using organic substances based on sodium acetate and sodium methanesulfonate as phase transition temperature standards for candidate materials to certified reference materials (CRMs) for the phase transition temperature traceable to the SI unit "temperature." The procedure for measuring the phase transition temperature (melting point) was performed by differential scanning calorimetry using the thermal analyzer STA 449 F5 JUPITER from the State Primary Standard GET 173-2017. The determination of the CRM certified value was carried out in accordance with GOST ISO Guide 35-2015; the contributions to the uncertainty due to heterogeneity of the starting materials were evaluated; the short- and long-term stabilities of the materials were studied.

Keywords Reference material · Thermal analysis · Phase transition temperature · Melting point · Anhydrous sodium acetate · Sodium methanesulfonate · Measurement procedure · Calibration

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Abbreviations

TA	Thermal analysis
DSC	Differential scanning calorimetry
FIF EUM	Federal information fund for ensuring the uniformity of measurements
CRM	Certified reference material
RTD	Resistance temperature detector

Introduction

TA is a popular type of measurement in the pharmaceutical, chemical, and other industries [1]. In particular, one of the most effective TA methods is a DSC method [2]. In addition to its wide application for determining the temperature and specific melting enthalpy of organic substances, the DSC method is used to study polymers [3–5] and inorganic substances [6]. Melting point measuring instruments are used by research and testing laboratories to determine and control the melting, dropping, and softening points used in the development and production of a wide range of materials. The obtained values of thermophysical quantities are important for choosing the optimal temperature of technological processes, determining the best storage conditions for materials, their operating temperature during operation, as well as for testing cosmetic products, fats, and substances with a waxy consistency. The requirements for TA instruments are constantly increasing; this is related to their indispensability in solving most scientific, engineering, and manufacturing problems [7]. In particular, this applies to various types of phase transition temperature analyzers, including melting, boiling, and various intermediate states of substances and materials. The analysis of the data in the FIF EUM[1] revealed that currently more than 10 types of analyzers of such companies as Mettler-Toledo GmbH, Bibby Scientific Limited, BUCHI Labortechnik AG, etc. are registered in the Russian Federation.

The need to create certified reference materials for the phase transition temperature of organic substances is related to the fact that some types of analyzers (for example, reg. No. 59055-14 and reg. No. 67807-17 in the FIF EUM) with an error in the measurement range from 180 to 400 °C was not standardized due to the lack of relevant certified reference materials (GSO). Metrological support was provided by melting point reference materials based on benzophenone (melting point ~ 48 °C) and benzoic acid (melting point ~ 122 °C). Metrological support for other analyzers (for example, reg. No. 41280-14, reg. No. 71833-18)[2] was carried out using the

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19. Accessed 10 October 2022 (In Russ.).

[2] FIF 59055-14 Instruments for measuring the melting point. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/372174. Accessed 10 October 2022 (In Russ.).

FIF 67807-17 Instruments for measuring the melting point. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/381782. Accessed 10 October 2022 (In Russ.).

CRM based on caffeine (melting point ~ 238 °C) in addition to benzophenone and benzoic acid, as well as by using a compact platinum resistance temperature detector (RTD). At the same time, metrological support using the CRM has a number of advantages compared to temperature measurement by comparison with the RTD (especially during calibration): ease of measurements, enhanced measurement rate, direct contact of the CRM with the measurement cell (the absence of temperature gradients associated with the placement of the RTD near the walls of the instrument cell).

Thus, the study of determining the temperature and specific enthalpy of melting of organic substances is of particular relevance for the metrological support of MI for measuring higher temperatures and traceability to the base physical units.

The purpose of the research is to test the use of organic substances based on anhydrous sodium acetate and sodium methanesulfonate as phase transition temperature standards for candidate materials to certified reference materials for the phase transition temperature traceable to the SI unit "temperature" (K (°C)).

Materials and Methods

Reagents

Anhydrous sodium acetate ($C_2H_3O_2Na$) and sodium methanesulfonate (CH_3NaO_3S) are a powdered substance with a mass fraction of the main component (98.0–98.5)%.

Substances with first-order phase transition (melting) temperatures closest to the upper limit of the measurement range of measuring instruments were selected as candidate materials to CRMs.

Equipment

The thermal analyzer STA 449 F5 JUPITER (NETZSCH, Germany) from the State Primary Standard of units of mass fraction, mass (molar) concentration of water in solid and liquid substances and materials GET 173-2017[3] implements the DSC method.

The mass of the starting material was measured on a laboratory balance of special accuracy class I (Sartorius, Germany) with a weighing resolution of 10 µg and an

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[3] GET 173-2017 State primary standard of units of mass fraction, mass (molar) concentration of water in solid and liquid substances and materials: UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397857. Accessed 10 October 2022 (In Russ.).

Table 1 Measurement ranges, values for accuracy, correctness, repeatability, intralaboratory precision, combined standard and expanded uncertainty

Measurement range, °C	Repeatability, °C	Intralaboratory precision, °C	Correctness, °C	Accuracy, °C	Combined standard uncertainty, °C	Absolute expanded uncertainty, °C $P = 0.95$ and k = 2
From 200 to 400 incl.	0.01	0.05	0.12	0.17	0.085	0.17

expanded uncertainty of 80 μg. The mass of the crucible and the weight of the crucible with a sample were obtained in ten parallel measurements. The sample mass was determined by the mass differences.

Research Methods and Procedure

The DSC method is widely used to determine the temperature and specific enthalpy of melting of organic substances [8]. The heat-accumulating materials are being intensely researched [9]. In particular, other thermophysical characteristics of organic substances are being studied [12–14] based on such organic compounds as myristic, palmitic, and stearic acids [10], esters and fatty acids [11]. In addition, this method is supported by the fact that materials based on benzophenone (GSO 11070-2018[4]) and benzoic acid (GSO 11071-2018[5]) were characterized by the same method.

Studies of CRMs were performed using the certified measurement procedure (MP) M.241.0036/RA.RU.311866/2022[6]; the MP measurements are presented in Table 1.

The impact of factors affecting the measurement accuracy has been minimized, namely:

- the crucible: manufacturers of thermal analysis instruments recommend the use of Pt crucibles for organic measurements to avoid interaction of the test material with crucible material in accordance with ASTM E 967-08 [15]. Closed crucibles were used for more even heat distribution in the sample;

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[4] GSO 11070-2018 Reference materials benzophenone melting point type-approved (CO C13H10O). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/593689. Accessed 10 October 2022 (In Russ.).

[5] GSO 11071-2018 Reference materials of the approved type of melting point of benzoic acid (CO $C_7H_6O_2$). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/394949. Accessed 10 October 2022 (In Russ.).

[6] M.241.0036/RA.RU.311866/2022 Method for measuring the phase transition temperatures of sodium methanesulfonate (CH_3NaO_3S) and anhydrous sodium acetate ($C_2H_3O_2Na$) by differential scanning calorimetry: UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/1399257. Accessed 10 October 2022 (In Russ.).

- the sample: good contact between the material and the bottom of the crucible is essential to increase the heat transfer and, therefore, the measurement accuracy. In this regard, flat-bottomed crucibles were used. The starting material was placed in the crucible so as to completely cover the bottom; then the compaction was carried out using special equipment, which is a common technique in sample preparation [14]. The mass of samples did not exceed 20 mg to reduce the "mass effect" and increase the signal-to-noise ratio > 10 [10, 12, 14] as specified in GOST R 57931-2017 [16];
- the atmosphere: it is necessary to use a gas with low thermal conductivity for precision measurements, therefore, measurements were carried out in N_2 atmosphere with a purity of 99.99% at a flow rate of 40 ml/min;
- the temperature calibration of MI: a thermal analyzer calibrated using GSO 2312-82/2316-82 CRMs for temperatures and phase-transition points (set SOTSF) was used. In particular, Ga, In, and Sn have accuracy indicators that correspond to working standards of the 1st category according to the second part of the State verification schedule for means measuring temperature (GOST 8.558-2009 [17]). This procedure is widely used by research organizations to calibrate instruments before precision measurements, in particular for In and Sn [12–14, 18], according to the recommendations of ASTM E 967-08;
- the sensitivity of measuring instruments: a DSC/TG sample holder (not DTA/TG) with a sensitivity of 1.2 μV/mW was applied to increase the sensitivity;
- the heating rate: in view of the fact that higher heating rates led to a shift in heat effects to higher temperatures, a heating mode at a rate of 1.0 °C/min was used during the study of CRMs. This value is indicated in the verification methods for MI (reg. No. 41280 and reg. No. 85498 in the FIF EUM[7]). An additional argument in favor of the selected rate was the fact that rates from 0.5 °C/min up to 2 °C/min are used worldwide in the analysis of similar materials [14, 18–20].

Thus, it can be argued that the establishment of certified values at different rates increases the complexity both for instrument users during calibration and for organizations verifying such instruments.

Results and Discussion

The experimental data in determining the certified values of CRMs are shown in Figs. 1 and 2.

The thermograms presented in Figs. 1 and 2 were processed in accordance with the MP. The obtained data show that the standard deviation of the measurement results does not exceed 0.01 °C, which meets the requirements of the MP.

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[7] FIF 41280-14 Melting point analyzers. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/351558. Accessed 10 October 2022 (In Russ.).

FIF 85498-22 Melting point analyzers. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/4/items/1399531. Accessed 10 October 2022 (In Russ.).

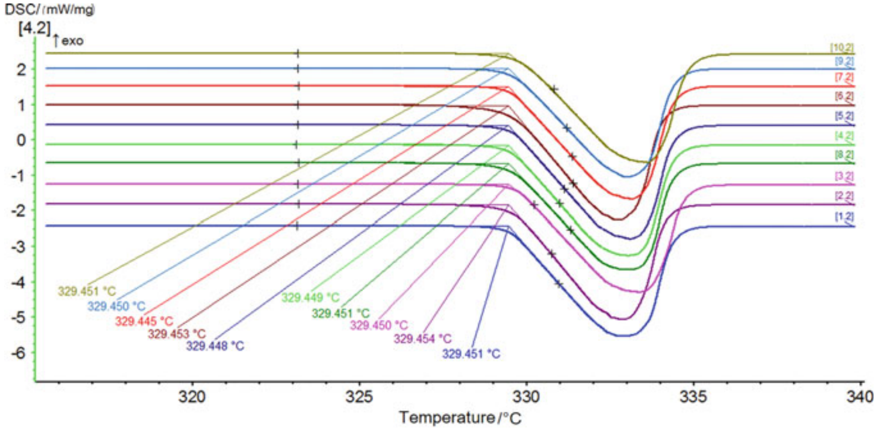

Fig. 1 Thermogram of anhydrous sodium acetate

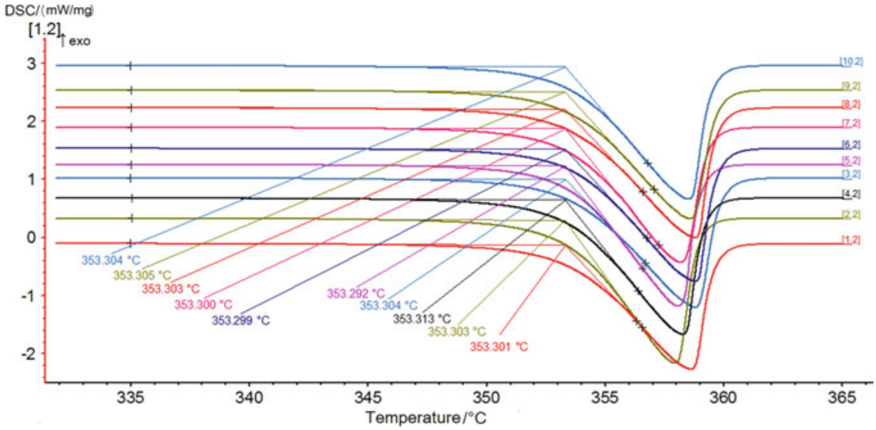

Fig. 2 Thermogram of sodium methanesulfonate

Taking into account the fact that many organic substances actively sublime at high temperatures [21], the mass loss of the studied materials was measured. The results for anhydrous sodium acetate are shown in Fig. 3.

Based on the data presented on the thermogram (Fig. 3), the mass loss during 6 h of heating, 2.5 h of which the CRM was at a temperature above the melting point, changed by 5%, which is 0.66 mg. The melting temperature value changed within 0.03 °C, which indicates an insignificant effect of the mass loss on the certified value of the CRM and a low sublimation rate.

The obtained metrological characteristics of the investigated batch of CRMs are presented in Table 2.

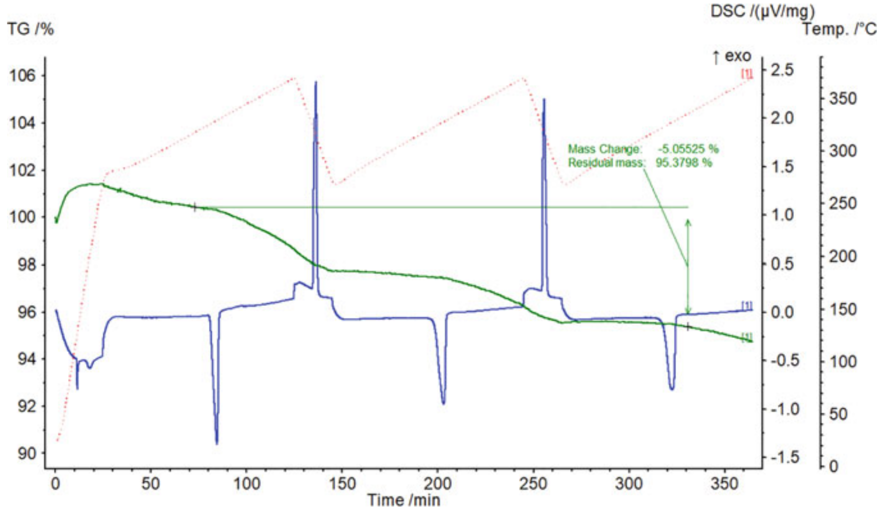

Fig. 3 The mass loss of anhydrous sodium acetate

Table 2 Metrological characteristics of certified reference materials for the phase transition temperature (melting point)

Certified characteristics of the phase transition temperature	Certified value*	Absolute error limits at $P = 0.95$	Absolute expanded uncertainty at $P = 0.95$ and k = 2
Melting point of anhydrous sodium acetate ($C_2H_3O_2Na$), °C	329.45	± 0.20	0.20
Melting point of sodium methanesulfonate (CH_3NaO_3S), °C	353.30	± 0.20	0.20

*The metrological characteristics of CRMs are given for the heating mode at a rate of 1.0 °C/min

The range of values indicated in the type specification is from 328.35 to 330.35 °C for anhydrous sodium acetate, from 352.05 to 354.05 °C for sodium methanesulfonate, which is due to the difference in the mass fraction of the main component from batch to batch during the subsequent certification of materials as certified reference materials.

The obtained certified values of the melting point of the CRM based on anhydrous sodium acetate are consistent within ± 1.4 °C with the reported reference values [22–24] as shown in Table 3.

Data on the study of sodium methanesulfonate with a similar chemical composition are not available. Taking into account the results obtained for anhydrous sodium acetate, and the correctness of the selected method and experimental conditions, we believe that the obtained values are representative.

Table 3 Comparison of the certified values of the CRM melting point of anhydrous sodium acetate with the reported reference values (the values are indicated in °C)

The certified value of UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology	[18]	[19]	[20]
329.45	329.62	328.10	328.20

The traceability of the certified values of the phase transition temperature of anhydrous sodium acetate and sodium methanesulfonate is provided to the SI unit "temperature" reproduced by GET 34-2020[8] by using certified reference materials (GSO 2312-82/2316-82) in phase transition temperature measurements.

The procedure described above can be applied for determining the thermophysical characteristics of similar substances, such as saccharin, vanillin, etc.

Conclusion

The purpose of this work was to test the results of research on the use of organic substances based on anhydrous sodium acetate and sodium methanesulfonate as phase transition temperature standards for the development of certified reference materials for the phase transition temperature traceable to the SI unit "temperature."

In the course of experimental studies, the possibility of using the procedure for measuring the phase transition temperature (melting point) by differential scanning calorimetry using the thermal analyzer STA 449 F5 JUPITER from GET 173-2017 was proved. The procedure was performed in accordance with GOST ISO Guide 35-2015 [25]. The contributions to the uncertainty due to heterogeneity of the starting materials were evaluated; the short- and long-term stabilities of the materials were studied, and the certified values of CRMs were determined.

The obtained metrological characteristics of the investigated batch of CRMs are as follows: the range of permissible certified characteristics of the melting point of the phase transition is (328.35–330.35) °C for anhydrous sodium acetate, (352.05–354.05) °C for sodium methanesulfonate. It was shown that the certified values of the melting point of the developed CRMs are consistent within ± 1.4 °C with the reference values presented in IUPAC.

The developed CRMs passed the metrological examination and were included in the Register of approved types of reference materials of the FIF EUM as a set of certified reference materials for the phase transition temperature (set of SS TPKR) GSO 11928-2022/GSO 11929-2022.[9]

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[8] GET 34-2020 State primary standard of temperature unit in the range from 0 to 3200 °C: D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1385580. Accessed 10 October 2022 (In Russ.).

[9] GSO 11928-2022/GSO 11929-2022 reference materials of phase transition temperatures (set of SS TPKR). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1399806. Accessed 10 October 2022 (In Russ.).

The theoretical significance of the obtained results lies in the proof of the possibility of applying differential scanning calorimetry for type approval tests to determine the thermophysical characteristics of organic substances based on anhydrous sodium acetate and sodium methanesulfonate, as well as similar substances, such as saccharin, vanillin, etc.

The practical significance of the obtained results lies in the possibility of establishing and monitoring the stability of the calibration dependence of thermal analysis measuring instruments; certification of measurement procedures (methods) and accuracy control of the measurement results of the phase transition temperature of metals, metal salts, metal oxides, polymeric materials, organic and inorganic substances.

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Author Contributions Shipitsyn A. P.—development of a methodology/procedure, implementation of a formal analysis, writing a draft version of the article, conducting research work; Nepomiluev A. M.—development of the research concept, conducting research work; Tyurnina A. E.—manuscript revision and editing.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Certified Reference Materials for the Phase Transition Temperature (Curie Temperature) Based on Alumel, Nickel, and Iron Silicide

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Abstract The requirements for thermal analysis instruments are increasing in terms of their metrological support with the development of scientific and technological progress in the field of metrological supervision and electronics industry. The study of determining the phase transition temperature, namely the Curie temperature, is of particular relevance for the metrological support of the applied thermal analysis instruments and traceability to the base physical units. The purpose of the research was to test the possibility of using materials based on alumel, nickel and iron silicide (trafoperm) as phase transition temperature standards for candidate materials to certified reference materials (CRMs) for the phase transition temperature traceable to the SI unit "temperature." The procedure for measuring the phase transition temperature (Curie temperature—T_C) was performed by the thermomagnetometric method using the thermal analyzer STA 449 F5 JUPITER from the State Primary Standard GET 173-2017. The determination of the CRM certified value was carried out in accordance with GOST ISO Guide 35-2015; the contributions to the uncertainty due to heterogeneity of the starting materials were evaluated; the short- and long-term stabilities of the materials were studied.

Keywords Reference material · Thermogravimetric analysis · Phase transition temperature · Curie temperature · Measurement procedure

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Abbreviations

TA	Thermal analysis
DSC	Differential scanning calorimetry
TGA	Thermo-gravimetric analysis
FIF EUM	Federal information fund for ensuring the uniformity of measurements
CRM	Certified reference material
TM	Thermomagnetometry

Introduction

Interest in the study of the thermophysical properties of substances and materials appeared already in the sixteenth century, and to date, numerous methods of thermal analysis (TA) remain an integral part of world metrology [1].

According to ASTM E 473-16 [2], thermogravimetric analysis (TGA) is a method where the mass of a substance is measured as a function of temperature or time, while the substance is subjected to a controlled temperature under a defined atmosphere. This method of analysis has found wide application in almost all areas of chemistry. Thermogravimetric analyzers or devices for simultaneous thermal analysis (STA) are used, which, in addition to the TGA method, implements differential scanning calorimetry (DSC). More than 20 types of analyzers of such companies as Mettler-Toledo GmbH, NETZSCH, Perkin Elmer, TA Instruments, are included in the FIF EUM.[1]

One of the types of the TGA method in determining the characteristics of substances and materials is thermomagnetometry (TM), where an increase or decrease in the mass of a sample is determined depending on the direction of the magnetic field gradient [3]. The reason for the change in the mass of the sample is a second-order phase transition, where the sample changes from the ferromagnetic state to the paramagnetic one. This transition is called the “Curie point” (Curie temperature). TM is mainly used to characterize ferromagnetic compounds, because they are key elements of most modern radio engineering, electronic, and computing devices. Lithium-titanium ferrites [4], lithium-zinc ferrites [5], nickel-zinc ferrites [6], magnetite [7], Heusler alloys [8], and other ferromagnetic compounds [9–11] are being studied.

The relevance of thermal analysis, in particular, the development of reference materials for the phase transition temperature is confirmed by the European roadmap for thermophysical properties metrology [12]. In the Russian Federation, the main means of ensuring the uniformity of measurements of TGA and STA analyzers are reference materials (RMs) for the first-order phase transition temperature [13].

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[1] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19. Accessed 10 October 2022 (In Russ.).

However, if these RMs are applicable for metrological support of STA (testing, verification, and calibration), then there are some difficulties for TGA devices. The main difficulty is related to the fact that the c-DTA method (calculated differential thermal analysis) is used to calibrate the instruments, which is not standardized, as opposed to the method based on the Curie temperature (second-order phase transition temperature) described in ASTM E 1582-00 [14]. Various groups of researchers have carried out and are carrying out work on the creation of CRMs for the method based on the Curie temperature [15]. As a result of the research work, a set of certified reference materials for the Curie temperature (CRM TC) was developed, which consists of three materials: alumel ($NiMn_3Al$, T_c ~ 163 °C), nickel (Ni, T_c ~ 352 °C), and trafoperm (SiFe, T_c ~ 751 °C).

Materials and Methods

Reagents

The starting material for the research was:

- an alumel alloy ($NiMn_3Al$) with a chemical composition (Ni 94%, Mn 2.1%, Al 1.7%, Si 1.5%, Fe 0.3%, Mg 0.15%, Cu 0.1%, C 0.05%);
- a metal (Ni) with a chemical composition (Ni 99.97%, Cu 0.003%, Mn 0.002%, C 0.005%, Si 0.002%, S 0.002%, Ti 0.001%, Mg 0.003%, Fe 0.01%);
- a silicon iron alloy (SiFe) with a chemical composition (Fe 95%, Si 4.5%, Mn 0.02%, Cu 0.2%, C 0.07%, Cr 0.06%, P 0.03%, S 0.02%).

The International Confederation for Thermal Analysis and Calorimetry (ICTAC) has recommended the use of a set of the following 5 materials as certified reference materials (CRMs) for metrological support of the temperature measurement range from 100 to 1,000 °C: permanorm 3 (T_c ~ 267 °C), nickel (T_c ~ 355 °C), mumetal (T_c ~ 386 °C), permanorm 5 (T_c ~ 458 °C), trafoperm (T_c ~ 751 °C) [16]. Subsequently, the measurement range was expanded (from 25 to 1,000 °C), and the list was adjusted: alumel (T_c ~ 153 °C), nickel (T_c ~ 358 °C), $Ni_{0.83}Co_{0.17}$ alloy (T_c ~ 554 °C), $Ni_{0.63}Co_{0.37}$ alloy (T_c ~ 746.4 °C), $Ni_{0.37}Co_{0.63}$ alloy (T_c ~ 930.8 °C) [17].

Three materials (alumel, nickel, and trafoperm) were selected for research for the following reasons:

- these materials can metrologically provide a temperature measurement range (from 25 to 770 °C) of existing TA devices (FIF EUM);
- these materials have been studied and certified as CRMs in other countries;
- the main source of temperature measurement error in TGA devices is the thermocouple error, which has a linear statistical characteristic. This allows using a smaller number of certified reference materials with characteristics located at the beginning, middle, and end of the specified measurement range, thereby saving time spent on verification/calibration of TA devices.

Equipment

The thermal analyzer STA 449 F5 JUPITER (NETZSCH, Germany) from the State Primary Standard of units of mass fraction, mass (molar) concentration of water in solid and liquid substances and materials GET 173-2017,[2] which implements simultaneous thermal analysis (STA) that combines differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA).

The mass of the starting material was measured on a laboratory balances of special accuracy class I (Sartorius, Germany) with a weighing resolution of 10 µg and an expanded uncertainty of 80 µg. The mass of the crucible and the mass of the crucible with a sample were obtained in ten parallel measurements. The sample mass was determined by the mass differences.

Research Methods and Procedure

Magnetometers [8, 10] or thermomagnetic analysis measuring instruments [18] are used to determine the Curie temperature. The main advantage of TM compared to other methods is the ability to measure the Curie temperature on a device that is calibrated and measures the melting point of materials that are fixed points of the International Temperature Scale (ITS-90), which increases the reliability of the measured values due to the same measurement conditions (the same temperature sensor, measurement rate, atmosphere).

It should be noted that the TM method was used in the certification of materials as CRMs in other countries [15].

The wide application of the TM method in the characterization of the substances and materials mentioned in the Introduction also confirms the correctness of the selected method for the certification of reference materials for the Curie temperature.

The method is sensitive and allows measuring the Curie temperature of materials containing magnetic ferrite phases with a mass fraction of more than 2% of the main component [4].

Some researchers process thermograms based on the peak of the derivative of the TG signal [5, 19], but the generally accepted and recommended in ASTM E 1582-00 is the assessment of the end point of the phase transition temperature.

A schematic representation of the installation implementing the TM method is shown in Fig. 1.

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[2] GET 173-2017 State primary standard of units of mass fraction, mass (molar) concentration of water in solid and liquid substances and materials: UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397857. Accessed 10 October 2022 (In Russ.).

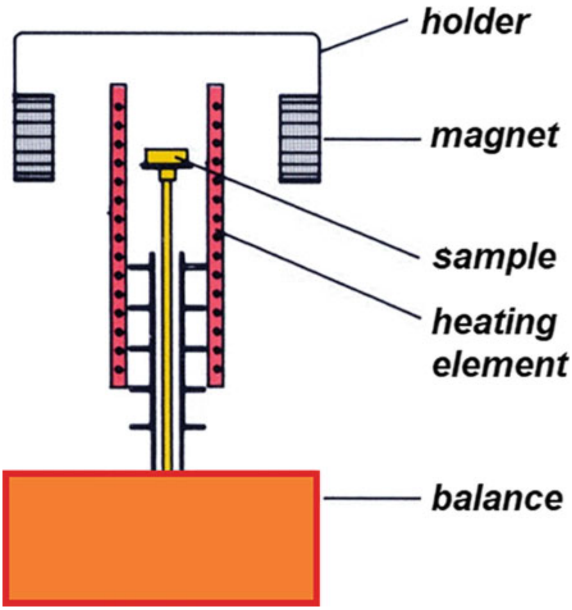

Fig. 1 The schematic representation of the installation implementing the TM method for determining the phase transition temperature (Curie temperature)

CRMs were studied using the certified measurement procedure M.221.0029/RA.RU.311866/2021[3] (MP), the accuracies of which are presented in Table 1.

The impact of factors affecting the measurement accuracy has been minimized, namely:

- the crucible: manufacturers of thermal analysis instruments recommend the use of Al_2O_3 crucibles for measurements of metals and their alloys to avoid interaction of the test material with crucible material as specified in ASTM E 967-08 [20]. Closed crucibles were used for more even heat distribution in the sample;
- the sample: there are increased requirements for the samples under study to determine the first-order phase transition temperature (melting, crystallization), namely, the geometry of the sample (a good thermal contact between the sample and the crucible is important), the weight of the samples (it should not exceed 20 mg to reduce the influence of the "mass effect"). These requirements become irrelevant for determining the second-order phase transition due to the fact that only the magnetic properties of materials change. The weight of samples varied from 20 to 40 mg;

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[3] M.241.0036/RA.RU.311866/2022 Method for measuring the phase transition temperatures of sodium methanesulfonate (CH_3NaO_3S) and anhydrous sodium acetate ($C_2H_3O_2Na$) by differential scanning calorimetry: UNIIM—Affiliated Branch of the D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/16/items/1399257. Accessed 10 October 2022 (In Russ.).

Table 1 Measurement ranges, values for accuracy, correctness, repeatability, intralaboratory precision, combined standard and expanded uncertainty

Measurement ranges, °C	Repeatability, °C	Intralaboratory precision, °C	Correctness, °C	Accuracy, °C	Combined standard uncertainty, °C	Absolute expanded uncertainty, °C at $P = 0.95$ and $\varkappa = 2$
From 28 to 200 incl.	0.01	0.05	0.06	0.11	0.055	0.11
From 200 to 400 incl.	0.01	0.05	0.12	0.17	0.085	0.17
From 400 to 700 incl.	0.02	0.07	0.26	0.33	0.165	0.33
From 700 to 1600 incl.	0.02	0.07	0.60	0.67	0.335	0.67

- the atmosphere: it is necessary to use a gas with low thermal conductivity for precision measurements, therefore, measurements were carried out in N_2 atmosphere with a purity of 99.99% at a flow rate of 250 ml/min;
- the temperature calibration of MI: a thermal analyzer was used that was calibrated using certified reference materials for temperatures and phase-transition points (set SOTSF) GSO 2312-82/2316-82[4] (in particular, In, Sn, Zn), and certified reference materials for temperature and specific enthalpy of phase transitions (set SOTSF-2) GSO 11890-2022/11896-2022[5] (in particular Al, Ag, Au), the accuracy indicators of which correspond to the working standards of the 1st category according to the second part of the State verification schedule for means measuring temperature (GOST 8.558-2009 [21]). This procedure is widely used by research organizations [7, 15, 22, 23] to calibrate instruments before precision measurements traceable to ITS-90 according to the recommendations of ASTM E 1582-00;
- the sensitivity of MI: a DSC/TG sample holder (rather than DTA/TG) with a sensitivity of 1.2 μV/mW was used to increase the sensitivity;
- the magnetic device: Ne–Fe–B magnets were used and placed on the device in such a way that the mass change was ~ 5% (ASTM E 1582-00 recommendation $\geq 2\%$);
- the heating rate: in view of the fact that higher heating rates lead to a shift in heat effects to higher temperatures, a heating mode at a rate of 10 °C/min was used

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[4] GSO 2312-82/2316-82 Reference materials of the approved type of temperatures and heats of phase transitions (set SOTSF). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/393963. Accessed 10 October 2022 (In Russ.).

[5] GSO 11890-2022/GSO 11896-2022 Reference materials of the approved type of temperature and specific enthalpy of phase transitions of metals and metal salts (set of CO SOTSF-2). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1398408. Accessed 10 October 2022 (In Russ.).

during the study of CRMs. An additional argument in favor of the selected rate was the fact that rates from 1.0 °C/min up to 20 °C/min are used worldwide in the analysis of materials [4, 7, 19]. At the same time, the rate of 10 °C/min is standardized in the verification methods for TA MI (FIF EUM). Chinese colleagues used a similar rate in the development of CRMs [15].

Results and Discussion

The thermograms of the experiments in determining the certified values of CRMs are shown in Figs. 2, 3 and 4.

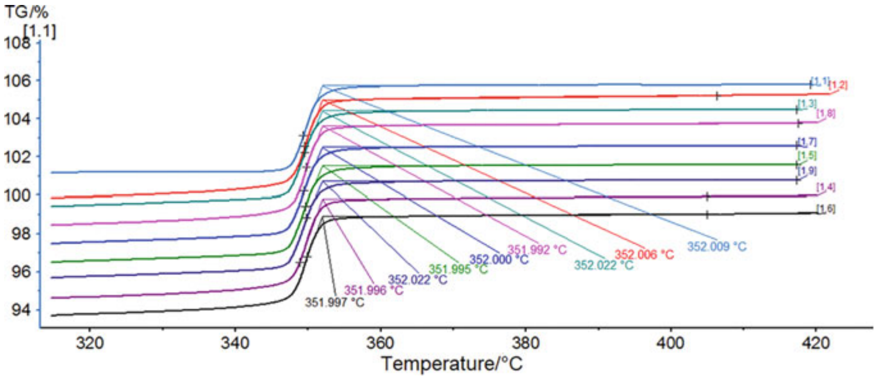

Fig. 2 Thermogram of nickel with a purity of 99.97%

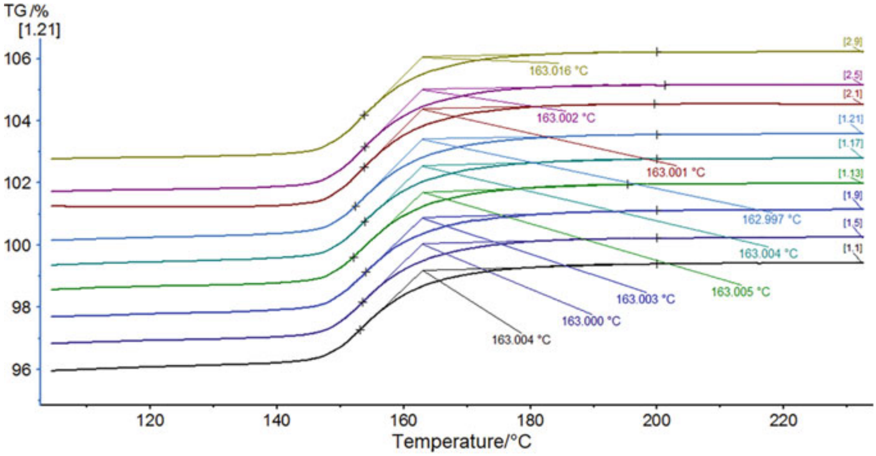

Fig. 3 Thermogram of alumel

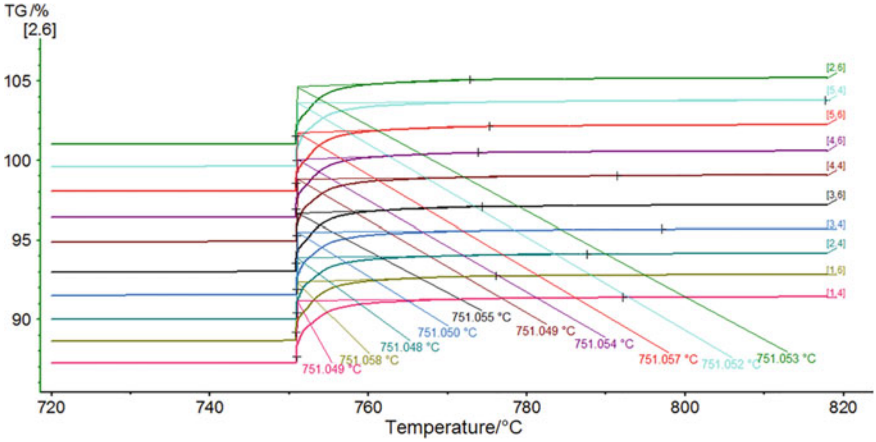

Fig. 4 Thermogram of trafoperm

The thermograms were processed in accordance with the MP; one sample was used for each material. The obtained data show that the standard deviation of the measurement results does not exceed 0.02 °C, which meets the requirements of the MP.

The obtained metrological characteristics of the investigated batch of CRMs are presented in Table 2.

The range of values indicated in the type specification is from 160 to 170 °C for alumel, from 350 to 360 °C for nickel, from 745 to 755 °C for trafoperm, which is due to the difference in the mass fraction of the main component from batch to batch during the subsequent certification of materials as certified reference materials. The variation in temperature values is also determined by the heating rate, the atmosphere, and the type of crucibles; therefore, all conditions for certification of RMs are indicated in the CRM certificate.

Particular attention should be paid to nickel, since the obtained certified value differs considerably from the values specified in other sources [15, 23]. The difference is mainly due to the purity of nickel: it was 99.99% in the above sources, and it was 99.97% in the study.

In this regard, additional measurements of high-purity nickel with a mass fraction of the main substance of 99.99% (ES-1.3-176-029-2018-Ni) were performed. The measurement results are presented in Table 3, and thermograms are shown in Fig. 5. The processing was carried out by the TG signal.

The thermograms presented in Fig. 5 are processed in accordance with the MP similarly to Figs. 2, 3 and 4. The obtained data show that the standard deviation of the measurement results does not exceed 0.02 °C, which indicates a good reproducibility of the measured values.

Table 2 Metrological characteristics of certified reference materials for the phase transition temperature (Curie temperature)

Certified characteristic	Certified value	Absolute error limits at $P = 0.95$	Absolute expanded uncertainty at $P = 0.95$ and $k = 2$
Phase transition temperature (Curie temperature of alumel), °C	163.00	± 0.30	0.30
Phase transition temperature (Curie temperature of nickel), °C	352.00	± 0.40	0.40
Phase transition temperature (Curie temperature trafoperm), °C	751.00	± 0.70	0.70

Note

- the metrological characteristics of CRMs were obtained under the following conditions
- heating rate—10.0 °C/min
- furnace atmosphere—inert (nitrogen)
- crucible material—Al_2O_3
- chemical composition of CRMs—indicated in the CRM certificate
- change in the mass of the sample when applying a magnetic field—5%

Table 3 The measurement results for nickel with a purity of 99.99%

Measurement No.	Measured value, °C	Average value, °C
1	358.301	358.32
2	358.342	
3	358.304	

The measurement conditions are similar to [15], namely:

- the change in the mass of the sample when applying a magnetic field is ~ 5%;
- the heating rate is 10 K/min;
- the purity of the material is 99.99%.

The results of T_c measurements by the DSC signal were additionally processed similarly to studies [23]. The measurement results are presented in Table 4, and thermograms are shown in Fig. 6. Nickel with a purity of 99.97% was selected as the material for testing.

The approaches and methods for determining, as well as the characteristics of the developed CRMs, are consistent with other groups of researchers and reference values:

- for nickel with a purity of 99.99%: the results obtained are consistent within ± 0.05 and ± 0.04 °C with the data of NIM (China) [15] and Université Paris-Sud

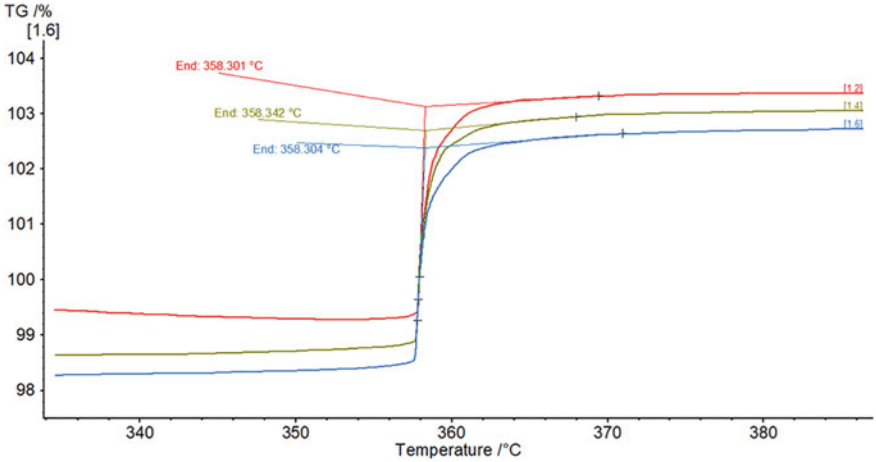

Fig. 5 Termogram of nickel with a purity of 99.99%

Table 4 The measurement results for nickel with a purity of 99.97%

Measurement No.	Measured value, °C	Average value, °C
1	352.155	352.18
2	352.187	
3	352.188	

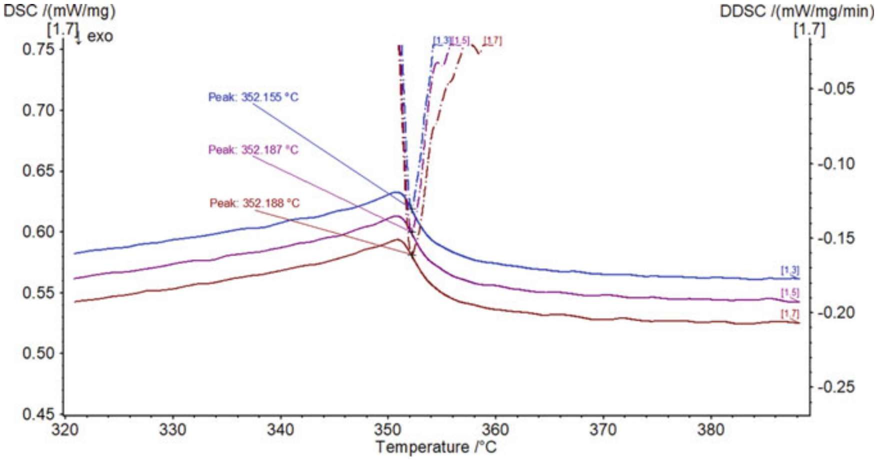

Fig. 6 Thermogram of nickel with a purity of 99.97%

(France) [19], respectively, and within ± 0.1 °C with [17, 22]. The difference with the data given in [24] was within ± 1.1 °C, which is associated with different changes in the mass of the sample associated with application of a magnetic field and different masses of samples.

- for trafoperm: the data are consistent within ± 1.5 °C [16];
- for alumel and nickel with a purity of 99.97%: data on the study of materials are not available. Taking into account the results obtained for other materials and the correctness of the selected method, as well as the experimental conditions, we believe that the obtained values are representative.

The results obtained by processing the DSC signal (Fig. 6) are consistent with the certified values within ± 0.2 °C, which indicates the possibility of applying this method to determine the Curie temperature of various substances and materials.

The traceability of the obtained certified values for the phase transition temperature is provided to the temperature unit (°C) reproduced by the State primary standard of temperature unit in the range from 0 to 3200 °C GET 34-2020,[6] and provided through the use of reference materials of In, Sn, Zn, Al, Ag, and Au, which are the fixed points of the International Temperature Scale (ITS-90), when measuring the phase transition temperature. The procedure described above can be applied for determining the characteristics of other ferromagnetic compounds, as well as for certification of RMs based on them.

Conclusion

The purpose of this work was to test the results of research on the use of materials based on alumel, nickel, and iron silicide (trafoperm) as phase transition temperature standards for the development of certified reference materials for the phase transition temperature (Curie temperature) traceable to the SI unit "temperature."

In the course of experimental studies, the possibility of using the procedure for measuring the phase transition temperature (Curie temperature) by thermomagnetometric and differential scanning calorimetry methods using the thermal analyzer STA 449 F5 JUPITER from GET 173-2017 was proved. The certified values of CRMs were established; the procedure was performed in accordance with GOST ISO Guide 35-2015 [25]; the contributions to the uncertainty due to heterogeneity of the starting materials were evaluated; short- and long-term stabilities were studied.

Comparison of the certified values of the Curie temperature of the developed CRMs with the reference values of the Curie temperature showed that the certified characteristics of the CRMs are well consistent.

The theoretical significance of the obtained results lies in the proof of the possibility of applying thermomagnetometric and differential scanning calorimetry

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[6] GET 34-2020 State primary standard of temperature unit in the range from 0 to 3200 °C: D. I. Mendeleyev Institute for Metrology. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1385580. Accessed 10 October 2022 (In Russ.).

methods for the development of certified reference materials for the phase transition temperature (set SO TK) GSO 12005-2022/GSO 12007-2022.[7]

The practical significance of the obtained results expand the possibility of establishing and monitoring the stability of the calibration dependence of measuring instruments for thermogravimetric analysis, as well as to increase the precision of measurements of the Curie temperature of various substances and materials.

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Contribution of the Authors Shipitsyn A. P.—development of methodology/procedure, implementation of a formal analysis, writing a draft version of the article, conducting research work, preparing/creating visual materials; Nepomiluev A. M.—research concept development, conducting research work, preparing/creating visual materials; Tyurnina A. E.—manuscript revision and editing.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference “Reference Materials in Measurement and Technology” (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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[7] GSO 12005-2022/GSO 12007-2022 Reference materials phase transition temperatures (set SO TK). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1403745. Accessed 10 October 2022 (In Russ.).

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Reference Materials of Absorbed Dose: Expanding Dynamic Range and Improving Measurement Accuracy

Vladimir P. Tenishev

Abstract Establishment and control of metrological characteristics of measurements of absorbed ionizing radiation doses in the range of 0.01 and 200 kGy by reference materials is an urgent task due to their wide application in various industries. The most convenient means of metrological support for transferring a unit of absorbed dose rate of intense photon, electron, and beta radiation to measuring instruments in radiation technologies are reference materials with established metrological traceability to the International System of Units (SI). In the present study, a method for expanding the dynamic range of measuring the absorbed dose of high-intensity ionizing radiation by radiochromic film dosimetry systems was considered and tested. The accuracy (uncertainty) of dose measurements was estimated depending on the initial optical density of the radiation-sensitive layer of the radiochromic composition. The possibility of expanding the dose characteristics and improving the metrological characteristics of the existing reference materials of absorbed dose (in water) for use as secondary standards (Measures) of the absorbed dose of ionizing radiation reproducing and (or) storing one or more points of the selected measurement scale of the absorbed dose with increased accuracy (uncertainty) of the measured values of the absorbed dose (in water) in an extended dynamic range was shown.

Keywords Radiochromic film · Radiation-sensitive composition · Optical density · Spectrophotometer · Reference material · Absorbed dose · Multilayer combination of absorbed dose reference materials

Abbreviations

IOD	Induced optical density
OD	Optical density
AD	Absorbed dose

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https://doi.org/10.1007/978-3-031-49200-6_25

RDF	Radiochromic dye film
RSC	Radiation-sensitive composition
RSL	Radiation-sensitive layer
SD	Standard deviation
RM	Reference material

Introduction

A reliable assessment of the absorbed dose measurements (in water) of ionizing radiation in reference materials in the range of 0.01 and 200 kGy in various radiation-technological processes determines the quality of products, their safety, the efficiency of the radiation-technological installation, and the technical and economic indicators of the entire radiation process in general [1]. In the Russian Federation, including the countries of the Customs Union, the traceability of the uniformity of the AD measurements in radiation technologies is carried out based on GET 209-2014[1] [2]. According to the State verification schedule [3], the transfer of an absorbed dose rate unit of intense photon, electron, and beta radiation in radiation technologies to measuring instruments should be carried out using secondary standards (***comparators***) or working standards indicating the range, accuracy (uncertainty) of measurements, including the main methods of verification (calibration). Reference materials of AD based on a radiochromic dye film (RDF) with a layer of radiation-sensitive composition (RSC) [4–10] were approved as working standards and dose measuring instruments. An important characteristic of radiochromic film reference materials (RMs) is the absence of temperature dependence and insensitivity to ambient light during processing and measurements.

According to the FIF EUM,[2] no secondary absorbed dose measurement standard has been registered as of May 2022. Thus, the purpose of this study is to analyze the possibility of expanding the dose characteristics and improving the metrological characteristics of the existing reference materials of AD for using RMs as secondary standards (**comparators**) of AD of ionizing radiation reproducing and (or) storing one or more points of the selected scale of the AD measurements with increased accuracy (uncertainty) of the measured values of absorbed dose in the extended dynamic range.

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[1] GET 209-2014 State Primary Special Standard of the Unit of Absorbed Dose Rate of Intense Photon, Electronic and Beta Radiation for Radiation Technologies: Custodian Institute All-Russian Scientific Research Institute of Physical Technical and Radio Technical Measurements Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397897. Accessed 4 August 2022 (In Russ.).

[2] Federal Information Fund for Ensuring the Uniformity of Measurements. Available via. https://fgis.gost.ru/fundmetrology. Accessed 4 August 2022 (In Russ.).

Theoretical Background

In this work, we consider a methodology for creating **comparators** based on certified reference materials of absorbed dose SO PD(E)-1/10 [5] for the range (1–10) kGy.

As a rule, a batch of films from one release prepared based on specifications [4] in accordance with the certificate of certified reference materials [5] contains samples with an optical density A in the range:

$$A_{0i} - 0.01 \leq Ai \leq A_{0i} + 0.01, \tag{1}$$

where A_{0i} is the average values of optical density (OD) (from 0.15 to 0.2) of the i-th batch of raw film.

Then separate sub-batches (sets) are selected from different batches according to the value of the initial optical density A_k satisfying the condition:

$$A_{k0} - 0.001 \leq A_k \leq A_{k0} + 0.001, \tag{2}$$

where A_k is the average optical density of a separate k-th sub-batches (k-th set).

Thus, each new k-th sub-batch is completed with the values of the initial OD in the range defined by the formula (2). In other words, we will obtain sub-batches of films-blanks with initial OD values ten times narrowed than in the initial batch.

It is easy to estimate that this procedure will select sub-batches of film samples that have a standard uncertainty ***u*** based on the initial OD that is significantly better than the standard uncertainty of a set of samples based on the initial film batch (not selected based on the OD). The proposed procedure will lead to the creation of a next generation of high-precision film reference materials of AD that significantly exceed the existing certified reference materials both in terms of dynamic range and accuracy characteristics. Such RMs can also be used as **Measures** for reproducing and (or) storing one selected value of the absorbed dose scale, as well as a set of **Measures** reproducing and (or) storing two or more points of the scale, for example, when calibrating the RM within the entire selected AD range.

Materials and Methods

Preparation of Film Samples-Blanks

The main idea of the proposed method for expanding the dynamic range and improving the measurement accuracy of film dosimeters is to select films with known OD values in a significantly narrower OD range than a very wide range of OD values of the initial batches of raw film from a set of blanks for RMs. The main objective is to minimize the source of measurement uncertainty in terms of the initial optical

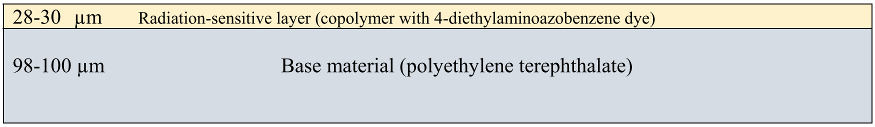

Fig. 1 The design of films-blanks of reference materials of absorbed dose

Fig. 2 Manufacture of a batch of blanks of reference materials of absorbed dose: 1 is a raw film roll; 2 is cut long (up to 2 m) strips ~ (10–12) mm wide; 3 is blanks of a reference material with a length of (30–35) mm; 4 is a batch of a reference material sealed in paper containers, 3–5 pieces per batch

density. Although this process significantly increases the time and effort for each case, but at the same time allows to achieve the desired objective.

RMs of absorbed dose SO PD(E)-1/10 are made from a single-use polymeric film material "Colored radiation-sensitive film of the CRF type" manufactured according to specifications [8] with a radiation-sensitive layer (RSL) (copolymer with 4-diethylaminoazobenzene dye) with a thickness of ~ 28 μm applied on a polyethylene terephthalate support with a thickness of ~ 100 μm (Fig. 1).

Then CRFs were cut into rectangular fragments (10–12) × (30–35) mm^2 and hermetically packed (thermally sealed) in 3–6 pieces in a container made of ~ 50 μm thick paper (Fig. 2) laminated with polyethylene (LDPE) grade 15803-020 or 10803-020. This paper was selected due to its weak susceptibility to ionizing radiation.

Selection of Films by the Value of the Initial Optical Density

The first step was to determine the distribution of the real OD range of long films or a set of unirradiated samples-blanks by measuring the OD on a Specord-210 Plus spectrophotometer (Analitik Jena AG, Germany) (Fig. 3). The optical density of RMs of AD was measured on long strips 10–12 mm wide (see Fig. 2, position 2) randomly selected from a batch.

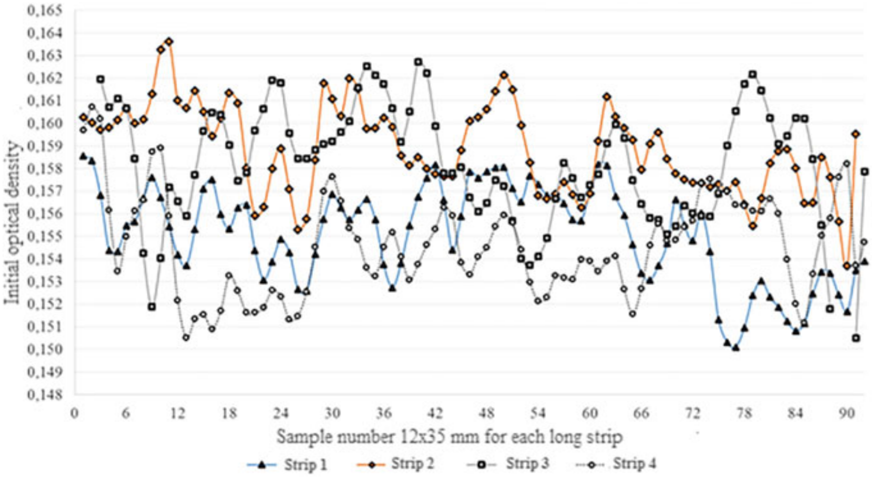

Fig. 3 Optical density distribution of one batch of unirradiated films

As shown in the figure, the initial OD is distributed in the range from 0.150 to 0.164 over the area of the raw roll for a given batch of film. This figure corresponds to the one shown in Fig. 4 distribution function of the number of film samples-blanks depending on the optical density.

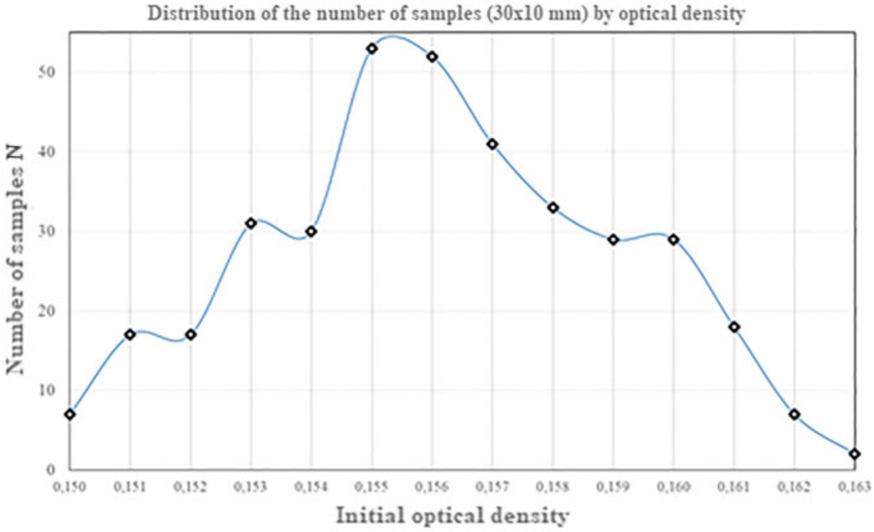

Fig. 4 Distribution of the number of samples-blanks by the optical density in a raw film batch

Thus, the OD (density and thickness of the RSL, respectively) is very inhomogeneous over the area of the raw film roll for the manufacture of RMs, which leads to a large inaccuracy (uncertainty) in the optical density value and, accordingly, a large inaccuracy in the AD measurement after irradiation of films in the ionizing radiation field.

As can be seen from the measurement results of the initial OD of the films (Fig. 4), the maximum amount of film occurs for an OD of ~ 0.156 with an uneven decrease in the number of films with distance from the average value of the OD of the entire batch.

Sets of five films with similar OD values within the OD values were collected from the obtained samples:

$$A_k - 0.001 \leq A_{ko} \leq \mathrm{A}_k + 0.001, \tag{3}$$

where A_k was selected through a step of 0.001 in the range of optical densities 0.150–0.164 of the selected batch (see Fig. 3).

Irradiation Facilities of the State Standard GET 209-2014 with Radionuclide Sources

Each set containing five identical RDF fragments packed in a sealed moistureproof paper container and selected in accordance with Fig. 5 was irradiated with doses in the range from 200 to 25,000 Gy in a cylindrical aluminum phantom with a height of 90 mm, diameter of 30 mm, and thickness of 1.8 mm at the laboratory ^{137}Cs-machine for microbiological and biochemical investigation LMB-γ-1 M (Russia) with a dose rate of 0.5 Gy/s and a laboratory ^{60}C0-machine for microbiological and radiation-chemical investigation MPX-γ-100 (Russia) with a dose rate of 2.5 Gy/s. In these experiments, the phantom with films was at a distance of 40 mm in the center of capsules with IR sources arranged in the form of a cylindrical squirrel wheel (ø ~ 80 mm).

Means for Measuring Optical Density

The optical density of films was measured in different modes of the Specord-210 Plus spectrophotometer in the carousel mode (OD of 15 samples was measured sequentially at one time). It has been experimentally established that the induced optical density (IOD) saturates at level 3 in the absorption measurement mode, and its spatial resolution is no worse than 1 mm (optical beam size is ~ 7×1 mm^2). The maximum sensitivity of the selected samples of the RDF blanks, as for the initial one, is observed at a wavelength of 550 nm, at which the IOD was read. The IOD of

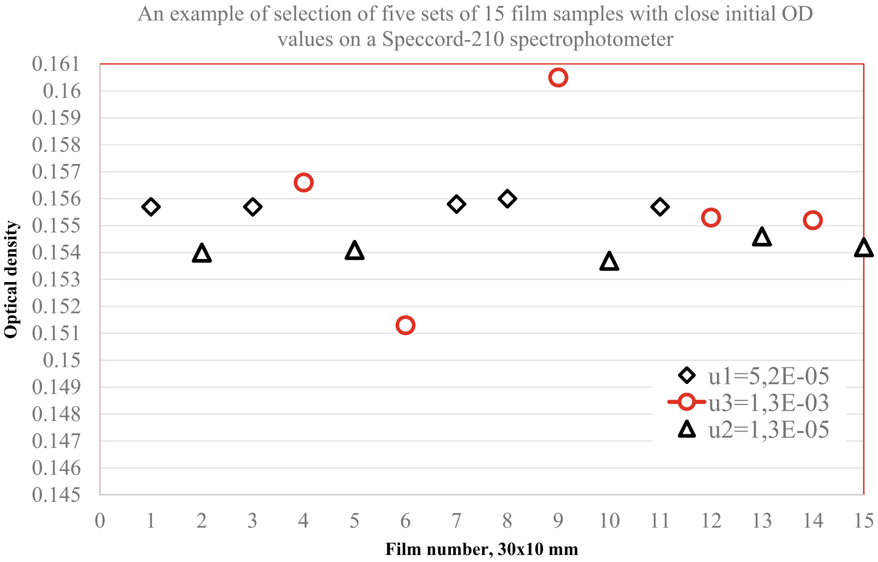

Fig. 5 Selection of sets of five pieces of film samples from 15 blank samples with similar OD values. The inset shows estimates of the uncertainty ***u*** for each of the three sets of selected films

all sets of irradiated films was started ~ 5 min after the end of irradiation (the time spent on removing the samples from the irradiation chamber, delivering, and placing them in the spectrophotometer holder).

Initially, we measured the OD spectral distribution irradiated with an absorbed dose of up to 10 kGy in order to establish the wavelength at which films show the maximum sensitivity (the highest sensitivity is in the range of 540–560 nm with a maximum at 550 nm). Then, the optical densities of the irradiated sets of films at values of 200, 250, 750, 1000 Gy and at 5, 15, 20, 25 kGy (in water) were measured at a fixed wavelength of 550 nm. The measurements were also carried out in the time scanning mode at fixed intervals in order to estimate the change in OD after irradiation (post-effect). The linearity of the optical density of film depending on the absorbed dose was estimated by a graphical method in the Excel program. Stability over time, the dependence of readings on the IR energy of film radiochromic systems will be published separately.

Results and Discussion

Dependence of the Induced Optical Density of the Selected Sets of Films on the Absorbed Dose

Figure 6 shows the experimental dependence induced with [11–13].

In the entire range of irradiated doses from 200 to 20 kGy, the IOD *A* value is mathematically described by a linear function:

$$A = 0.908 \cdot D\ ,\ (R^2 = 0.998), \tag{4}$$

where D is the absorbed dose, R^2 is the coefficient of linear determination (Fig. 6).

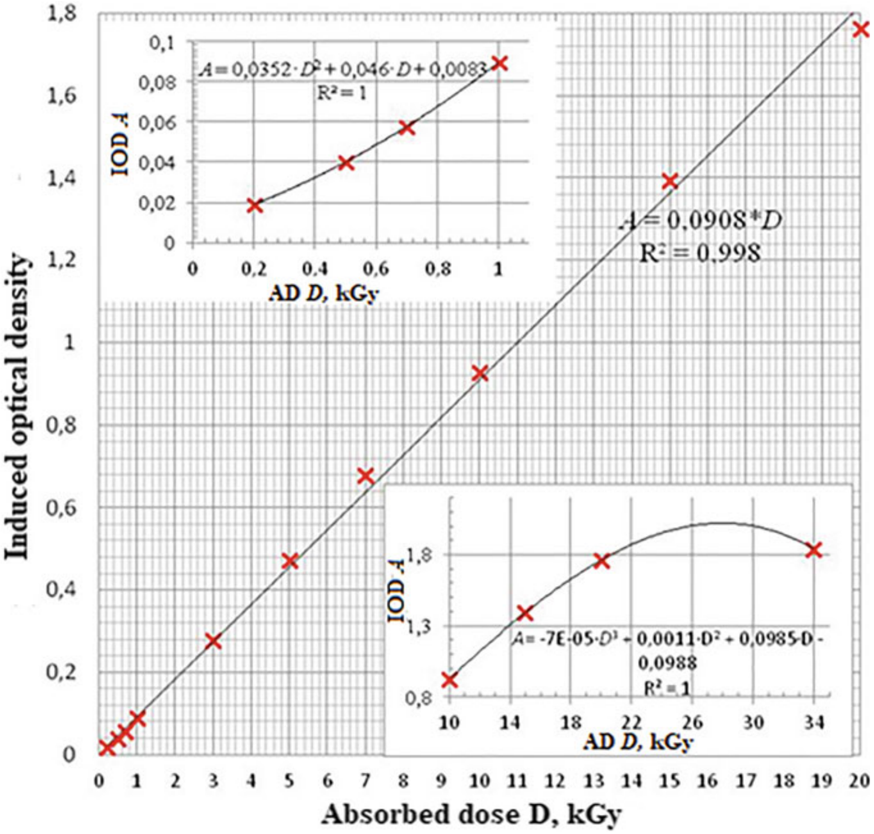

Fig. 6 Diagram of the dependence of the induced optical density of the selected sets of films on the absorbed dose *D*

However, as follows from the data in the insets to Fig. 6, the OD dependence on the AD at low (200–800 Gy) and high (15 kGy and above) is non-linear. This behavior of the OD at low doses is associated with insufficiently efficient absorption of the RSL energy of ionizing radiation, and the non-linearity at high doses is apparently due to the saturation of the optical density of the RSL based on a copolymer with 4-diethylaminoazobenzene dye.

This study compared the performance of the described method (with film sampling at initial ODs) of dosimetry with a conventional non-selective film dosimetry procedure with a wide range of initial ODs. Figure 7 shows the results of a comparison of the relative values of the IOD $\boldsymbol{u/A_{avg}}$ for different batches of films (with and without selection of sets according to the initial values of the OD of unirradiated films) in the entire range of absorbed doses studied.

Discussion and Conclusions

In this research, we proposed a new method for expanding the dynamic range of OD measurements by radiochromic film dosimeters and improving their accuracy characteristics. Verification experiments showed that the range of measured doses from blanks for reference materials of absorbed dose SO PD(E)-1/10 (Fig. 7) for sets with selected samples of films with initial optical densities in the range $A_0 - 0.001 \leq A \leq A_0 + 0.001$ is from 200 Gy up to 20 kGy with a standard uncertainty of no

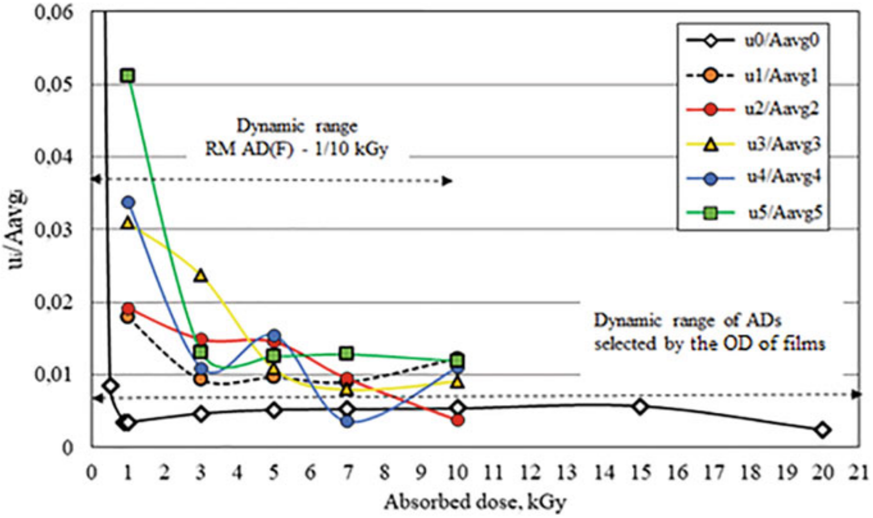

Fig. 7 Relative uncertainty (u_i/A_{angi}) of the arithmetic mean values of OD for six batches for each value of AD, where index $i = 0$, which corresponds to sets of films selected according to the initial OD, and indices i = 1–6 correspond to sets with unselected OD

worse than 0.8%, while sets of samples not selected according to OD with values in the range $A_0 - 0.01 \leq A \leq A_0 + 0.01$ exhibit a standard uncertainty of no better than 2% in the approved range of the AD (1–10) kGy.

Thus, the method of sampling sets of films in a narrow OD interval, in contrast to sets of films in a wide OD range (without sampling), gives a significantly better result in estimating both the dynamic range and the standard uncertainty of the measured doses.

The results indicate that:

1. Certified reference materials of absorbed dose SO PD(E)-1/10 can be used in a significantly wide range of absorbed doses from 200 to 25 kGy and with a standard measurement uncertainty up to 0.8%.
2. According to the State verification scheme [3], measuring instruments with a total relative standard deviation of the measurement results (0.8–2.5)%, including instability for the calibration interval, are allowed as secondary standards and ADR comparators. The research results showed that radiochromic film certified reference materials under certain conditions for selecting film sets can be used as secondary standards—**ADR comparators**.

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Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized and the review procedure was carried out.

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Development of Measures for Metrological Support of Raman Spectroscopy

Anna A. Yushina, Vladimir A. Aseev, and Alexander D. Levin

Abstract Raman spectroscopy is widely used in various industries and requires the use of special means for metrological support. In this study, prototype measures made from inorganic glasses activated with metal ions to excite fluorescence lines at specific wavelengths were developed for calibrating Raman spectrometers and microscopes on a scale of relative intensities with metrological traceability to SI base units. The study established the metrological characteristics of prototype measures, where the certified characteristic is the relative intensity of the reproduced fluorescence radiation. The maximum expanded uncertainty of measuring the relative fluorescence intensity at a coverage factor $k = 2$ is 9.4%, 5.2%, and 2.8% for prototype measures designed to reproduce the relative fluorescence intensity when excited at wavelengths of 532 nm, 633 nm, and 785 nm, respectively. By certifying the measures on the laser Raman confocal microscope Confotec NR 500, part of the GET 196-2015, metrological traceability can be established through the scale of relative intensities of the GET 86-2017 microscope, providing traceability to SI units of the "(light) energy flux" value. These findings have practical significance, since they allow the calibration characteristics of Raman microscopes and spectrometers to be established and monitored on a scale of relative intensities.

Keywords Reference material · Raman spectroscopy · Relative intensity calibration · Metrological support · Measures · Fluorescence line

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Abbreviations

GET 86-2017	State Primary Standard of Units of Radiometric Quantities and Units of Spectroradiometric Quantities in the Range of Wavelengths from 0.2 to 25.0 μm
GET 196-15	State Primary Standard of Units of Mass (Molar) Fraction and Mass (Molar) Concentration of Components in Liquid and Solid Substances and Materials Based on Spectral Methods
RM	Reference material
SI	International System of Units
SRM	Standard reference material

Introduction

Raman spectroscopy is a rapidly developing technique for analyzing the chemical composition of samples of natural and artificial origin, including biological samples, by determining the vibrational modes of molecules. In recent years, the method has gained popularity due to the improvement of equipment, the development of compact and inexpensive devices, and the growing interest in the rapid determination of substances [1] in various fields, including biological and medical diagnostics [2–4], pharmaceuticals and the chemical industry [5–8], criminalistics and forensic examination [9, 10], and environmental and food safety [11–13].

It is important to note that Raman spectra obtained using different instruments may exhibit significant variations in the relative intensities of the peaks of the analyzed compounds (Fig. 1). This is mainly due to the specific design of the instruments. The recorded spectral intensities are influenced by the transmission functions of optical components, the diffraction efficiency of the grating, and the spectral sensitivity of the photodetector, with the contribution of each component varying for different spectrometers. Furthermore, variations in the instrument response, which affect the shape of the Raman spectra, can occur on the same instrument following the replacement of a component or maintenance work [14, 15].

The broad application of Raman spectroscopy necessitates obtaining precise and instrument-independent spectra. These criteria are crucial for identifying substances by comparing experimental spectra with reference spectra obtained on different instruments. Therefore, it is essential to use specialized metrological means, such as measures to calibrate Raman microscopes and spectrometers by the spectral shape, i.e., the relative spectral sensitivity.

An example of such measures for calibrating Raman spectrometers and microscopes by the spectral shape are NIST SRM 2241-2246 (USA), based on glasses activated by various metal ions. These RMs are designed to correct the relative intensities of Raman spectra obtained using devices with laser excitation at the following wavelengths: 488, 514.5, 532, 633, 785, 830, and 1064 nm. The RMs are calibrated

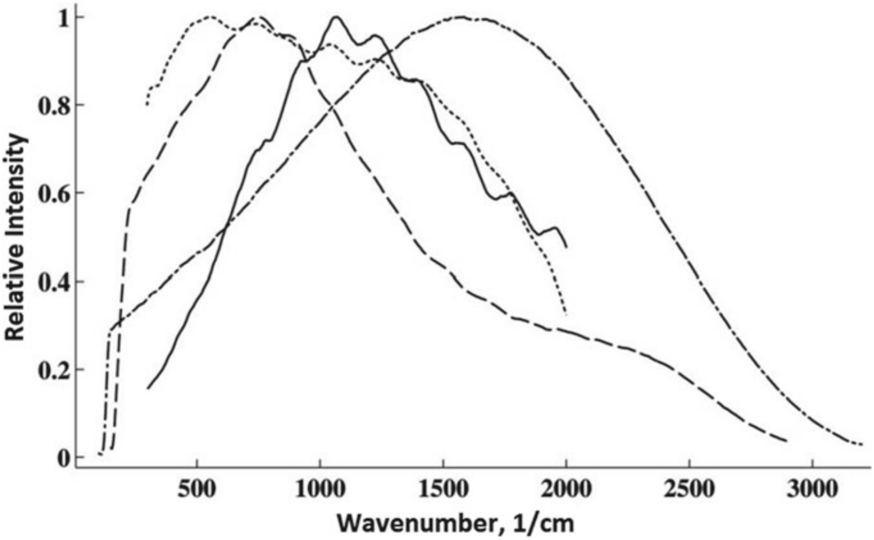

Fig. 1 Fluorescence spectra of NIST SRM 2241 reference material obtained on four Raman spectrometers with an excitation wavelength of 785 nm [17]

on a spectrometer traceable to a reference light source (radiometric standard). The advantage of using glass-based RMs (in comparison with measures based on liquids and polymeric materials) is their resistance to thermal, chemical, and photochemical effects, as well as their long shelf life.

Until recently, the metrological support of Raman microscopes and spectrometers in the Russian Federation heavily relied on the NIST RMs mentioned earlier. However, due to the necessity of timely maintenance of industrial production lines and the limited availability of the required RMs, it is necessary to carry out active import substitution, along with the development of new systems for the calibration of spectrometers and Raman microscopes. Moreover, these systems must ensure the metrological traceability to the SI units of the "(light) energy flux."

In Russia, measuring means that reproduce optical characteristics of samples such as transmission coefficient, optical density, and fluorescence intensity are commonly referred to as "measures" rather than "RMs." Therefore, the aim of this study was to develop prototype measures for calibrating Raman spectrometers and microscopes using a scale of relative intensities that are ensured by metrological traceability to the basic SI units of the "(light) energy flux."

The aims of this research are as follows: development of glasses with specific fluorescent properties; development of prototype measures for calibrating Raman spectrometers and microscopes on a relative intensity scale when excited at 532, 633, and 785 nm using these glasses; determination of the metrological characteristics of these systems; and substantiation of the metrological traceability of the system to SI units of "(light) energy flux."

Materials and Methods

Materials

The prototype measures were developed using three types of special fluorescent glasses based on an oxide matrix activated by metal ions, which were developed at the National Research University ITMO [16]. The glasses were activated with manganese (Mn_2O_3, 1 wt%), bismuth (Bi_2O_3, 1 wt%), and chromium (Cr_2O_3, 0.02 wt%) metals. The synthesis of the glasses involved using the following systems: $42B_2O_3$–27MgO–$18Al_2O_3$–13CaO (mol%), $72.4P_2O_5$–11.6ZnO–$15.7Al_2O_3$–$0.3Bi_2O_3$ (mol%), and $20K_2O$–Al_2O_3–$60B_2O_3$ (mol%).

Apparatuses and Laboratory Ware

The measurements were carried out using a high-precision, fully automated 3D-scanning Confotec NR 500 confocal Raman microscope (SOL Instruments, Belarus), which is included in the GET 196-2015[1] State primary standard of units of mass (molar) fraction and mass (molar) concentration of components in liquid and solid substances and materials based on spectral methods.

The scale of relative intensities of the microscope is traceable to the GET 86-2017[2] State primary standard of units of radiometric and spectroradiometric values in the wavelength range from 0.2 to 25.0 μm. In order to ensure this traceability, a radiation source based on a 64,250 HLX halogen lamp (OSRAM, Germany) was used as a comparator.

Glass Synthesis

Glasses intended to reproduce the relative intensity of fluorescence when exposed to laser radiation at a wavelength of 532 nm were activated using manganese ions. Bismuth was selected as the activating metal for glasses intended to induce fluorescence under laser irradiation at 633 nm, while chromium ions were used to activate

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[1] GET 196-2015 State Primary Standard of Units of Mass (Molar) Fraction and Mass (Molar) Concentration of Components in Liquid and Solid Substances and Materials Based on Spectral Methods: All-Russian Research Institute for Optical and Physical Measurements. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397882. Accessed 31 October 2022 (In Russ.).

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Table 1 Technical characteristics of prototype measures for metrological support of Raman spectroscopy

Parameter	Value		
	Manganese-activated glasses	Bismuth-activated glasses	Chromium-activated glasses
Characteristic excitation wavelength, nm	532	633	785
Cross section, mm	10 × 10	10 × 10	10 × 10
Height, mm	2.71	2.07	2.77
Fluorescence quantum yield	34	12	14

glasses designed to reproduce the relative fluorescence intensity when excited at a wavelength of 785 nm.

Manganese-activated glasses were synthesized using the $42B_2O_3$–$27MgO$–$18Al_2O_3$–$13CaO$ (mol%) system. The synthesis was carried out in a platinum crucible with a platinum stirrer at 1300 °C for 1.5 h. The glass was subsequently cast onto a metal plate and annealed at 550 °C for 3 h.

Bismuth-activated glasses were synthesized using the $72.4P_2O_5$–$11.6ZnO$–$15.7Al_2O_3$–$0.3Bi_2O_3$ (mol%) system. The synthesis was carried out in a quartz crucible using a quartz stirrer at 1450 °C for 8 h. The glass was further cast onto a metal plate and annealed at 600 °C for 4 h.

Chromium-activated glasses were synthesized in the system $20K_2O$–Al_2O_3–$60B_2O_3$ (mol%) at chromium Cr_2O_3 concentration of 0.02 wt%. The synthesis was carried out in a platinum crucible with a platinum stirrer at 1400 °C for 1.5 h. The melt was cast into a metal mold and annealed at 450 °C for 1 h.

The prototypes of measures comprised glass plates with technical specifications listed in Table 1. The dimensions of the samples were determined by the intended use of the measures on devices with a scattering angle of 180°. The prototypes are shown in Fig. 2.

Determination of Metrological Characteristics and Estimation of Expanded Uncertainty

The metrological characteristics of the prototype measures were determined using a laser microscope that conforms to the specifications outlined in GET 196-2015. The microscope scale of relative intensities is traceable to GET 86-2017. In order to ensure the traceability of the prototype measures to the basic SI units of "energy (light) flux," a halogen incandescent lamp was used as a comparator.

The operating modes and parameters of the microscope used to measure the fluorescence intensity of the prototype measures were carefully selected through

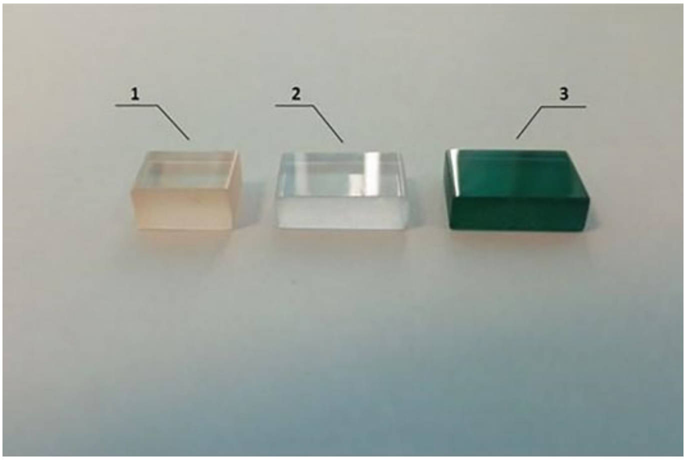

Fig. 2 Prototype measures for metrological support of Raman spectroscopy, where: 1—manganese-activated glasses; 2—bismuth-activated glasses; 3—chromium-activated glasses

experimental testing. The main criterion for selecting the parameters was to ensure that the microscope sensitivity range corresponded to the fluorescence intensity of the studied sample, with an optimal range exceeding the upper limit of the maximum fluorescence intensity by 20%. The main characteristics of the microscope operating modes and parameters are listed in Table 2.

The metrological characteristics of the prototype measures were determined in accordance with GOST 8.009-84 as follows.

The sample was placed on the stage of the microscope, and the operating modes and parameters of the microscope specified in Table 2 were set.

Ten measurements were carried out under the repeatability of the dependence of the fluorescence intensity signals for the measure on the wavenumber [$F_{n\_meas}(k_i)$, instrument-depended units (i.d.u.)], where k_i is the wavenumber, i is the order number of the wavenumber, n is the spectrum number measured under repeatability conditions.

Table 2 Operating modes and parameters of the Raman microscope when measuring the intensity of fluorescence radiation of prototype measures for metrological support of Raman spectroscopy

Parameter	Value
Diffraction grating, groove/mm	200
Mirror type	Ag
Detector type	CCD
Magnification of an objective lens of a Raman microscope	5
Aperture diaphragm, mm	100
Number of consecutive spectral measurements	10

On the basis of the measurement results, the relative intensity of fluorescence emission with respect to the maximum within a given wavenumber range was calculated for each measured spectrum using the following formula:

$$F_{n\_rel}(k_i) = \frac{F_{n\_meas}(k_i)}{F_{n\_max}(k_i)}, \quad (1)$$

where $F_{n\_rel}(k_i)$ is the relative intensity of Raman scattering with respect to the maximum; n is the spectrum number under repeatability conditions; i is the order number of wavenumber; k is the wavenumber, cm^{-1}; $F_{n\_meas}(k_i)$ is the measured value of the Raman scattering intensity for the n-th measurement, i.d.u.

The average relative fluorescence intensity of the sample at its excitation scattering wavelength was calculated using the formula:

$$\bar{F}_{rel}(k_i) = \frac{1}{N} \cdot \sum_{n=1}^{N} F_{n\_rel}(k_i), \quad (2)$$

where $\bar{F}_{rel}(k_i)$ is the average relative intensity of fluorescence emission of the measure at the operating excitation wavelength, r.u.; N is the number of measured spectra under repeatability conditions (here $N = 10$); $F_{n\_rel}(k_i)$ is the relative intensity determined based on the results of the n-th measurement, calculated according to Eq. (1).

The type A standard uncertainty $u_A(k_i)$ was estimated as the standard deviation (SD) of the mean value in the series of measurements using the formula:

$$u_A(k_i) = \frac{\sqrt{\frac{\sum_{n=1}^{N}[F_{n\_rel}(k_i) - \bar{F}_{rel}(k_i)]^2}{N(N-1)}}}{\bar{F}_{rel}(k_i)} \cdot 100. \quad (3)$$

The main source of type B uncertainty is the calibration inaccuracy of the scale for relative intensities, which is influenced by the uncertainty in measuring relative intensities using the microscope. This uncertainty was estimated during the calibration of the microscope, which ensures its traceability to the GET 86-2017 standard. In the development of the prototype measure, only this component was considered. Additional sources of type B uncertainty include the heterogeneity of the measure, instability in microscope calibration on relative intensity scale, and error in polynomial approximation of the relationship between fluorescence intensity and wavenumber. In subsequent preparations for type approval tests, the contribution of each of these factors to the type B uncertainty will be assessed.

The type B standard uncertainty for the reproducibility of the relative fluorescence intensity by the measure was estimated by the formula:

$$u_B = \frac{U_{con}}{2}, \quad (4)$$

where U_{con} is the expanded uncertainty of measuring relative intensities on the microscope determined through its calibration as part of the GET 196-15, which includes an estimation of the uncertainty of unit transfer from the radiometric standard of GET 86-2017 using the comparator, i.e., a halogen lamp-based radiation source mentioned above.

The combined standard uncertainty of the reproducibility of the relative fluorescence emission intensity by measures was calculated by the formula:

$$u_C(k_i) = \sqrt{u_A^2(k_i) + u_B^2(k_i)}, \tag{5}$$

where $u_A(k_i)$ is the type A standard uncertainty of the reproducibility of relative fluorescence intensity, $u_B(k_i)$ is the type B standard uncertainty of the reproducibility of the relative fluorescence intensity.

The expanded uncertainty of measuring the relative intensity of Raman scattering with the coverage factor $k = 2$ was calculated by the formula:

$$U_P(k_i) = 2 \cdot u_C(k_i), \tag{6}$$

Results and Discussion

Prototype measures were developed in order to provide metrological support for Raman spectroscopy, specifically to calibrate Raman spectrometers and microscopes on a relative intensity scale at specified excitation wavelengths within ranges of wavenumbers characterizing the shift in fluorescence emission frequency relative to the frequency of excitation emission. The range of wavenumbers for the measure intended to excite radiation at a wavelength of 532 nm (Sample 1) was 200–4000 cm^{-1}, for the measure intended to excite radiation at 633 nm (Sample 2), it was 200–3500 cm^{-1}, and for the measure intended to excite radiation at 785 nm (Sample 3), it was 200–3000 cm^{-1}.

The metrological properties of the developed prototype measures comprise the relative fluorescence intensity and wavenumber-dependent values of the expanded uncertainty of the relative emission intensity, at a coverage factor of $k = 2$. The highest values of uncertainties are shown in Table 3.

Figure 3 presents the fluorescence spectrum of prototype measure 1, normalized to unity and approximated by two 7th-order polynomials, with an approximation error of less than 3%. The fluorescence spectra of prototype measures 2 and 3 are shown in Figs. 4 and 5, respectively. The spectrum of prototype measure 2 was approximated using an 8th-order polynomial, while a 9th-order polynomial was used to approximate the spectrum of prototype measures 3.

The dependence of the spectra of the developed prototype measures from laser irradiation power was evaluated. Figure 6a illustrates the fluorescence spectra of

Table 3 Uncertainty characteristics of prototype measures for metrological support of Raman spectroscopy

Characteristics of uncertainty	Value of uncertainty, %		
	Sample 1 Manganese-activated glasses	Sample 2 Bismuth-activated glasses	Sample 3 Chromium-activated glasses
Type A uncertainty	≤ 4.5	≤ 1.1	≤ 0.2
Type B uncertainty	1.4	1.4	1.4
Combined standard uncertainty	≤ 4.7	≤ 2.6	≤ 1.4
Expanded uncertainty at $k = 2$	≤ 9.4	≤ 5.2	≤ 2.8

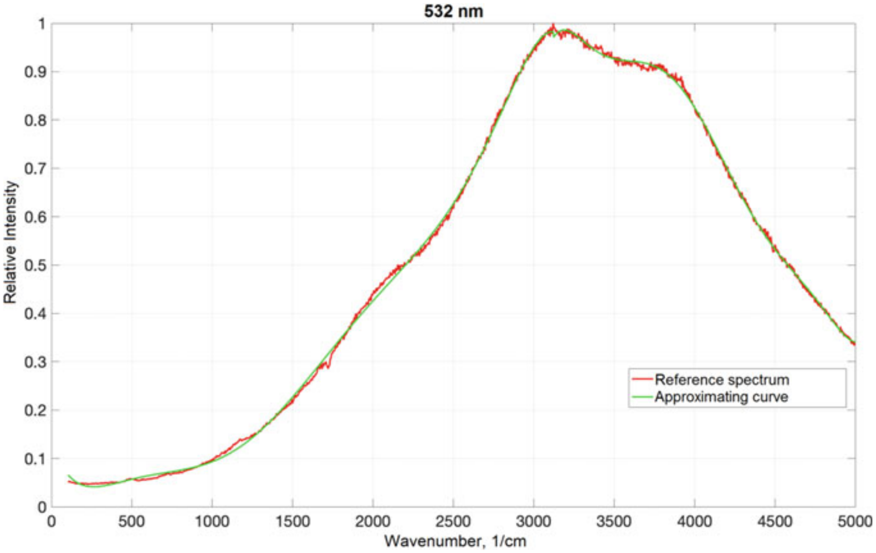

Fig. 3 Fluorescence spectrum of prototype measure 1 normalized to unity and approximated by 7th-order polynomials

prototype measure No. 1 at different laser powers, while Fig. 6b presents the same spectra normalized to a maximum. As shown, the spectra normalized to a maximum are almost identical, indicating that the shape of the spectra of the prototype measures is independent of the intensity of the laser radiation.

Device-independent Raman spectra can be ensured by a spectral correction function, which is represented by the ratio of the certified spectrum of the measure (approximated as a function) to the spectrum measured on a particular instrument. This is shown in the following formula:

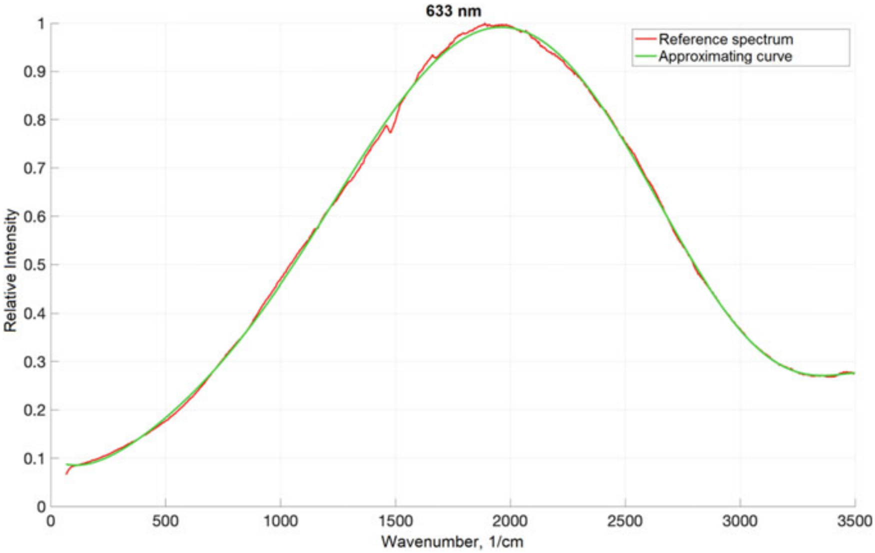

Fig. 4 Fluorescence spectrum of prototype measure 2 normalized to unity and approximated by 8th-order polynomials

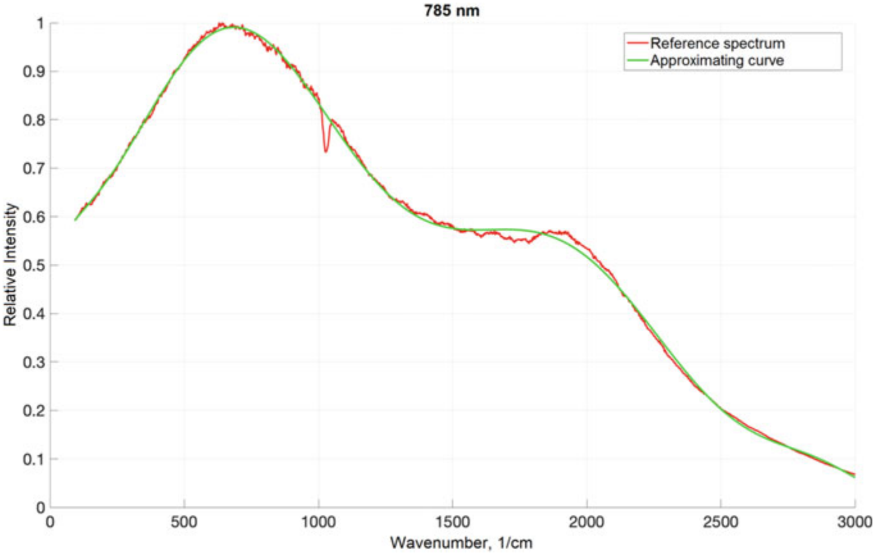

Fig. 5 Fluorescence spectrum of prototype measure 3 normalized to unity and approximated by 9th-order polynomials

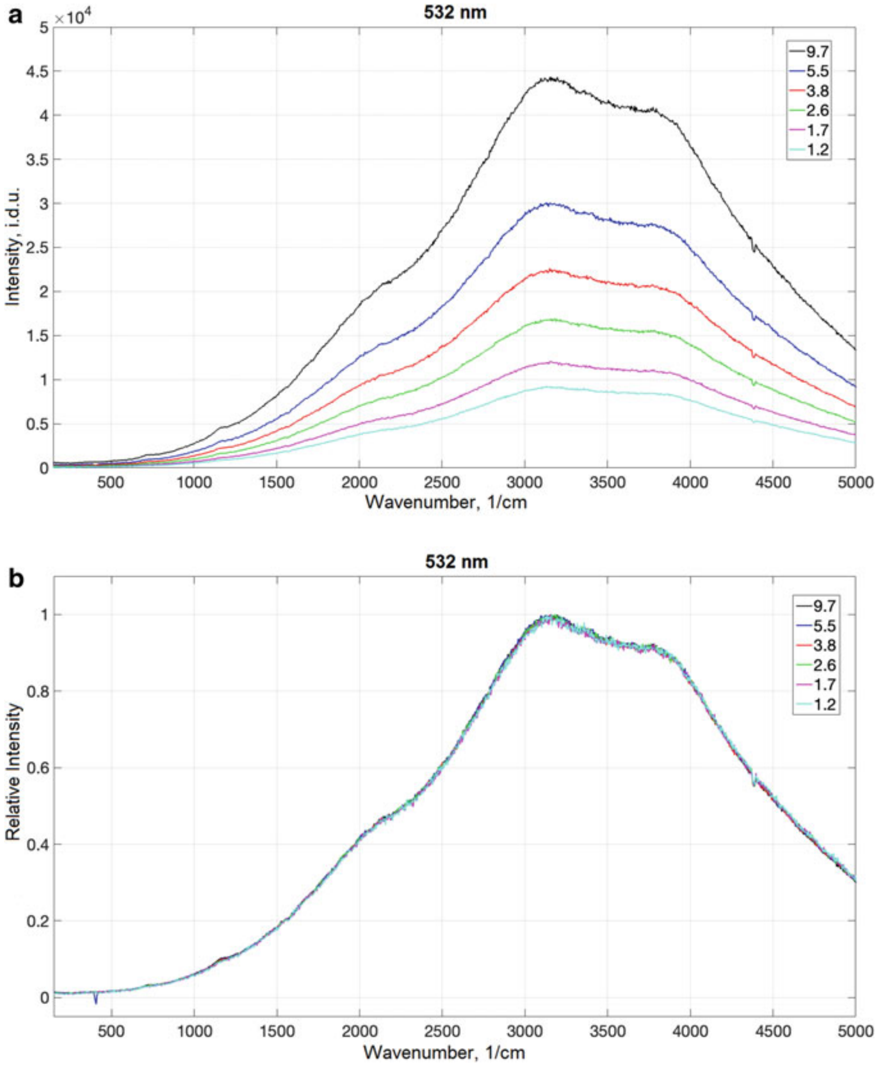

Fig. 6 **a** Fluorescence spectra of the prototype measure No. 1 taken at different laser powers (mW), **b** fluorescence spectra of the prototype measure No. 1 taken at different laser powers (mW) and normalized to a maximum

$$Corr(\Delta\nu) = \frac{I_{cert.meas}(\Delta\nu)}{I_{m.meas}(\Delta\nu)}, \tag{7}$$

where $Corr(\Delta\nu)$ is the spectral correction function; $I_{cert.meas}(\Delta\nu)$ is the certified spectrum of measure traceable to the state primary radiometric standard GET 86-2017; $I_{m.meas}(\Delta\nu)$ is the spectrum measured on a particular instrument.

By introducing a correction equal to the value of the spectral correction function, device-independent Raman spectra can be obtained in further work with the instrument, as shown in the formula:

$$I_{corr.sample}(\Delta\nu) = Corr(\Delta\nu) \cdot I_{meas.sample}(\Delta\nu), \tag{8}$$

where $I_{meas.sample}(\Delta\nu)$ is the spectrum of a sample recorded on the instrument; $I_{corr.sample}(\Delta\nu)$ is the corrected spectrum of a sample.

The measures will be certified on the Confotec NR 500 laser Raman confocal microscope, introduced to the GET 196-15 standard. The traceability of the intensity scale of this microscope to GET 86-2017 will be ensured by using an emission source based on a halogen lamp as a comparator.

In conclusion, the developed measures can satisfy the requirements for providing metrological support to Raman spectroscopy and can be used for calibrating Raman spectrometers and microscopes on a relative intensity scale, with laser excitation at 532, 633, and 785 nm in a wide range of wavenumbers.

Conclusion

Prototype measures made of inorganic glasses based on an oxide matrix activated with different metal ions were developed to excite a broad fluorescence line when emitting at specific wavelengths: manganese ions for 532 nm, bismuth ions for 633 nm, and chromium ions for 785 nm.

The metrological characteristics of the prototype measures were established, with the certified characteristic being the relative intensity of the reproducible fluorescence radiation. The maximum expanded measurement uncertainty of the relative fluorescence intensity at a coverage factor $k = 2$ for prototype measures designed to reproduce relative fluorescence intensity at 532 nm, 633 nm, and 785 nm amounted to 9.4%, 5.2%, and 2.8%, respectively.

Certifying the measures on the Confotec NR 500 laser Raman confocal microscope, as part of the GET 196-2015 standard, can establish metrological traceability through the scale of relative intensities of the GET 86-2017 microscope, providing traceability to SI units of the "energy (light) flux" value.

Therefore, the developed measures allows the traceability of Raman spectra to GET 196-2015 to be established using the spectral correction function. This expands the ability to establish and monitor the stability of calibration characteristics of Raman microscopes and spectrometers, i.e., calibration using the scale of relative intensities, which promotes obtaining accurate and device-independent Raman spectra.

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Author Contributions Yushina A. A.—research work, implementation of formal analysis, writing a draft version of the article; Aseev V. A.—provision of materials for research; Levin A. D.—development of the concept of research, implementation of formal analysis, control of research, creation of visual materials.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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Estimation of Uncertainty in Charpy Pendulum Impact Test Using the Reference Material

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Abstract An urgent task for accredited testing laboratories is to estimate the uncertainty of test results. In Charpy pendulum impact test the sources of the main contributions from the measuring instrument to the uncertainty of the result are: the angle of rise and the angle of fall of the pendulum, force exerted by the pendulum, and the distance from the axis of rotation to the center of strike. The aim of the work is to describe the method for estimating the uncertainty of the results of Charpy pendulum impact test based on information about the calibration of the impact testing machine and traceability to the reference material of impact energy (absorbed energy) of steel grade 45 GSO 11934-2022. The uncertainty estimation method is based on the statistical model from ISO 21748-2017. Uncertainty estimation was carried out for a pendulum impact tester TE JBW-300 using GSO 11934-2022 type 11 samples according to GOST 9454-78 with a type V-notch concentrator at room temperature. The study found that the greatest contribution to the uncertainty of test results is made by a systematic error associated with traceability to CRM GSO 11934-2022 and a random component of measurements. Accounting of traceability to the CRM GSO 11934-2022 increases the estimate of the uncertainty of the result, but makes it possible to correctly compare the results of two different laboratories, which is of fundamental importance in arbitration tests.

Keywords Mechanical properties · Impact bending · Impact energy · Metrological traceability · Reference material

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Introduction

Structural steel standards and material properties databases generally do not contain information on the uncertainties of the given impact strength values (impact energy, absorbed energy). Therefore, it is important to know the uncertainty of the result when deciding on the conformity of metal products based on the results of an impact bending test.

Formulas for the uncertainty of the impact bending test result are given in [1, 2]. However, ISO 148-1-2016 [1] considers the uncertainty of the average result based on the results of testing five samples, and the interstate standard GOST 9454-78 [3] does not provide for processing the results and calculating the average value.

It should be noted that when estimating the uncertainty in ISO 148-1-2016 [1], the uncertainty of the calibration of the pendulum impact tester is not taken into account, but only its error.

The classical approach to estimating uncertainty according to GOST 34100.3-2017/ISO/IEC Guide 98-3:2008 [4] is based on estimating the uncertainty of the quantities included in the measurement equation. The measured values in the impact test are: the angle of rise and the angle of fall of the pendulum, force exerted by the pendulum, and the distance from the axis of rotation to the center of strike. A modern automated testing machine determines the required values and calculates the impact energy (absorbed energy) according to the underlying algorithm.

The proposed method for estimating the uncertainty of the result of a single impact test includes accounting of the uncertainty of the quantities included in the measurement equation using the results of the pendulum testing machine calibration. The measurement equation additionally takes into account the contribution of traceability to the GSO (CRM) 11934-2022.[1]

The aim of the study is to describe the method for estimating the uncertainty of the results of Charpy pendulum impact test based on information about the testing machine calibration and traceability to the reference material of impact energy (absorbed energy) of steel grade 45 GSO 11934-2022.

Materials and Methods

GSO 11934-2022 was obtained as a result of research and characterization of hot-rolled square section steel according to GOST 2591-2006 [5] from steel grade 45 according to GOST 1050-2013 [6].

Characterization of rolled products in terms of Charpy impact strength testing (absorbed energy, impact energy) was carried out in accordance with the algorithms

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[1] GSO 11934-2022 Reference materials impact work (absorbed energy) steel grade 45. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/19/items/1400120. Accessed 4 August 2022 (In Russ.).

Table 1 Metrological characteristics of the reference material

Certified characteristic	Certified value	Limits of the values of the absolute error at $P = 0.95$
Charpy impact strength testing (impact energy, absorbed energy) at temperature (23 ± 5) °C, J	25.90	± 1.60

and provisions of GOST 8.532-2002 [7] by an interlaboratory experiment. 16 laboratories with experience in studying the mechanical properties of steel were involved. Within 2 months, samples of the reference material were sent to each laboratory for testing in accordance with GOST 9454-78 [3].

The error characteristic of the interlaboratory certification was 1.01 J. The error due to the heterogeneity of the reference material was set to 0.70 J to calculate the limits of the values of the absolute error of the reference material during type approval tests.

The values of the metrological characteristics of the reference material of the impact energy (absorbed energy) of steel grade 45, established as a result of testing batch No. 1, are shown in Table 1.

The testing machine *TE JBW-300* JINAN TESTING EQUIPMENT IE CORPORATION (China) is designed to measure the fracture energy of specimens during Charpy pendulum impact test with two-bearing.

The operating principle of an testing machine is based on measuring the amount of energy spent on the fracture of a sample by a single impact. The amount of energy is defined as the difference between the values of the potential energy of the impact pendulum before impact and after the fracture of the sample.

Elevation and angle of fall measurement readings are calculated by a signal measuring device with an optical-to-electrical transducer, which provides the calculation of the result using a special computer program and displays numerical values on a personal computer monitor with the possibility of further saving and printing the results.

The testing machine TE JBW-300 was calibrated according to ISO 148-2-2016 [8] with traceability to the State primary standard of mass unit GET 3-2008,[2] the State primary standard of the unit of length GET 2-2021,[3] the State primary standard of the flat angle unit GET 22-2014.[4]

A modeling approach based on ISO 21748-2017 [9] was used to estimate measurement uncertainty. A mathematical model has been created, which is an equation that determines the quantitative relationship between the measured value and all the values on which it depends, including all components that contribute to the measurement uncertainty. The standard uncertainties of all individual uncertainty components have

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[2] GET 3-2008 State primary standard of mass unit (kilogram). Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1385582. Accessed 4 August 2022 (In Russ.).

[3] GET 2-2021 State primary standard of the unit of length—meter. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/1387037. Accessed 4 August 2022 (In Russ.).

[4] GET 22-2014 State primary standard of the flat angle unit. Available via FIF EUM. https://fgis.gost.ru/fundmetrology/registry/12/items/397907. Accessed 4 August 2022 (In Russ.).

been estimated. The standard deviations from repeated measurements are the standard uncertainties for the respective components (if a normal distribution can be assumed). The total standard uncertainty is calculated by applying the law of propagation of uncertainty which depends on the partial derivatives for each input value. The expanded uncertainty U is calculated (providing an interval from (y − U) to (y + U) for the measured value y). The coverage factor k = 2 is usually chosen for a normal distribution. The measurement result, together with its uncertainty, is presented in accordance with GOST 34100.3-2017/ISO/IEC Guide 98-3:2008 [4].

Results

The statistical model on which the methods for estimating uncertainty are based is written as Eq. (1) ISO 21748-2017 [9]:

$$KV = FL(\cos\beta - \cos\alpha) + \xi_{GSO} + g_{KV} + e_{KV}, \tag{1}$$

where

F	is the force exerted by the pendulum, N;
L	is the distance from the axis of rotation to the center of strike (point of strike F), m;
β	is the angle of fall of the pendulum, °;
α	is the angle of rise of the pendulum, °;
ξ_{GSO}	is the bias due to traceability;
g_{kv}	is the rounding of the measurement result;
e_{KV}	is the random component of measurement uncertainty under repeatability conditions.

Taking into account the model described by Eq. (1), the measurement uncertainty of KV was estimated using Eq. (2):

$$\begin{aligned} u^2(KV) = {} & \left(\frac{\partial KV}{\partial F}\right)^2 u^2(F) + \left(\frac{\partial KV}{\partial L}\right)^2 u^2(L) \\ & + \left(\frac{\partial KV}{\partial \beta}\right)^2 u^2(\beta) + \left(\frac{\partial KV}{\partial \alpha}\right)^2 u^2(\alpha) \\ & + u^2(\xi_{GSO}) + u^2(g_{KV}) + u^2(e_{KV}) \end{aligned} \tag{2}$$

where

$u^2(F)$	is the uncertainty associated with the contribution due to the force exerted by the pendulum;
$u^2(L)$	is the uncertainty associated with the distance from the axis of rotation to the center of strike;

$u^2(\beta)$	is the uncertainty associated with the angle of fall of the pendulum;
$u^2(\alpha)$	is the uncertainty associated with the angle of rise of the pendulum;
$u^2\left(\xi_{\sigma_B}\right)$	is the uncertainty caused by the uncertainty of the estimate obtained on the basis of measurements of a reference material with a certified value;
$u^2(g_B)$	is the uncertainty due to the rounding of the measurement result;
$u^2(e_{KV})$	is a random component of measurement uncertainty of a reference material under repeatability conditions.

In this example, the measurement error is calculated for a single sample made from the material under test. The measurement result of 105.5 J was obtained on the testing machine TE JBW-300, which was calibrated according to ISO 148-2-2016 [8].

Impact energy measurement result KV with expanded measurement uncertainty U(KV)

$$KV = (105.5 \pm 5.0)\ \text{J}.$$

Discussion

By analyzing the uncertainty budget presented in Table 2, the two largest contributions to the combined standard uncertainty of the result can be distinguished: the uncertainty due to traceability to a certified reference material and the random component of measurements under repeatability conditions.

The contribution due to the random component of measurements of a reference material under repeatability conditions describes the generally random effects occurring in the laboratory.

Since the absolute value (0.6 J) of the random component of the measurement uncertainty of the reference material is comparable to the uncertainty of the reference material (0.8 J), it can be assumed that it is formed due to the heterogeneity of the reference material.

The contribution due to the traceability of the reference material includes a systematic component associated with the implementation of the test procedure in the laboratory, for example, the V-trough concentrator, the coincidence of the length of the pendulum with the center of impact on the sample, etc.

The contribution due to the uncertainty components associated with the values standardized for the impact tester and measured by the automated impact tester system is insignificant for the studied copy of the impact tester.

It should be noted that the last conclusion is based on the calibration results of the impact tester according to the ISO 148-2-2016 method [8] and may be inaccurate for the tested impact testers.

Table 2 Measurement uncertainty budget calculated using pendulum impact tester calibration data

№.	Source of uncertainty	Unit designation	Formula
1	Certified value of the reference material and its uncertainty (based on the reference material certificate)	$KVGSO$ $u(KVGSO)$	$u(KV_{GSO}) = \frac{\Delta(KV_{GSO})}{1.96}$
2	Traceability to the reference material	$u(\xi_{KV})$	$u(\xi_{KV}) = \sqrt{u^2(KV_{GSO}) + \frac{(KV_{GSO} - \overline{KV})^2}{3}}$
3	Random component of the measurement uncertainty of the reference material under repeatability conditions	$u(e_{KV})$	$u(e_{KV}) = \frac{s_{KV} t(P, f)}{\sqrt{n}} = \frac{s_{KV} t_{0.6827.5}}{\sqrt{5}}$
4	Force exerted by the pendulum	$u(F)$ $c(F)$	$\mathrm{c}(F) = KV/F$ $\mathrm{c}(F)u(F)$
5	Distance from the axis of rotation to the center of strike	$u(L)$ $c(L)$	$\mathrm{c}(L) = KV/F$ $\mathrm{c}(L)u(L)$
6	Angle of fall of the pendulum	$u(\beta)$ $c(\beta)$	$\mathrm{c}(\beta) = -FL\sin\beta$ $\mathrm{c}(\beta)u(\beta)$
7	Angle of rise of the pendulum	$u(\alpha)$ $c(\alpha)$	$\mathrm{c}(\alpha) = FL\sin\alpha$ $\mathrm{c}(\alpha)u(\alpha)$
8	Rounding of the measurement result	$u(g_{KV})$	$u(g_{KV}) = \frac{Lc}{2\sqrt{3}}$

(continued)

Table 2 (continued)

№.	Source of uncertainty	Unit designation	Formula
9	Combined standard uncertainty	$u(KV)$	$u(KV) = \sqrt{c^2(F)u^2(F) + c^2(L)u^2(L) + c^2(\beta)u^2(\beta) + c^2(\alpha)u^2(\alpha) + u^2(g_{KV}) + u^2(\xi_{KV}) + u^2(e_{KV})}$
10	Effective number of degrees of freedom	ν_{eff}	$\nu_{eff} = \frac{u^4(KV)}{\frac{u^4(F)}{\nu(F)} + \frac{u^4(L)}{\nu(L)} + \frac{u^4(\beta)}{\nu(\beta)} + \frac{u^4(\alpha)}{\nu(\alpha)} + \frac{u^4(g_{KV})}{\nu(g_{KV})} + \frac{u^4(\xi_{KV})}{\nu(\xi_{KV})} + \frac{u^4(e_{KV})}{\nu(e_{KV})}}$
11	Expanded uncertainty	$U(KV)$	$U(KV) = t_{0.95,\nu_{eff}} \cdot u(KV)$

№.	Used variables, parameters	Example
1	$KV_{GSO} = 25.9\,\text{J}$ $\Delta(KV_{GSO}) = 1.6\,\text{J}$—error value of the certified value of the reference material according to the certificate	$u(KV_{GSO}) = \frac{1.6}{1.96} = 0.816\,\text{J}$
2	$\overline{KV}$—average value of measurements on the reference material	Measured values: 27.4, 26.7, 28.3, 26.3, 25.0 J Average value $\overline{KV} = 26.74\,\text{J}$ $u(\xi_{KV}) = \sqrt{0.816^2 + \frac{(25.9-26.74)^2}{3}} = 0.949\,\text{J}$
3	s_{KV}—standard deviation of measurement results on the reference material $t_{0.6827.5} - Student\prime scoefficient = 1.14$ for n = 5	$s_{KV} = 1.2\,\text{J}$ $u(e_{KV}) = \frac{1.14 \cdot 1.2}{\sqrt{5}} = 0.612\,\text{J}$
4	From the calibration certificate $u(F) = 0.35\text{N}$ $F = 217.82$ N The result for the test material $KV = 105.5$ J	$c(F) = 105.5/217.82 = 0.484$ $c(F)u(F) = 0.484 * 0.35 = 0.169\,\text{J}$
5	From the calibration certificate $u(L) = 0.1$ mm $L = 0.741$ m The result for the test material $KV = 105.5$ J	u(L) = 0.0001 m $c(L) = 105.5/0.741 = 142$ $c(L)u(L) = 0.0001 * 142 = 0.0142\,\text{J}$

(continued)

Table 2 (continued)

№.	Used variables, parameters	Example
6	From the calibration certificate $u(\beta) = 0.06°$ $L = 0.741$ m $F = 217.82$ N For the test material $KV = 105.5$ J $\beta = 109°$	$0.06° = 0.00105$ rad $\mathrm{c}(\beta) = -217.82 \cdot 0.741 \cdot \sin 109° = -153$ $\mathrm{c}(\beta)u(\beta) = 153 \cdot 0.00105 = 0.153$ J
7	From the calibration certificate $u(\alpha) = 0.06°$ $L = 0.741$ m $F = 217.82$ N For the impact tester $\alpha = 160°$	$0.06° = 0.00105$ rad $\mathrm{c}(\alpha) = 217.82 \cdot 0.741 \cdot \sin 160° = 54.9$ $u(\alpha) = 54.9 \cdot 0.001 = 0.0549$ J
8	Lc = 0.1 J	$u(g_{KV}) = \frac{0.1}{3.46} = 0.0289$ J
9		$u(KV) =$ $\sqrt{0.169^2 + 0.0142^2 + 0.153^2 + 0.0549^2 + 0.0289^2 + 0.949^2 + 0.612^2} =$ 1.17 J
10	$\nu(F) = \infty$ $\nu(L) = \infty$ $\nu(g_{KV}) = \infty$ $\nu(\xi_{KV}) = 2 - 1 = 1$ $\nu(e_{KV}) = 5 - 1 = 4$	$\nu_{eff} = \frac{1.2^4}{0+0+0+\frac{0.996^4}{1}+\frac{0.612^4}{4}} = 2.035$
11	$t_{0.95,\nu_{eff}} = 4.30$	$U(KV) = 1.17 \cdot 4.30 = 5.03$ J

Conclusion

The uncertainty of the result of the impact test according to GOST 9454-78 [3] for the pendulum impact tester TE JBW-300 was estimated, taking into account the traceability to the GSO (CRM) 11934-2022.

The proposed method for estimating the uncertainty of the impact test result can be used in the event that the calibration results of the pendulum impact tester according to ISO 148-2-2016 [8] are available.

The main contributor to the uncertainty of the impact test result is the contribution due to the traceability to a certified reference material, which can be explained at least by the shape of the notch that the laboratory makes and the mismatch between the impact center of the pendulum and the location of the sample; these factors cannot be estimated by directly calibrating the impact tester.

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Author Contributions Chentsova Iu. S.—implementation of a formal analysis, conducting research work; Tolmachev V. V.—development of the research concept, implementation of a formal analysis; Zabelina A. A.—conducting research work.

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Mathematical Methods for Characterization of Reference Materials and Risks of False Solutions

New Algorithms for Estimating the Certified Characteristic of CRMs for Substances and Materials Using Interlaboratory Certification

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Abstract In this work, algorithms for specifying certified reference materials (CRMs) are developed based on the data modeling of interlaboratory experiments containing hidden uncertainties. These algorithms aid in estimating hidden uncertainties and, thus, in correcting the data and obtaining a consistent value of the certified characteristic. Using the Monte–Carlo method, the properties of hidden uncertainty estimates are studied. The proposed algorithms are compared with those conventionally used.

Keywords Certified reference materials · CRMs · Interlaboratory experiment · Statistical model of interlaboratory experiments · Uncertainty of interlaboratory experiments results · Consistent value of CRMs characteristic · CRMs specification

Introduction

Currently, interlaboratory experiments are widely used to determine the values of certified reference materials (CRMs).

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The corresponding mathematical algorithms for evaluating the value of the CRMs characteristic are given in the GOST 8.532-2002 [1] and are based on the assumption that the experimental data obtained by various analytical methods from different laboratories represent a sample from one general totality and have a normal (even if contaminated) probability distribution. However, based on international comparisons of CRMs performed by national metrological institutes when assessing the reference value of the comparison, the need for considering the uncertainty of measurement results in a specific laboratory has been established [2]. A possible statistically significant influence of laboratory effects on the evaluation of the reference value has been established in association with a deviation from the normal distribution of random effects which can be described by the Laplace distribution, leading to hidden uncertainties or displacements in interlaboratory experiments (see the summary of such works [3]).

This paper proposes a general statistical model presenting the measurement results of interlaboratory experiments as a set of representatives from different general populations.

The data obtained from the laboratories is a set of the pairs $\{x_i, u_i\},\ i = \overline{1, n}$,

$$x_i = x + \Delta_i + \xi_i, \quad i = \overline{1, n}, \tag{1}$$

where x_i is the result of measurements in the ith laboratory, x is the true value of the measured value; Δ_i is an unknown bias of the result in the ith laboratory; ξ^i is a random error in the result of the ith laboratory, leading to a standard uncertainty u_i of the type A (considered known), n is the number of laboratories participating in the interlaboratory experiment.

Note that for the data model of Eq. (1), the squared standard uncertainty of the ith laboratory result has the form

$$u^2(x_i) = E(x_i - x)^2 = \Delta_i^2 + u_i^2, \tag{2}$$

that is, it contains a hidden part in the form of a squared bias.

Due to the central limit theorem of the probability theory, random variables, as well as the measurement results obtained by processing repeated measurements (such as the artithmetic mean, sample median, etc.) have a distribution close to normal. If the laboratory data are consistent, that is, they satisfy the chi-squared test, then the optimal estimate (maximum likelihood estimate) is the weighted mean of the laboratory results with weights inversely proportional to their variances. However, hidden biases in the results of the majority of cases, which leads to hidden uncertainties, make the laboratory data inconsistent.

Based on this model, the methods for estimating hidden uncertainties and biases of the results of interlaboratory experiments have been developed. Data correction algorithms have been created to make the data consistent and apply the optimal procedure for evaluating the value of the CRMs characteristic and their uncertainty.

Description of Algorithms for Processing the Results of Interlaboratory Experiments

The developed algorithms are based on the procedure for finding the largest consistent data subset [4, 5] submitted by laboratories, as well as the necessary for consistency correction of the data from the laboratories that were not included in this subset. This has required calculating the weighted average.

$$\overline{x} = \left(\sum_{i=1}^{n} u_i^{-2}\right)^{-1} \sum_{i=1}^{n} u_i^{-2} x_i, \tag{3}$$

and conducting a data consistency test—checking the fulfillment of the condition

$$\sum_{i=1}^{n} \frac{(x_i - \overline{x})^2}{u_i^2} \le \chi^2(P; n-1), \tag{4}$$

where χ^2 $(P;$ n − 1) the P-quantile of the chi-squared distribution with n − 1 degrees of freedom. If the condition (4) is met, then the data of all the laboratories are consistent, the estimate (3) is optimal and has a standard uncertainty

$$u = \left(\sum_{i=1}^{n} u_i^{-2}\right)^{-\frac{1}{2}}. \tag{5}$$

Otherwise, the numbering of laboratories is organized in such a way that

$$\frac{(x_1 - \overline{x})^2}{u_1^2} \le \frac{(x_2 - \overline{x})^2}{u_2^2} \le \cdots \le \frac{(x_n - \overline{x})^2}{u_n^2}. \tag{6}$$

The laboratory data with the number n is excluded and the values (3) and (4) are recalculated, now for n − 1 participants. The consistency condition (4) is checked for them. The process is repeated until for the remaining k participants, the consistency condition is finally met:

$$\sum_{i=1}^{k} \frac{(x_i - \overline{x}_k)^2}{u_i^2} \le \chi^2(P; k-1), \overline{x}_k = \left(\sum_{i=1}^{k} u_i^{-2}\right)^{-1} \sum_{i=1}^{k} u_i^{-2} x_i. \tag{7}$$

Thus, the maximum consistent data subset is found. To reconcile the excluded data, the latter must be corrected.

Consider the data of the last excluded participant with the number $k + 1$, for which the value of

$$\frac{(x_{k+1}-\overline{x}_{k+1})^2}{u_{k+1}^2}, \tag{8}$$

is too big for the chi-squared test to be performed. To include the data of this participant into the consistent subset, they need to be adjusted so that the value (8) decreases to the value at which the chi-squared test can be performed. This can be achieved in two ways:

(1) by increasing the uncertainty of the data of the $(k+1)$th participant, which leads to an increase in the denominator in (8);
(2) by correcting the measurement result in the data of the $(k+1)$th participant, leading to a decrease in the numerator in (8).

Consider the first method.

Build a function

$$g_{k+1}(\lambda)=\sum_{i=1}^{k}\frac{(x_i-\overline{x}_{k+1}(\lambda))^2}{u_i^2}+\frac{(x_{k+1}-\overline{x}_{k+1}(\lambda))^2}{u_{k+1}^2+\lambda},\ \ \lambda\geq 0, \tag{9}$$

where

$$\overline{x}_{k+1}(\lambda)=\left(\sum_{i=1}^{k}u_i^{-2}+(u_{k+1}^2+\lambda)^{-1}\right)^{-1}\left(\sum_{i=1}^{k}u_i^{-2}x_i+(u_{k+1}^2+\lambda)^{-1}x_{k+1}\right). \tag{10}$$

The function $g_{k+1}(\lambda)$ monotonically decreases since

$$\frac{dg_{k+1}(\lambda)}{d\lambda}=-\frac{(x_{k+1}-\overline{x}_{k+1}(\lambda))^2}{(u_{k+1}^2+\lambda)^2}<0. \tag{11}$$

The lower limit of the function (9) values at $\lambda\to\infty$ is defined by the left side of the inequality (7), and it does not exceed $\chi^2(P;k-1)$. Since $\chi^2(P;k-1)<\chi^2(P;k)$, there is a value $\lambda=\sigma_{k+1}^2$, at which

$$g_{k+1}(\sigma_{k+1}^2)=\sum_{i=1}^{k}\frac{(x_i-\overline{x}_{k+1}(\sigma_{k+1}^2))^2}{u_i^2}+\frac{(x_{k+1}-\overline{x}_{k+1}(\sigma_{k+1}^2))^2}{u_{k+1}^2+\sigma_{k+1}^2}\leq\chi^2(P;k). \tag{12}$$

This value can be found by sequentially calculating the values of the function $g_{k+1}(\lambda)$ with some step along λ, starting with $\lambda=0$. Thus, by increasing the standard uncertainty of the $(k+1)$th participant of the interlaboratory experiment, a consistent subset of now $k+1$ participants is obtained.

Continuing this process, including by correcting the results of all new participants from the excluded data when building the largest consistent subset, we receive a consistent value of the CRMs characteristic

$$\bar{x}_n(\sigma_{k+1}^2, ...\sigma_n^2) = \left(\sum_{i=1}^{k} u_i^{-2} + \sum_{i=k+1}^{n} (u_i^2 + \sigma_i^2)^{-1}\right)^{-1}$$
$$\left(\sum_{i=1}^{k} x_i u_i^{-2} + \sum_{i=k+1}^{n} x_i (u_i^2 + \sigma_i^2)^{-1}\right), \tag{13}$$

the squared standard uncertainty of which has the form

$$u^2(\overline{x}_n(\sigma_{k+1}^2, ...\sigma_n^2)) = \left(\sum_{i=1}^{k} u_i^{-2} + \sum_{i=k+1}^{n} (u_i^2 + \sigma_i^2)^{-1}\right)^{-1}. \tag{14}$$

The found parameter values $\{\sigma_i^2\}_{i=k+1}^n$ in accordance with (2) are the estimates of the values Δ_i^2, and they characterize the hidden standard uncertainties of the laboratories participating in the interlaboratory experiment.

Consider now the second algorithm for matching the data of the interlaboratory experiment by correcting the results of the participants who did not fall into the largest consistent subset. Write the correcting parametric family as

$$\hat{x}_{k+1}(\mu) = x_{k+1} - \mu \cdot \mathrm{sign}(x_{k+1} - \overline{x}_k), \quad \mu \geq 0, \tag{15}$$

as well as the function

$$f_{k+1}(\mu) = \sum_{i=1}^{k} \frac{(x_i - \overline{x}_{k+1}(\mu))^2}{u_i^2} + \frac{(\hat{x}_{k+1}(\mu) - \overline{x}_{k+1}(\mu))^2}{u_{k+1}^2}, \tag{16}$$

where

$$\overline{x}_{k+1}(\mu) = \left(\sum_{i=1}^{k+1} u_i^{-2}\right)^{-1} \left(\sum_{i=1}^{k} x_i u_i^{-2} + \hat{x}_{k+1}(\mu) u_{k+1}^{-2}\right). \tag{17}$$

It can be shown that $\frac{df_{k+1}(\mu)}{d\mu} < 0$ and the function (16) decrease monotonously at $0 \leq \mu \leq |\overline{x}_k|$.

There is a value $\mu = \left|\hat{\Delta}_{k+1}\right|$ at which

$$f_{k+1}(\hat{\Delta}_{k+1}) \leq \chi^2(P; k). \tag{18}$$

This value can be found by sequentially calculating the values of the function $f_{k+1}(\mu)$ with some step along μ, starting with $\mu = 0$.

Thus, correcting the result of the $(k + 1)$th participant of the interlaboratory experiment,

$$\hat{x}_{k+1}(\hat{\Delta}_{k+1}) = x_{k+1} - \left|\hat{\Delta}_{k+1}\right| \mathrm{sign}(x_{k+1} - \overline{x}_k) = x_{k+1} - \hat{\Delta}_{k+1}, \tag{19}$$

a consistent subset of now $k + 1$ participants is obtained.

Continuing this process, as well as by adjusting the results of all the new participants from those excluded in the process of constructing the maximum consistent subset, one can obtain the consistent value of the certified characteristic;

$$\overline{x}_n(\hat{\Delta}_{k+1}, \ldots \hat{\Delta}_n) = \left(\sum_{i=1}^{n} u_i^{-2}\right)^{-1} \left(\sum_{i=1}^{k} x_i u_i^{-2} + \sum_{i=k+1}^{n} \hat{x}_i(\hat{\Delta}_i) u_i^{-2}\right), \tag{20}$$

the squared standard uncertainty of which has the form

$$u^2(\overline{x}_n(\hat{\Delta}_{k+1}, \ldots \hat{\Delta}_n)) = \left(\sum_{i=1}^{n} u_i^{-2}\right)^{-1}. \tag{21}$$

The parameters $\{-\hat{\Delta}_i\}_{i=k+1}^{n}$ estimate the hidden displacements of the measurement results from the laboratories participating in the interlaboratory experiment in accordance with the measurement results model (1).

Results

To study the properties of estimates of hidden uncertainties (the first algorithm, we name it "the weighted average with uncertainty correction") and the hidden biases (the second algorithm, "the weighted average with the correction of results"), the Monte–Carlo experiment was set up. The laboratory data were modeled according to the model (1).

$$x_i = 10 + \Delta_i + \xi_i, i = \underline{1.15}$$

where the biases were selected as

$$\Delta_i = \pm 0.1 \cdot i,$$

(the sign chosen randomly), and the random component of the margin of error was generated from a normal totality $N(0, u)$ with zero mathematical expectation and standard deviation $u = 0.5$. The estimates values for the hidden uncertainties and hidden displacements for the results from the maximum consistent subset were assumed to be zero. The data generation was performed 260 times. As typical examples, the histograms of estimates for the first six laboratories are shown in Fig. 1: the left column—for the algorithm with the uncertainty correction, the right—for the algorithm with the results correction. The red vertical line indicates the true values (i.e., the values of the parameters set in the software when modeling the distributions by

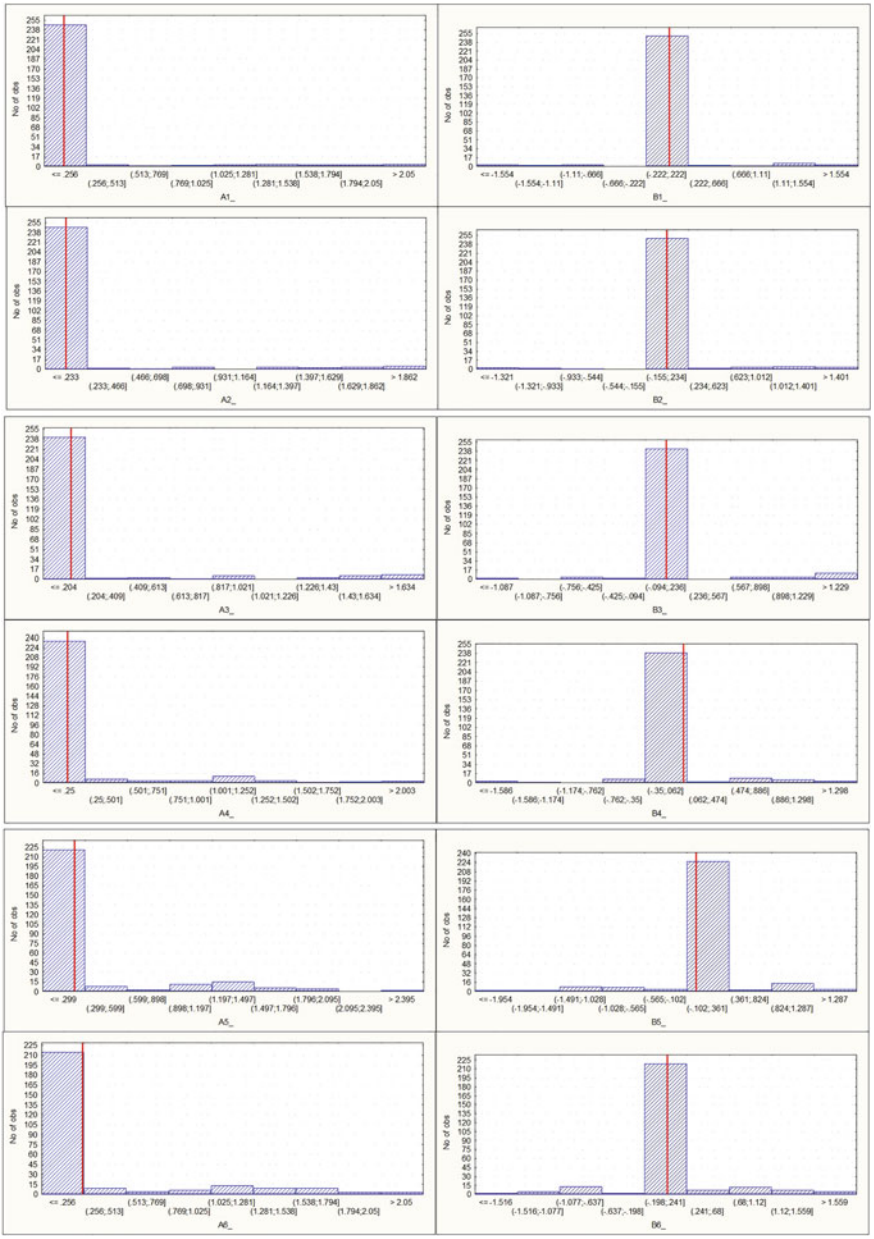

Fig. 1 Histograms of estimates of displacement modules for the first algorithm (left column) and displacements given the sign for the second algorithm (right column) (A1–A6, B1–B6—laboratory numbers; "No of obs"—number of results)

Table 1 The comparison of various estimates when processing the data from a model interlaboratory experiment

Evaluation	RMSD* result
Arithmetic mean	0.41
Median	0.25
Weighted mean without data correction	0.36
Weighted mean with uncertainty correction	0.27
Weighted mean with results correction	0.23

*Root-mean-square deviation

the Monte–Carlo method) of the displacement modules, for the first algorithm, and the displacements given the sign, for the second algorithm.

As the Fig. 1 shows, there is a good correspondence of the estimate values to the specified hidden parameters at the conditions when their values are comparable in the magnitude to random errors.

To compare the algorithms described above with the known estimation methods, another numerical experiment using the Monte–Carlo method was conducted. Laboratory data were modeled according to model (1).

Hidden displacements and random margin errors were selected from the normal aggregates

$$\Delta_i \sim N(0, \sigma_i), \xi_i \sim N(0, u_i),$$

where σ_i and u_i were randomly selected, the first—from the general totality with an exponential distribution density $e(x) = \exp(-x), x \geq 0$, the second—from the interval $[0.1, 0.5]$. The root-mean-square deviation was calculated for various estimates. The results are presented in Table 1.

As Table 1 shows, the new weighted mean algorithm with laboratory results correction has the least margin error among the compared estimates.

The developed algorithms were also tested on the real data. Figures 2 and 3 illustrate the work of algorithms in processing the results of interlaboratory experiments to measure the calorific value of coal. The blue indicates the maximum consistent subset of results, black—non–consistent results, green—adjusted uncertainties in Fig. 2 and the adjusted results in Fig. 3.

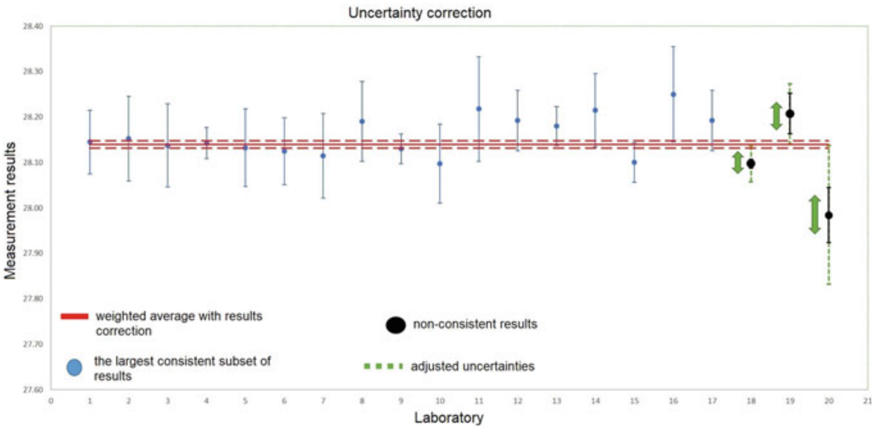

Fig. 2 Interlaboratory experiment results coordination by means of uncertainty correction

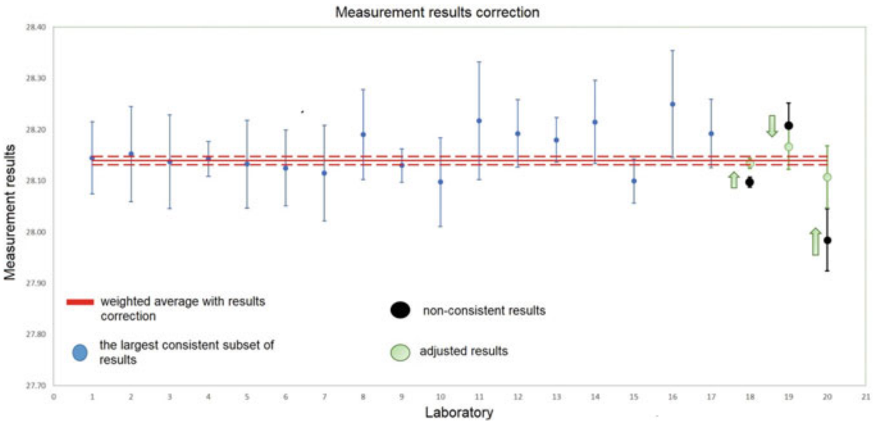

Fig. 3 Interlaboratory experiment results coordination by means of results correction

Conclusion

1. The model proposed in this paper for describing the results of interlaboratory experiments in the form of a set of representatives from various general aggregates allows, when evaluating the CRMs value, to account for the possibility of hidden uncertainties (or displacements) due to the influence of the measurement results in this laboratory by means of characteristic features of the methods, techniques and measuring instruments, CRMs, technical means, and reagents used for measuring the value of the CRMs characteristic.

2. This model is more accurate compared to the traditional statistical model of interlaboratory experiments based on the assumption of statistical averaging of possible displacements of measurement results obtained in different laboratories using various methods, procedures, and measuring instruments.
3. It is shown that the hidden uncertainties (or displacements) of the measurement results of interlaboratory experiments must be identified and considered when assessing the uncertainty of the CRMs value.
4. The research conducted in this paper has confirmed the possibility of using the proposed algorithms for processing measurement information to correct the data of interlaboratory experiments. These algorithms can be effective in interlaboratory experiments for a small number of laboratories, as well as for high financial, material, and time costs required for conducting experiments.
5. The new algorithms proposed in the paper for estimating the value of the CRMs characteristics undoubtedly require further study, but the existing research already shows they deserve attention when revising GOST 8.532-2002 State system for ensuring the uniformity of measurements. Certified reference materials of composition of substances and materials. Interlaboratory metrological certification.

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Contribution of the Authors Aronov P. M.—development and analysis of mathematical algorithms, conducting mathematical research, description of algorithms, text composing; Sobina E. P.—concept and initiation of research, methodological support, overall management of the work, analysis of results; Migal P. V.—methodological support, general management of work, analysis of results; Kremleva O. N.—participation in editing the article, provision of real experimental data in examples to assess the applicability of the proposed models and algorithms in metrological practice in the field of CRMs; Firsanov V. A.—programming of algorithms and numerical experiments; Medvedevskikh S. V.—participation in the research work in terms of statistical processing of the experimental data.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference "Reference Materials in Measurement and Technology" (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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On the Stability Assessment of Reference Materials

Pavel V. Migal, Egor P. Sobina, Petr M. Aronov, Olga N. Kremleva, Valeriya V. Studenok, Valery A. Firsanov, and Sergey V. Medvedevskikh

Abstract As well as presenting comparative characteristics of domestic and international approaches to assessing the instability of reference materials, the paper describes proposed changes in R 50.2.031–2003. A proposed mathematical apparatus is based on an algorithm of actions for estimating the standard uncertainty from instability and the validity period of a reference material (RM). Approaches to estimating uncertainty from instability in cases of the absence or presence of a significant trend in a RM's certified characteristic over time are considered. The minimum number of measurements necessary to study the stability of an RM is determined and justified. Smoothing of measurement results when assessing the stability of an RM is shown to lead to an overestimation of the period of validity.

Keywords Reference material · CRM · Stability study · Isochronous measurements · Uncertainty of reference materials

Abbreviations

RM	Reference material
SD	Standard deviation

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$X(\tau)$	Dependence of the certified value on time
a	Slope coefficient of linear equation $X(\tau)$
τ	Point in time of RM storage
X_0	Certified value (at time $\tau_0 X(\tau_0) = X_0$)
$\tilde{X}_n$	Result of determining the RM certified characteristic at the *n*th moment of time
$\hat{X}(\tau_n)$	Predicted value according to the estimated parameters of the linear model $(\hat{a}, \hat{X}_0)$
N	Number of measurement results obtained during the RM stability study $(n = 0, 1, \ldots, N - 1)$
S	Random component of the measurement error $\tilde{X}_n$
Δ_T	Target RM instability error value
$\hat{\Delta}_T$	Obtained RM instability error value
τ_Γ	Target RM expiration date value
$\hat{\tau}_\Gamma$	Obtained RM expiration date value
$S(\varepsilon)$	Estimated SD of regression residuals
$S\left(\hat{X}(\tau)\right)$	Estimated SD regression line
$t_{p,N-2}$	Two-sided Student’s coefficient for confidence probability p
$S(\tau_\Gamma)$	SD errors from instability
$u(\tau_\Gamma)$	Standard uncertainty from instability
τ_v	Moment of time at isochronous study
T_0, T_1	Storage temperatures of rms under normal conditions and under artificial deterioration

Introduction

Reference materials (RMs) generally represent a means of storing and transmitting a unit of physical quantity during verification and calibration of measuring instruments for the composition and properties of substances, i.e., they perform the functions of measurement standards. Obviously, in order to transfer a unit of a quantity stored by an RM, it is necessary to take into account the uncertainty of the value of this quantity. One of the sources of uncertainty of the certified value of the RM is its instability over time. In 2003, a recommendation was developed to evaluate the stability characteristics of RMs R 50.2.031–2003 [1]. Currently, some approaches described in R 50.2.031–2003 are not fully consistent with international practice, which is based on the application of approaches described in ISO Guide 35:2017 [2].

Comparative Analysis

The approaches described in R 50.2.031–2003 and ISO Guide 35 are based on an obvious assumption, which is that the certified value of the composition or a property of the RM may vary over time. Based on this statement, a linear model of such behavior is proposed, for example

$$X(\tau) = X_0 + a \cdot \tau, \tau \geq 0, \tag{1}$$

where X_0 is the established certified value of the RM; a is the slope coefficient of the linear equation; τ is the time interval.

To assess the characteristics of the error from instability for a certain period, N measurement results of the certified characteristic $\tilde{X}_n$ ($n = 0, 1, \ldots, N - 1$) are obtained at time points $\tau_0 = 0, \tau_1, \ldots, \tau_{N-1}$.

The main difference between the mathematical apparatus in R 50.2.031–2003 and ISO Guide 35:2017 is that the Russian recommendation assumes smoothing of measurement results. The smoothing coefficient, which depends on the ratio of the random component of the error of the measurement technique used to the limit of the permissible error of the studied RM, can take values from 0.1 to 0.3 ($\alpha\left(\frac{S}{\Delta}\right) \in 0.1 \ldots 0.3$, $\frac{S}{\Delta} \leq 2$). It is known that any smoothing, by reducing the random error, introduces a systematic error. The developers of R 50.2.031–2003 probably introduced such an approach to reduce the impact of emissions that may be present among the measurement results, as well as to reduce the impact of random error of the methodology on the evaluation of the shelf life of the RM. At the same time, the use of smoothing in some cases (for example, at $a \to 0$) can lead to a significant overestimation of the period of validity. To illustrate this effect, the authors conducted a synthetic test with the following parameters: $\frac{S}{\Delta} \approx 1$; $N = 21$; $a \to 0$. The results of the validity period assessment are presented graphically in Fig. 1, where it can be seen that the confidence bounds of Eq. (1) exceed the target error from instability for a period of 27 months, while the calculation according to R 50.2.031–2003 leads to a validity period of more than 50 months.

Selecting the Number of Dimensions

ISO Guide 35:2017 does not set out clear criteria for choosing the duration of RM studies or the required number of measurement results to assess the stability of an RM. This is in contrast to R 50.2.031–2003, where recommendations are given for the choice of N based on the ratio of the random component of the error of the measurement technique used to the limit of the permissible error of the studied RM. These recommendations are given in the form of a table without explaining the underlying rationale of the numerical value of N.

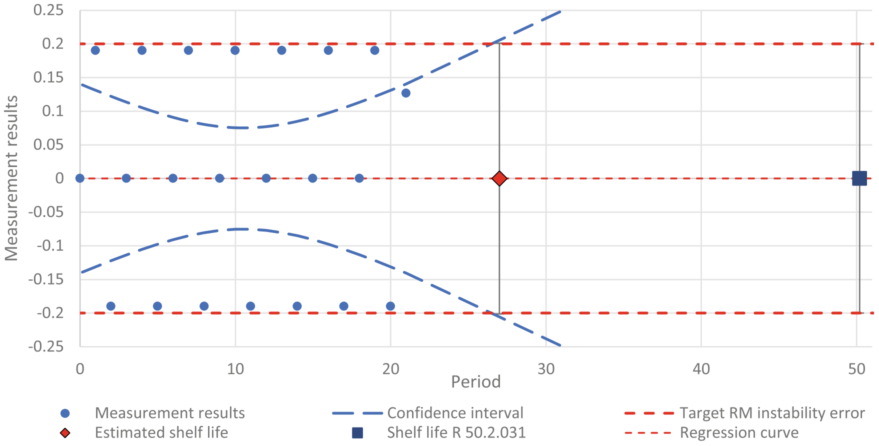

Fig. 1 Graphical representation of the results of the validity period evaluation with the following parameters: $\frac{S}{\Delta} \approx 1;\ \ N = 21;\ \ a \to 0$

As a result of research aimed at updating R 50.2.031–2003, the present authors decided to mathematically substantiate the basic procedures of the experiment to study the stability of the RM. Thus, it is established:

$$\frac{S}{\Delta_T} \le 2,\, N > t_{p,N-2}^2 \cdot \left(1 + 3 \cdot \frac{N-1}{N+1}\right) \cdot \left(\frac{S}{\Delta_T}\right)^2, \tag{2}$$

where S is the random component of the error of the measurement technique;

Δ_T is the target RM instability error value;

$t_{p,N-2}$ is Student's coefficient for the confidence level p.

The meaning of the condition for N is that for a stable RM ($a = 0$), the instability detected due to the influence of random errors in the measurement technique with a probability of p will not exceed the target value. Essentially, meeting this condition provides a confidence interval for regression (1) within the band $\pm\hat{\Delta}_T$ at least for a measurement period to detect instability, as well as to determine the validity period of the RM outside this period.

Estimation of Regression Parameters

To obtain the described confidence interval, estimates of the parameters of the linear model (1) are found using the least squares method:

$$\hat{a} = \frac{\sum_{n=0}^{N-1}\left(\tilde{X}_n - \overline{X}\right)(\tau_n - \overline{\tau})}{\sum_{n=0}^{N-1}(\tau_n - \overline{\tau})^2}, \tag{3}$$

$$\hat{X}_0 = \overline{X} - \hat{a} \cdot \overline{\tau}, \tag{4}$$

where $\overline{X} = \frac{1}{N}\sum_{n=0}^{N-1} \tilde{X}_n$, $\overline{\tau} = \frac{1}{N}\sum_{n=1}^{N-1} \tau_n$.

And statistical estimates of standard deviations (SD) of measurement errors and the constructed dependence [3]:

$$S(\varepsilon) = \sqrt{\frac{1}{N-2}\sum_{n=0}^{N-1}\left(\tilde{X}_n - \hat{X}(\tau_n)\right)^2}, \tag{5}$$

$$S\left(\hat{X}(\tau)\right) = S(\varepsilon) \cdot \sqrt{\frac{1}{N} + \frac{(\tau - \overline{\tau})^2}{\sum_{n=0}^{N-1}(\tau_n - \overline{\tau})^2}}. \tag{6}$$

It is obvious that the certified value of the RM for the duration of the entire validity period (τ_Γ) must be within the confidence interval taking into account the predicted value of the certified characteristic, i.e., the inequality must be fulfilled

$$\hat{X}(\tau) - t_{p,N-2} \cdot S\left(\hat{X}(\tau)\right) \le X(\tau) \le \hat{X}(\tau) + t_{p,N-2} \cdot S\left(\hat{X}(\tau)\right). \tag{7}$$

RM Stability Characteristics

Based on the above, it is possible to evaluate the stability characteristics of the RM:

(a) determine the **error from instability** based on the target expiration date according to the formula

$$\hat{\Delta}_T(\tau_\Gamma) = \left|\hat{a}\right| \cdot \tau_\Gamma + t_{p,N-2} \cdot S\left(\hat{X}(\tau_\Gamma)\right), \tag{8}$$

(b) determine the **expiration date** ($\hat{\tau}_\Gamma$) based on the target error from instability (Δ_T) relatively as the positive root of the quadratic equation τ_Γ

$$\frac{1}{N} + \frac{(\tau_\Gamma - \overline{\tau})^2}{\sum_{n=0}^{N-1}(\tau_n - \overline{\tau})^2} = \frac{(\hat{\Delta}_T - \left|\hat{a}\right| \cdot \tau_\Gamma)^2}{(t_{p,N-2} \cdot S(\varepsilon))^2}. \tag{9}$$

To illustrate the calculation, let us consider the modified example from R 50.2.031–2003 (Fig. 2), which presents results of measurements of the mass fraction of crude fat during half of the expected period of validity (24 months). Based on these measurement results, the dependence $X(\tau) = -2.6923 \cdot 10^{-3} \cdot \tau + 8.1656$ is constructed along with the confidence limits $t_{p,N-2} \cdot S\left(\hat{X}(\tau)\right)$ estimated at $P = 0.95$.

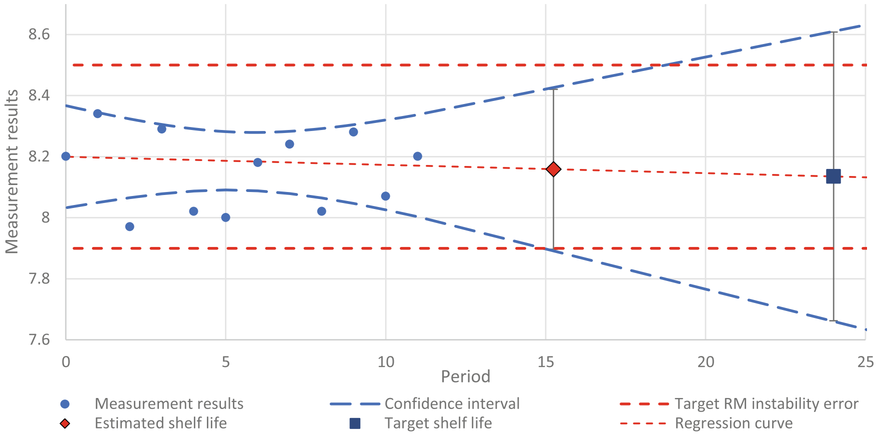

Fig. 2 Example of an estimate of the period of validity and target error from RM instability according to the proposed algorithms

Table 1 shows the initial data and calculation results for the example described above. The values of the required parameters are rounded to 6 digits for the purpose of validating calculations using the algorithms proposed in the article.

Figure 2 depicts the meaning of formula (8): this is the point at which inequality (7) ceases to be fulfilled; that is, the predicted certified value exceeds the target error from instability, taking into account the confidence boundaries of the regression line according to (6) [4]. This approach is justified when the error/uncertainty from instability makes a significant contribution to the accuracy of the certified value. In

Table 1 Initial data and calculations

Input data		Calculated parameters	
Period, month	Result, %	Parameter	Value
0.00	8.20	SD of regression errors $S(\varepsilon)$	0.134408
1.00	8.34	Number of degrees of variance $N-2$	10
2.00	7.97	Student's coefficient t	2.23
3.00	8.29	Estimation of the SD regression line $S(X)$	0.212235
4.00	8.02	Target period of validity value τ_Γ	24
5.00	8.00	Confidence limits of error from instability $\hat{\Delta}_T(\tau_\Gamma)$	0.537505
6.00	8.18	Standard uncertainty from instability $u(\tau_\Gamma)$	0.213053
7.00	8.24	Permissible limits of error from instability Δ_T	0.3
8.00	8.02	Obtained RM expiration date value $\hat{\tau}_\Gamma$	15.24669
9.00	8.28	Confidence limits of error from instability $\hat{\Delta}_T\left(\hat{\tau}_\Gamma\right)$	0.302870
10.00	8.07	Standard uncertainty from instability $u\left(\hat{\tau}_\Gamma\right)$	0.118103
11.00	8.20	–	–

such cases, it is advisable to sacrifice the period of validity to ensure the specified accuracy.

On the other hand, a long validity period may sometimes prevail over the accuracy of the certified value, in which case the error/uncertainty from instability will not affect the consumer properties of the RM. Then it will be possible to estimate the contribution to the accuracy of the certified value from instability using the formula (9).

Period of Stability Research

Based on the analysis of the components of the instability error (see (8) and the previously described components), two main sources can be identified:

- The first term at $a \to 0$ also tends to zero; however, at the significance of a it can become the main source of error from instability, since, in this case, the change in the properties/composition of the material over time is obvious;
- The second term is smaller with a greater the value of N and the research period, and a smaller value of $S(\varepsilon)$, which characterizes the regression errors SD.

Figures 1 and 2 show that the confidence interval has the lowest value in the middle of the research period; however, this value increases when predicting the expiration date.

In this regard, it is possible to proceed as follows.

The initial duration of the studies is chosen to be at least half of the expected validity period. After estimating the required number N, at least three measurements X are carried out. Based on the obtained results, preliminary estimates of the instability error are made either based on the target expiration date or on the expiration date based on the target instability error as described above. The measurements are repeated (preferably at regular intervals) until the instability error or the expiration date value meet the established requirements (for example, described in the technical specification for the development of the RM) or until further research becomes impractical (for example, instability of the RM material is detected, which will prohibit the use of this material for the target destination).

Using this approach, it becomes possible to adjust the experimental plan at the early stages of research and more correctly evaluate the accuracy characteristics from the instability of the RM.

Another important component of the duration of instability studies is the dependence of the rate of degradation of the RM on the magnitude of the external influencing factor. If there is such a dependence, then knowledge of this dependence can be used to validate research carried out under conditions of isochronous stability studies [5]. The duration of the study time with accelerated aging of the RM is determined based on the estimated validity period of the RM instance. If the factor of RM instability is the RM storage temperature, then the period of study of RM instability during accelerated aging is determined according to the Van't Hoff rule using the formula

$$\tau_v = \frac{\tau_\Gamma}{\gamma^{\frac{T_1 - T_0}{10}}}, \tag{10}$$

where τ_v is the period of study of RM instability during accelerated aging; τ_Γ is the estimated validity period of RM at storage temperature; T_0, T_1 is the storage temperature of the RM and storage temperature of RM during accelerated aging;

γ is the reaction acceleration coefficient (assumed to be equal to 2 or estimated experimentally).

When using accelerated aging in calculations, the values of $\tau_1, \ldots, \tau_{N-1}$ must be normalized using the equation

$$\tau_n = \tau_{v,n} \cdot \gamma^{\frac{T_1 - T_0}{10}}. \tag{11}$$

In order to estimate the coefficient γ based on the Van't Hoff rule, it is necessary to determine the values τ_v and τ_Γ. For this purpose, an experiment it carried out at different RM storage temperatures [6]. We select such a T_x that $T_0 \le T_x \ll T_1$. Thus

$$\gamma = \left(\frac{\tau_v}{\tau_x}\right)^{\frac{10}{T_1 - T_x}}. \tag{12}$$

Since T_x can be equal to T_0, it is possible to combine classical and accelerated RM stability studies to estimate the acceleration coefficient.

By analogy with the approach described above, an experiment is carried out to assess stability at T_x and T_1. The choice of the duration of studies τ_v at T_1 is made according to the formula (10) where $\gamma = 2$. N measurement results are obtained at T_x and T_1 during τ_v. The corresponding linear regression coefficients $\hat{a}_x$ and $\hat{a}_v$ are calculated by analogy with (3).

The calculation of γ is carried out according to the formula

$$\gamma = \left(\frac{\hat{a}_v}{\hat{a}_x}\right)^{\frac{10}{T_1 - T_x}}. \tag{13}$$

It should be noted that $\hat{a}_x$ may be insignificant (for example, according to Student's criterion) according to the results of the experiment. This may be caused by an insufficiently high value of T_x or a short duration of studies. In such a situation, it is advisable to increase the value of T_x or τ_v.

The known value of the acceleration coefficient makes it possible to more correctly determine the RM stability characteristics, as well as helping to study of RM stability even faster during periodic release and monitoring the stability of subsequent batches of this RM or other analogous RMs. For example, in [7], a study of the stability of

RM solutions of chemical elements was described in which the value $\gamma = 3.2$ was set to significantly reduce the duration of stability studies of similar RMs, as well as to develop an express method for assessing the stability of new RM batches of the same type.

Standard Uncertainty from Instability

Various options for converting the error from instability ($\hat{\Delta}_T(\tau_\Gamma)$) into the corresponding standard uncertainty are possible depending on the properties of the constructed dependence of the certified value from time to time. For example, ISO Guide 35:2017 paragraphs 8.6, 8.7.4 describes a case when a significant trend of linear regression is observed; here, it is recommended to convert the trend into standard uncertainty and include, together with the uncertainty of the expected degradation, the uncertainty of the assigned value (for example, using a uniform distribution if a linear trend is observed). Although a mathematical apparatus for the described recommendations is not given in ISO Guide 35:2017, it is indicated that "the resulting uncertainty for a time-dependent certified value can be applied only to one side of the interval due to the fact that degradation tends to spread only in one direction, which leads to asymmetric uncertainties."

The authors do not deny the possibility of using various options for converting the error from instability to the corresponding standard uncertainty. Specific options should be based on the principles set out in the JCGM 100:2008 Manual on the Expression of Measurement Uncertainty [8]. However, assuming a uniform distribution of the magnitude associated with the trend of linear regression, the standard deviation of the error and the standard uncertainty from the instability of the RM can be calculated by the formula

$$S(\tau_\Gamma) = u(\tau_\Gamma) = \sqrt{\left(\frac{\hat{a} \cdot \tau_\Gamma}{\sqrt{3}}\right)^2 + S\left(\hat{X}(\tau_\Gamma)\right)^2}, \quad (14)$$

where $S(\tau_\Gamma)$ is the standard deviation of error from instability; $u(\tau_\Gamma)$ is the standard uncertainty from instability.

Conclusion

As a result of the work carried out on the revision of R 50.2.031–2003:

- the approaches have been harmonized with international practice described in ISO Guide 35:2017;
- algorithms for estimating the standard deviation and standard uncertainty from the instability of the certified RM characteristic are given;

- technical inaccuracies in algorithms and examples have been eliminated;
- new algorithms have been developed to adequately assess the RM period of validity;
- a specific algorithm of actions for assessing the stability characteristics of the RM is provided;
- criteria data for selecting the duration of RM studies or the required number of measurement results to assess the stability of an RM are given.

Separately, it can be noted that the described algorithms can be used to study both the long-term and short-term stability of an RM [9].

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Author Contributions Migal P. V.—definition of the idea and methodology of the article, literature analysis, experimental data processing, work with the text of the article; Sobina E. P.—concept and initiation of research, methodological support, general management of work, analysis of results; Aronov P. M.—analysis of mathematical algorithms, conducting mathematical research, description of algorithms; Kremleva O. N.—participation in the general editing of the article, methodological support of the research underlying the article; Studenok V. V.—editing the article, providing experimental data in examples to assess the applicability of the proposed models and algorithms in metrological practice in the field of reference materials; Firsanov V. A.—programming algorithms for processing experimental data for evaluation of the stability of standard samples; Medvedevskikh S. V.—participation in research work in terms of statistical processing of experimental data.

Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference “Reference Materials in Measurement and Technology” (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

The authors Medvedevskikh S. V., Sobina E. P., Kremleva O. N. are editors of the book “Reference Materials in Measurement and Technology. RMMT 2022.”

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Appendix 1: Determination of Inequality (2)

The target value of the error from RM instability $\hat{\Delta}_T$ is assumed to be set. Then from the formulas (3), (5) and (7), the equality follows

$$\left|\hat{a}\right| \cdot \tau_\Gamma + t_{p,N-2} \cdot S(\varepsilon) \cdot \sqrt{\frac{1}{N} + \frac{(\tau_\Gamma - \overline{\tau})^2}{\sum_{n=0}^{N-1}(\tau_n - \overline{\tau})^2}} = \hat{\Delta}_T, \tag{15}$$

as well as the inequality

$$t_{p,N-2} \cdot S(\varepsilon) \cdot \sqrt{\frac{1}{N} + \frac{(\tau_\Gamma - \overline{\tau})^2}{\sum_{n=0}^{N-1}(\tau_n - \overline{\tau})^2}} \leq \hat{\Delta}_T. \tag{16}$$

Squaring both parts (16), we get

$$t_{p,N-2}^2 \cdot \frac{S^2(\varepsilon)}{N} \cdot \left(1 + \frac{(\tau_{N-1} - \overline{\tau})^2}{S_\tau^2}\right) \leq \hat{\Delta}_T^2, \tag{17}$$

where

$$S_\tau^2 = \frac{1}{N}\sum_{n=0}^{N-1}(\tau_n - \overline{\tau})^2. \tag{18}$$

Given that the inequality $\tau_\Gamma > \tau_{N-1}$ is satisfied for the expiration date from (17), we obtain

$$t_{p,N-2}^2 \cdot \frac{S^2(\varepsilon)}{N} \cdot \left(1 + \frac{(\tau_{N-1} - \overline{\tau})^2}{S_\tau^2}\right) < \hat{\Delta}_T^2. \tag{19}$$

If measurements are made at regular intervals, that is

$$\tau_n = \Delta\tau \cdot n, n = \underline{0, N-1}, \tag{20}$$

then the values $\overline{\tau}$ and S_τ^2 can be calculated analytically

$$\overline{\tau} = \frac{1}{N}\sum_{n=1}^{N-1}\tau_n = \Delta\tau \cdot \frac{1}{N}\sum_{n=1}^{N-1} n = \Delta\tau \cdot \frac{N-1}{2}. \tag{21}$$

It follows that

$$\overline{\tau} = \Delta\tau \cdot \frac{N-1}{2} \quad \text{and} \quad \tau_{N-1} - \overline{\tau} = \Delta\tau \cdot \frac{N+1}{2}. \tag{22}$$

For the value (18), we obtain

$$S_\tau^2 = \frac{1}{N}\sum_{n=0}^{N-1}(\tau_n - \overline{\tau})^2 = \frac{1}{N} \cdot \sum_{n=1}^{N-1}\tau_n^2 - \overline{\tau}^2 = (\Delta\tau)^2 \cdot \frac{1}{N} \cdot \sum_{n=1}^{N-1} n^2 - (\Delta\tau)^2 \cdot \left(\frac{N-1}{2}\right)^2. \tag{23}$$

Taking into account the formula for summing squares of natural numbers [10], we obtain

$$\frac{1}{N} \cdot \sum_{n=1}^{N-1} n^2 = \frac{(N-1) \cdot (2 \cdot N - 1)}{6}, \tag{24}$$

and for magnitude (23)

$$S_\tau^2 = (\Delta\tau)^2 \cdot \frac{N^2 - 1}{12}. \tag{25}$$

Taking into account formulas (22) and (25), we obtain

$$\frac{(\tau_{N-1} - \overline{\tau})^2}{S_\tau^2} = 3 \cdot \frac{N-1}{N+1}. \tag{26}$$

Thus, for measurements carried out at regular intervals, the inequality (19) takes the form

$$t_{p,N-2}^2 \cdot \frac{S^2(\varepsilon)}{N} \cdot \left(1 + 3 \cdot \frac{N-1}{N+1}\right) < \hat{\Delta}_T^2. \tag{27}$$

It follows from (27) that the number of dimensions N should be chosen as the minimum for which condition (2) is satisfied.

Appendix 2: Quadratic Equation for Determining the RM Period of Validity

It is considered that the maximum allowable error value from instability $\hat{\Delta}_T$ is known. Then, during the expiration date, the error from instability should not exceed the maximum allowable value of $\hat{\Delta}_T(\tau) \leq \hat{\Delta}_T$; as a consequence of the monotonous increase of the function describing the error from RM instability for the moments of time $\tau > \tau_{N-1}$, the expiration date is defined as the positive root of the quadratic equation relative to τ_Γ

$$\hat{\Delta}_T\left(\hat{\tau}_\Gamma\right) = \hat{\Delta}_T. \tag{28}$$

Taking into account formulas (3) and (6), Eq. (8) takes the form

$$\left|\hat{a}\right| \cdot \hat{\tau}_\Gamma + t_{p,N-2} \cdot S(\varepsilon) \cdot \sqrt{\frac{1}{N} + \frac{(\hat{\tau}_\Gamma - \overline{\tau})^2}{\sum_{n=0}^{N-1} (\tau_n - \overline{\tau})^2}} = \hat{\Delta}_T. \tag{29}$$

By transferring the first of the terms from the left side to the right and squaring both parts of the equation, the following quadratic equation is obtained

$$\frac{1}{N} + \frac{(\hat{\tau}_\Gamma - \bar{\tau})^2}{\sum_{n=0}^{N-1}(\tau_n - \bar{\tau})^2} = \frac{\left(\hat{\Delta}_T - \left|\hat{a}\right| \cdot \hat{\tau}_\Gamma\right)^2}{\left(t_{p,N-2} \cdot S(\varepsilon)\right)^2}, \tag{30}$$

then τ_Γ can be expressed as

$$\hat{\tau}_\Gamma = \frac{q + w}{e}, \tag{31}$$

where

$$q = \sqrt{\begin{array}{l} S^2(\varepsilon) \cdot \sum\limits_{n=0}^{N-1}(\tau_n - \bar{\tau})^2 \cdot N \cdot t_{p,N-2}^2 \cdot \Big(N \cdot \hat{\Delta}_T \cdot \Big[\hat{\Delta}_T - 2 \cdot \bar{\tau} \cdot \left|\hat{a}\right|\Big] \\ \quad + \Big[\Big(\bar{\tau}^2 \cdot N + \sum\limits_{n=0}^{N-1}(\tau_n - \bar{\tau})^2\Big) \cdot \hat{a}^2 - S^2(\varepsilon) \cdot t_{p,N-2}^2\Big]\Big) \end{array}}, \tag{32}$$

$$w = S^2(\varepsilon) \cdot \bar{\tau} \cdot N \cdot t_{p,N-2}^2 - \sum_{n=0}^{N-1}(\tau_n - \bar{\tau})^2 \cdot N \cdot \left|\hat{a}\right| \cdot \hat{\Delta}_T, \tag{33}$$

$$e = S^2(\varepsilon) \cdot N \cdot t_{p,N-2}^2 - \sum_{n=0}^{N-1}(\tau_n - \bar{\tau})^2 \cdot N \cdot \hat{a}^2. \tag{34}$$

Appendix 3

Microsoft® Excel® "Calc.xlsm" file with calculation example is available via: https://uniim.ru/calculations/.

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Algorithms for Evaluating the Homogeneity of Reference Materials for the Composition and Properties of Dispersed and Monolithic Materials

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Abstract Reference material (RM) production, characterization, and certification are crucial to ensuring measurement traceability in various industries. One source of RM uncertainty is the standard uncertainty (SU) from heterogeneity. SU evaluation is based on the analysis of variance according to GOST 8.531–2002, ISO Guide 35:2017. The study aims to develop calculation algorithms for the SU from heterogeneity of RMs for the composition and properties of dispersed and monolithic materials. The research objectives: to analyze the algorithms outlined in GOST 8.531–2002 and ISO Guide 35:2017 and, based on them, to develop and test new algorithms for calculating the SU from heterogeneity. The developed algorithms differ from both standards. They were tested on various examples, including simulated data. Their applicability was proved. The research shows that ISO Guide 35:2017 helps to evaluate SU from heterogeneity more effectively. This approach is modernized and considers the smallest representative sample mass. Moreover, the developed algorithm is applicable to studying composition indicators, properties of solid and liquid substances and materials. The updated algorithms will be used to revise GOST 8.531–2002, to align the two standards, to increase confidence in the results of determining RM metrological characteristics in Russia, to ensure measurement traceability internationally.

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Keywords Standard uncertainty from heterogeneity · One-way ANOVA · Two-way ANOVA · Smallest representative sample

Introduction

Ensuring physical and chemical measurement traceability in a particular country is a major objective. Reference materials (RM) are one of the main physical and chemical measurement assurance means used to verify and calibrate measuring instruments. Other RM functions include constructing a graduation (calibration) curve and monitoring the accuracy of certified and standardized measurement procedures. RM production and characterization are central to ensuring the metrological traceability of measurement results while producing substances and materials. Additionally, they are key to maintaining the safety of human life and health, as well as the environment, and controlling product quality. The basic metrological requirements for RMs, their development and approval procedure are set forth in GOST 8.315–2019.

One source of RM uncertainty is the standard uncertainty from heterogeneity. Standard uncertainty evaluation is based on the analysis of variance (ANOVA) performed in accordance with GOST 8.531–2002, ISO Guide 35:2017, and is also considered in [1–6]. In Russia and the CIS countries, GOST 8.531–2002 is commonly used for heterogeneity evaluation. The GOST 8.531–2002 algorithms are widely and successfully adopted to assess RM heterogeneity. Fine examples are RMs for the chemical composition and properties of nanomaterials, the composition of gold-bearing ores, the composition (agrochemical indicators) of typical chernozem, as well as environmental and technogenic media [1–6]. The main advantage of GOST 8.531–2002 consists in describing specific algorithms (a sequence of formulas) with an indication of the minimum number of (a) samples taken and (b) repeated measurements made in them. Moreover, it also considers the mass of analyzed weighed samples and the minimum representative sample when calculating the standard uncertainty from heterogeneity. At the same time, other countries use ISO Guide 35:2017 when determining the metrological characteristics of RMs. This meta-standard differs markedly from the national document when processing measurement results to evaluate heterogeneity in the case when the standard uncertainty from heterogeneity is comparable to the type A standard measurement uncertainty.

We can identify the following basic prerequisites for modifying the algorithms provided in GOST 8.531–2002:

- the statistical models employed to estimate the standard deviation from heterogeneity are not supplied;
- when the standard deviation from heterogeneity is comparable to the random measurement error, the GOST 8.531–2002 algorithms differ from the international standard ISO Guide 35:2017. This leads to other, as a rule, underestimated values of the standard deviation from heterogeneity;

- to evaluate the homogeneity of monolithic materials, GOST 8.531–2002 gives only a particular data processing algorithm for the number of analytical surfaces and the number of repeated measurements equal to 2;
- it is unfeasible to process data with missing experimental values which inevitably appear in practice when outliers occur;
- application is only confined to RM for composition although the general algorithms can hold true for RMs for properties as well.

Thus, to increase confidence in the results of determining RM metrological characteristics in Russia at the international level, it is necessary to harmonize GOST 8.531–2002 and the international ISO Guide 35:2017. The article aims to develop specific algorithms suitable for evaluating the homogeneity of RMs for the composition and properties of dispersed media and monolithic materials. The objectives of the study include the following: (a) analyzing the algorithmic approach when calculating the standard uncertainty from heterogeneity in accordance with the interstate standard GOST 8.531–2002 and the international ISO Guide 35:2017; (b) developing algorithms for calculating the standard uncertainty from heterogeneity; (c) testing these algorithms on various examples, including simulated data, to prove their applicability.

Theoretical Part

Evaluation of Dispersed Media Homogeneity

To study RM heterogeneity, I RM samples are randomly selected. Sampling is performed after preparing and packaging the reference standard material. The mass of each RM sample must be sufficient to make J repeated measurements of the characteristic being certified. For repeated measurements, weighed samples with mass m are selected.

The statistical model of the measurement results on which the estimation algorithm is based is written as:

$$x_{ij} = x + b_i + e_{ij}, \quad i = \overline{1, I}, \quad j = \overline{1, J}, \tag{1}$$

where x is the average value of the characteristic being certified in the reference standard material;

b_i is the content deviation from the mean value in the ith RM sample;

e_{ij} is the random error in measuring the content in the ith RM sample during the jth repeated measurement.

It is assumed that $\{b_i\}$, $\{e_{ij}\}$ are statistical samples of independent random variables from various normal populations and, accordingly, (2) holds true:

$$E[b_i] = E\left[e_{ij}\right] = 0,$$

$$E\left[b_i^2\right] = \sigma_b^2,$$

$$E\left[e_{ij}^2\right] = \sigma_e^2, \quad i = \overline{1, I}, \quad j = \overline{1, J}, \tag{2}$$

where σ_b^2 is the variance from heterogeneity of RM samples with mass m of the reference standard material;

σ_e^2 is the variance of random measurement errors.

When evaluating the homogeneity characteristic, the measurement results are processed in the following order:

The arithmetic mean of the RM characteristic being certified is calculated

$$\hat{x} = \overline{x}_{**} = \frac{1}{I \cdot J} \sum_{i,j} x_{ij}. \tag{3}$$

The arithmetic mean values of the RM characteristics being certified are calculated in each RM sample

$$\overline{x}_{i*} = \frac{1}{J} \sum_{j} x_{ij}. \tag{4}$$

Estimated (standard) deviations of the characteristic being certified from the mean are calculated in RM samples

$$\hat{b}_i = \overline{x}_{i*} - \overline{x}_{**}, \quad i = \overline{1, I}. \tag{5}$$

Random error estimates for repeated measurements are calculated

$$\hat{\mathrm{e}}_{ij} = x_{ij} - \hat{x} - \hat{b}_i = x_{ij} - \overline{x}_{i*}, \quad i = \overline{1, I}, \quad j = \overline{1, J}. \tag{6}$$

The estimated variance (sample variance) of random errors is calculated

$$\hat{\sigma}_e^2 = \frac{1}{I \cdot (J-1)} \sum_{i,j} \hat{e}_{ij}^2 = S_e^2 = \frac{1}{I \cdot (J-1)} \sum_{i,j} (x_{ij} - \overline{x}_{i*})^2. \tag{7}$$

The estimate is unbiased, that is $E\left[S_e^2\right] = \sigma_e^2$.

The sample variance of the mean in RM samples is calculated

$$S_b^2 = \frac{1}{(I-1)} \sum_{i} (\hat{b}_i)^2 = \frac{1}{(I-1)} \sum_{i} (\overline{x}_{i*} - \overline{x}_{**})^2. \tag{8}$$

The sample variance (8) is a biased estimate of heterogeneity variance σ_b^2 since

$$E\left[S_b^2\right] = \sigma_b^2 + \frac{\sigma_e^2}{J}. \tag{9}$$

The difference $S_b^2 - \frac{S_e^2}{J}$ could serve as an unbiased estimate of variance from RM heterogeneity, but it is not positive definite and can take negative values.

Therefore, the estimated variance resulting from the heterogeneity of the RM component being certified is calculated by formula (10)

$$\hat{\sigma}_b^2 = \max\left(S_b^2 - \frac{S_e^2}{J}; \frac{S_e^2}{J} \cdot \sqrt{\frac{2}{I \cdot (J-1)}}\right), \tag{10}$$

where $u\left(\frac{S_e^2}{J}\right) = \frac{S_e^2}{J} \cdot \sqrt{\frac{2}{I \cdot (J-1)}}$ is an evaluation of the standard uncertainty of the value $\frac{S_e^2}{J}$ whose random fluctuations can make the difference $S_b^2 - \frac{S_e^2}{J}$ small positive or even negative when $\sigma_b^2 \approx u\left(\frac{S_e^2}{J}\right)$.

The standard uncertainty from the heterogeneity of the RM value being certified corresponds to the minimum representative RM sample with mass Δm and is evaluated by the formula

$$u_h = \sigma_h = \hat{\sigma}_b \sqrt{\frac{m}{\Delta m}}, \tag{11}$$

where Δm is the minimum RM representative sample.

Note that the GOST 8.531–2002 algorithm is identical to the one presented. However, when the difference $S_b^2 - \frac{S_e^2}{J}$ is negative, the evaluation of the standard uncertainty from heterogeneity is calculated by the formula

$$u_h = \sigma_h = 1/3 \cdot S_e \sqrt{\frac{m}{\Delta m}}. \tag{12}$$

Moreover, it should be noted that ISO Guide 35:2017 does not address the issue of the minimum representative sample and the weighed sample to be analyzed. By default, it is assumed that $\Delta m = m$. Since this condition is not always satisfied in practice, we have developed general formulae that consider the aspect.

Homogeneity Evaluation of Monolithic Materials

The approach described below can also be applied to studying the homogeneity of dispersed materials with measurement methods that require preliminary sample dissolution. This provides the opportunity to perform several repeated measurements in a solution to identify the random component of the error. To study the RM heterogeneity, I samples are randomly selected. $J \geq 2$ analytical surfaces are prepared by cutting the RM in random places lengthways (height-wise). Repeated measurements are performed on each analytical surface. The statistical model of the measurement results on which the evaluation algorithm is based is written as:

$$x_{ijn} = x + b_i + w_{ij} + e_{ijn}, \quad i = \overline{1, I}, \quad j = \overline{1, J}, \quad n = \overline{1, N}, \tag{13}$$

where x is the average content of the component being certified in the reference standard material;

b_i is the deviation of the average content of the component being certified in the ith selected RM sample from the mean value in the RM material;

w_{ij} is the deviation of the content of the component being certified on the jth analytical surface from the mean value in the ith RM sample;

e_{ijn} is the random error of the nth repeated measurement of the content of the component being certified on the jth analytical surface in the ith RM sample.

$\{b_i\}$, $\left\{w_{ij}\right\}$, $\left\{e_{ijn}\right\}$ are supposed to be samples of independent random variables from different normal populations and, therefore, (14) holds true:

$$E[b_i] = E\left[w_{ij}\right] = E\left[e_{ij}\right] = 0$$

$$E\left[b_i^2\right] = \sigma_b^2$$

$$E\left[w_{ij}^2\right] = \sigma_w^2$$

$$E\left[e_{ijn}^2\right] = \sigma_e^2, \quad i = \overline{1, I}, \quad j = \overline{1, J}, \quad n = \overline{1, N}, \tag{14}$$

where σ_b^2 is the variance of material heterogeneity between RM samples,

σ_w^2 is the material heterogeneity variance within each RM sample,

σ_e^2 is the variance of random measurement errors.

The measurement results of evaluating the homogeneity characteristic are processed as follows:

The arithmetic mean of the content of the RM component being certified is calculated

$$\hat{x} = \overline{x}_{***} = \frac{1}{I \cdot J \cdot N} \sum_{i,j,n} x_{ijn}. \tag{15}$$

The arithmetic mean of the content of the component being certified is calculated in different RM samples

$$\overline{x}_{i**} = \frac{1}{J \cdot N} \sum_{n,j} x_{ijn}. \tag{16}$$

The arithmetic mean of the content of the component being certified is calculated on different analytical surfaces of various RM samples

$$\overline{x}_{ij*} = \frac{1}{N}\sum_{n} x_{ijn} \tag{17}$$

The estimates of the heterogeneity magnitude within each RM sample are calculated

$$\hat{w}_{ij} = \overline{x}_{ij*} - \overline{x}_{i**}, \quad i = \overline{1, I}, \quad j = \overline{1, J} \tag{18}$$

The estimates of the heterogeneity magnitude between RM samples are calculated

$$\hat{b}_i = \overline{x}_{i**} - \overline{x}_{***}, \quad i = \overline{1, I} \tag{19}$$

The estimates of the magnitude of random errors in repeated measurements are calculated

$$\hat{e}_{ijn} = x_{ijn} - \hat{x} - \hat{b}_i - \hat{w}_{ij} = x_{ijn} - \overline{x}_{ij*}, \quad i = \overline{1, I}, \quad j = \overline{1, J}, \quad n = \overline{1, N} \tag{20}$$

The random error variance is estimated

$$\hat{\sigma}_e^2 = \frac{1}{I \cdot J \cdot (N-1)}\sum_{i,j,n} \hat{e}_{ijn}^2 = S_e^2 = \frac{1}{I \cdot J \cdot (N-1)}\sum_{i,j,n} (x_{ijn} - \overline{x}_{ij*})^2 \tag{21}$$

The estimated variance is unbiased, i.e. $E\left[S_e^2\right] = \sigma_e^2$.

The sample variance of heterogeneity within the samples is calculated

$$S_w^2 = \frac{1}{I \cdot (J-1)}\sum_{i,j} (\hat{w}_{ij})^2 = \frac{1}{I \cdot (J-1)}\sum_{i,j} (\overline{x}_{ij*} - \overline{x}_{i**})^2 \tag{22}$$

The sample variance (22) is the biased sample variance of the heterogeneity within the σ_w^2 samples since $E\left[S_w^2\right] = \sigma_w^2 + \frac{\sigma_e^2}{N}$.

The *heterogeneity variance within RM samples is estimated*

$$\hat{\sigma}_w^2 = \max\left(S_w^2 - \frac{S_e^2}{N}, \frac{S_e^2}{N} \cdot \sqrt{\frac{2}{I \cdot J(N-1)}}\right) = S_{mic}^2. \tag{23}$$

Here $u\left(\frac{S_e^2}{N}\right) = \frac{S_e^2}{N} \cdot \sqrt{\frac{2}{I \cdot J(N-1)}}$ is an estimate of the standard uncertainty (root-mean-square deviation, RMSD) of $\frac{S_e^2}{N}$.

The sample variance of heterogeneity between RM samples is calculated.

$$S_b^2 = \frac{1}{(I-1)}\sum_i (\hat{b}_i)^2 = \frac{1}{(I-1)}\sum_i (\overline{x}_{i**} - \overline{x}_{***})^2 \tag{24}$$

The sample variance (24) is the biased sample variance of the heterogeneity between the RM samples since $E\left[S_b^2\right] = \sigma_b^2 + \frac{\sigma_w^2}{J} + \frac{\sigma_e^2}{J \cdot N}$.

The *heterogeneity variance between RM samples is estimated*

$$\hat{\sigma}_b^2 = \max\left(S_b^2 - \frac{S_w^2}{J}, \frac{S_w^2}{J} \cdot \sqrt{\frac{2}{I \cdot (J-1)}}\right) = S_{mac}^2 \tag{25}$$

Here $u\left(\frac{S_w^2}{J}\right) = \frac{S_w^2}{J} \cdot \sqrt{\frac{2}{I \cdot (J-1)}}$ is an estimate of the standard uncertainty (RMSD) of $\frac{S_w^2}{J}$.

The standard uncertainty (standard deviation, SD) from the heterogeneity of the RM value being certified is calculated by the formula:

$$u_h = S_h = \sqrt{S_{mac}^2 + S_{mic}^2} \tag{26}$$

Mac (Rus мак)—between-bottle heterogeneity.

Mic (Rus мик)—within-bottle heterogeneity.

Experimental

Based on the developed algorithms, we evaluated standard uncertainty from RM heterogeneity on six typical examples. Each example had the following distinctive features:

Example 1. We considered actual experimental data obtained by analyzing a dispersed material. For this material, there is a significant standard uncertainty from heterogeneity;

Example 2. We considered actual experimental data obtained by analyzing a dispersed material. For this material, there is a standard uncertainty due to heterogeneity comparable to the type A standard measurement uncertainty;

Examples 3–5. We considered simulated data obtained by the Monte Carlo method for different quantities of dispersed RM material samples;

Example 6. We considered actual experimental data obtained by analyzing a monolithic material.

Let Us Consider Several Typical Examples When Evaluating the Heterogeneity of Dispersed Materials

Example 1 The RM material is potassium chloride produced by flotation. The component being certified is potassium ions (K+). The certified characteristic is the mass fraction of potassium ions, %. The number of selected RM samples is I = 10. The number of repeated measurements in the RM samples is J = 2. The mass of samples is m = 1 g. The measurement results are recorded in Table 1. In the example with the performed calculations, the final and intermediate numbers of calculations are intentionally written with 4 decimal places.

According to the results given in Table 1, the average results for samples $\overline{x}_{i*}$, $i = 1, \ldots, 10$ are calculated by formula (4) and written down in the last column of Table 1. The measurement error variance $S_e^2 = 0.0263$ is estimated by formula (7). The overall mean $\overline{x}_{**} = 47.5310$ is calculated by formula (3). The sample variance of the means between samples $S_b^2 = 0.0304$ is calculated by formula (8). Then the difference

$$S_b^2 - \frac{S_e^2}{J} = 0.0304 - \frac{0.0263}{2} = 0.0173 \text{ as well as}$$

$$u\left(\frac{S_e^2}{J}\right) = \frac{S_e^2}{J} \cdot \sqrt{\frac{2}{I \cdot (J-1)}} = \frac{0.0263}{2} \cdot \sqrt{\frac{2}{10 \cdot (2-1)}} = 0.0059 \text{ are calculated.}$$

Since 0.0173 > 0.0059, then $\hat{\sigma}_b^2 = 0.0173$ is obtained by formula (10) to estimate the heterogeneity variance. In this case, the SD due to material heterogeneity is indeed larger than the SD of the measurement precision. Thus, the algorithms provided in

Table 1 Results of mass fraction measurements of potassium ions (%)

RM sample number	Measurement result number		$\overline{x}_{i*}$
	1	2	
1	47.32	47.16	47.24
2	47.37	47.73	47.55
3	47.39	47.34	47.37
4	46.98	47.55	47.27
5	47.67	47.55	47.61
6	47.64	47.68	47.66
7	47.75	47.68	47.72
8	47.69	47.63	47.66
9	47.55	47.67	47.61
10	47.60	47.67	47.64

ISO Guide 35:2017 and GOST 8.531–2002 lead to the same estimates of the standard uncertainty from heterogeneity. Taking account of the minimum representative sample mass $\Delta m = m = 1$ g and formula (11), we obtain

$$u_h = S_h = \sqrt{0.0173 \cdot \frac{1.0}{1.0}} = 0.1314$$

for the maximum standard deviation of heterogeneity in the sample.

Example 2 The RM material is potassium chloride produced by flotation. The component being certified is potassium chloride (KCl). The certified characteristic is the mass fraction of potassium chloride, %. The number of selected RM samples is I = 10. The number of repeated measurements in the RM samples is J = 2. The mass of samples is m = 1 g. The measurement results are recorded in Table 2. In the example with the performed calculations, the final and intermediate numbers of calculations are intentionally written with 4 decimal places.

In the same way as in Example 1, we calculate

$$S_e^2 = 0.1367, \overline{x}_{**} = 95.5698, S_b^2 = 0.0639, S_b^2 - \frac{S_e^2}{J} = -0.0044,$$

$$u\left(\frac{S_e^2}{J}\right) = \frac{S_e^2}{J} \cdot \sqrt{\frac{2}{I \cdot (J-1)}} = 0.0306.$$

Thus, the $S_b^2 - \frac{S_e^2}{J}$ value is negative and cannot be used to estimate the heterogeneity variance. Therefore, according to ISO Guide 35:2017,

$$\hat{\sigma}_b^2 = \max(-0.0044, 0.0306) = 0.0306$$

Table 2 Results of mass fraction measurements of potassium chloride (%)

RM sample number	Measurement result number		$\overline{x}_{i*}$
	1	2	
1	95.32	94.92	95.120
2	95.44	95.50	95.470
3	95.32	96.415	95.868
4	94.90	95.40	95.150
5	95.51	95.77	95.640
6	95.65	95.94	95.795
7	95.40	95.81	95.605
8	95.51	95.94	95.725
9	95.37	95.95	95.660
10	95.40	95.93	95.665

$$u_h = S_h = 0.1749\%.$$

Moreover, if the calculation is performed according to GOST 8.531–2002, then formula (12) ought to be used. In this case the standard uncertainty from heterogeneity will be

$$u_h = S_h = 0.1233\%.$$

This example demonstrates that the underestimation of the standard uncertainty from heterogeneity according to GOST 8.531–2002 was 1.4 times larger compared to the algorithms set forth in ISO Guide 35:2017.

Let us now consider the ratio of standard uncertainty estimates based on ISO Guide 35:2017 (11) to those based on GOST 8.531–2002 when the difference $S_b^2 - \frac{S_e^2}{J}$ is negative

$$K(I, J) = \frac{u_h(ISO)}{u_h(GOST)} = 3 \cdot J^{-1/2} \cdot \left(\frac{2}{I \cdot (J-1)}\right)^{1/4} \tag{27}$$

The analysis of expression (27) indicates that this ratio depends only on the samples under study and the number of observations in them, i.e. on the scope of experimental research. The dependence K(I, J) is shown in Fig. 1.

In general, it is obvious from this graph that in a small-volume experiment, the estimates of the standard uncertainty from heterogeneity according to GOST 8.531–2002 will be underestimated. On the other hand, in a large-volume experiment, the reverse is true, and the estimates of the standard uncertainty from heterogeneity according to GOST 8.531–2002 will be overestimated. To definitively answer the question of what a large- and small-volume experiment is, we will employ the Monte Carlo method to numerically analyze several typical examples to compare the estimated uncertainty from heterogeneity according to ISO Guide 35:2017 and GOST 8.531–2002.

Example 3 I = 10, J = 2, N = 10^4, m = Δm = 1, $\mu(Sb)$ = 0.12–0.45; $\mu(Se)$ = 0.3; $\sigma(Sb)$ = 0.015; $\sigma(Se)$ = 0.015; the distribution is normal. Figure 2 shows the calculation results. As we have already shown in Example 2, the uncertainty from heterogeneity obtained according to GOST 8.531–2002 is understated by a factor of 1.4 in comparison with ISO Guide 35:2017.

Example 4 I = 50, J = 2, N = 10^4, m = Δm = 1, $\mu(Sb)$ = 0.12–0.45; $\mu(Se)$ = 0.3; $\sigma(Sb)$ = 0.015; $\sigma(Se)$ = 0.015. The calculation results in Fig. 3 indicate that the estimates of uncertainties from heterogeneity obtained in accordance with GOST 8.531–2002 and ISO Guide 35:2017 are similar.

Example 5 I = 100, J = 2, N = 10^4, m = Δm = 1, $\mu(Sb)$ = 0.12–0.45; $\mu(Se)$ = 0.3; $\sigma(Sb)$ = 0.015; $\sigma(Se)$ = 0.015. The calculation results presented in Fig. 4 indicate that

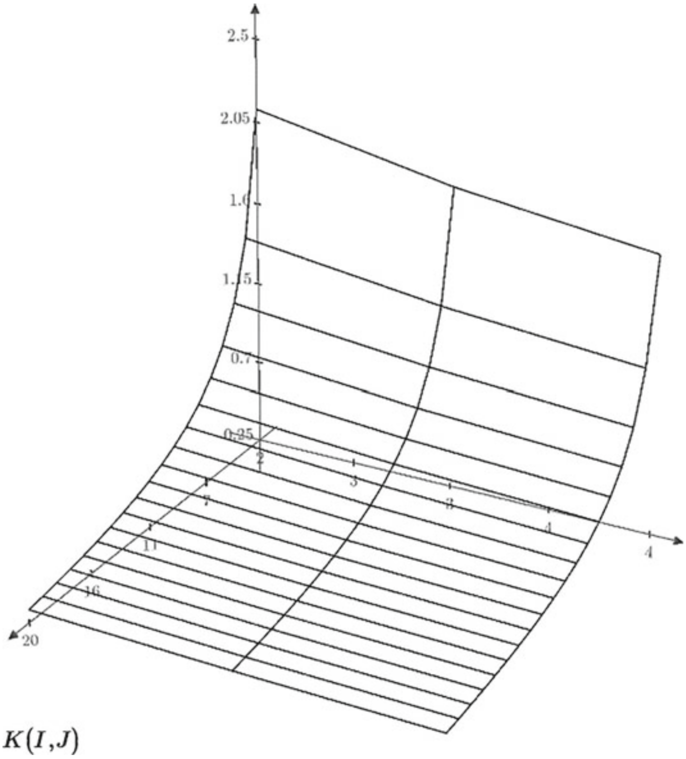

Fig. 1 Dependence of $K(I, J)$ on the number of selected RM samples I and the number of repeated observations in them J

the estimates of uncertainty from heterogeneity obtained according to GOST 8.531–2002 are overestimated in comparison with those obtained in accordance with ISO Guide 35:2017. Example 5 is more of a theoretical option since in practice in most cases experimental studies are performed in a much smaller volume, like in Example 3.

Let Us Consider a Typical Example When Evaluating the Heterogeneity of Monolithic Materials

Example 6 The RM material is bronze. The component being certified is tin. The RM homogeneity was investigated by atomic emission spectroscopy. The measurement results are recorded in Table 3. In the example with the performed calculations, the final and intermediate numbers of calculations are intentionally written with a large number of decimal places.

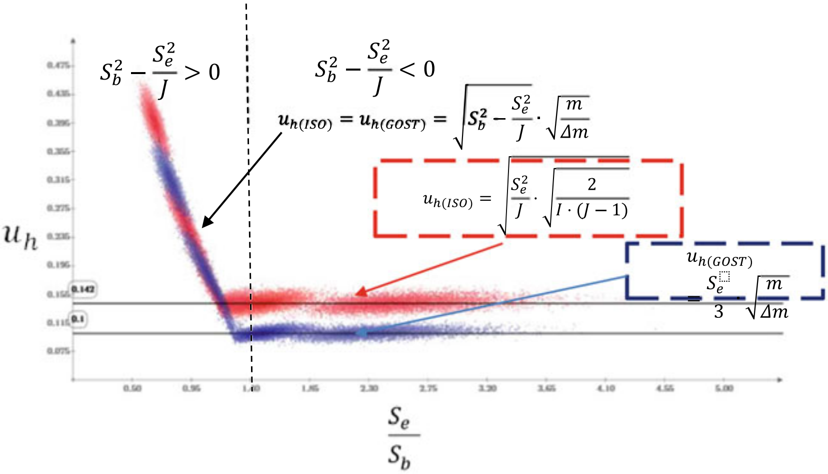

Fig. 2 Dependence of the standard uncertainty from heterogeneity on the ratio $\frac{S_e}{S_b}$ (I = 10, J = 2, N = 10^4, m = Δm = 1, μ(*Sb*) = 0.12–0.45; μ(*Se*) = 0.3; σ(*Sb*) = 0.015; σ(*Se*) = 0.015; normal distribution)

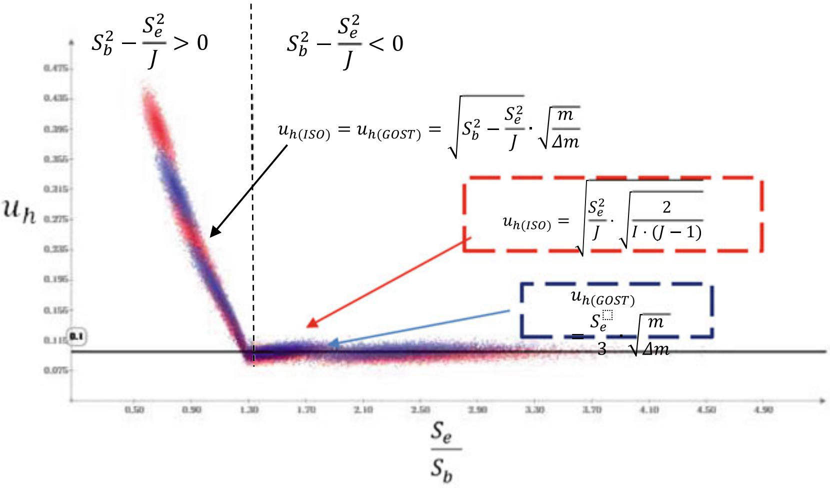

Fig. 3 Dependence of the standard uncertainty from heterogeneity on the ratio $\frac{S_e}{S_b}$ (I = 50, J = 2, N = 10^4, m = Δm = 1, μ(*Sb*) = 0.12–0.45; μ(*Se*) = 0.3; σ(*Sb*) = 0.015; σ(*Se*) = 0.015; normal distribution)

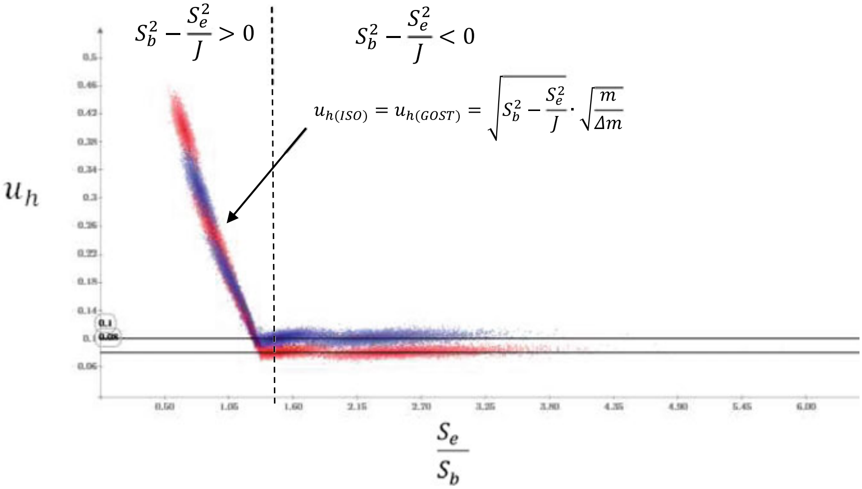

Fig. 4 Dependence of the standard uncertainty from heterogeneity on the ratio $\frac{S_e}{S_b}$ (I = 100, J = 2, N = 10^4, m = Δm = 1, $\mu(Sb)$ = 0.12–0.45; $\mu(Se)$ = 0.3; $\sigma(Sb)$ = 0.015; $\sigma(Se)$ = 0.015; normal distribution)

Based on the column sums in Table 3, the values are calculated by formulae (15)–(25)

$$\hat{x} = \overline{x}_{***} = 4.4449,\ S_e^2 = 0.0115850,\ S_w^2 = 0.03502950,\ S_b^2 = 0.017910146.$$

$$S_w^2 - \frac{S_e^2}{N} = 0.0029237, \text{ and also } u\left(\frac{S_e^2}{N}\right) = \frac{S_e^2}{N} \cdot \sqrt{\frac{2}{I \cdot J(N-1)}}$$

$$= \frac{0.01195850}{2} \cdot \sqrt{\frac{2}{2 \cdot 2(25-1)}} = 0.0011585$$

$$S_{mic}^2 = 0.0029237.$$

$$S_b^2 - \frac{S_w^2}{J} = 0.000395396,$$

$$\text{and also } u\left(\frac{S_w^2}{J}\right) = \frac{S_w^2}{J} \cdot \sqrt{\frac{2}{I \cdot (J-1)}} = \frac{0.0350295}{2} \cdot \sqrt{\frac{2}{25 \cdot (2-1)}} = 0.00495392$$

$$S_{mac}^2 = 0.00495392.$$

$$S_h = \sqrt{S_{mic}^2 + S_{mac}^2} = \sqrt{0.0029237 + 0.00495392} = 0.1849\%,$$

Table 3 Results of mass fraction measurements of tin (%)

Measurement results				$\overline{x}_{ij*}$	$\overline{x}_{i**}$	$\widehat{w}_{ij}^2$	$\widehat{b}_i^2$	$\hat{e}_{ijn}^2$	
RM number	Analytical surface number	Measurement number						Measurement number	
		1	2					1	2
1	1	4.06	4.06	4.060	4.1075	0.002256250	0.113838760	0.000000000	0.000000000
	2	4.21	4.10	4.155		0.002256250		0.003025000	0.003025000
2	1	4.29	4.04	4.165	4.21	0.002025000	0.055178010	0.015625000	0.015625000
	2	4.21	4.3	4.255		0.002025000		0.002025000	0.002025000
3	1	4.22	4.26	4.240	4.39	0.022500000	0.003014010	0.000400000	0.000400000
	2	4.40	4.68	4.540		0.022500000		0.019600000	0.019600000
4	1	4.19	4.29	4.240	4.2875	0.002256250	0.024774760	0.002500000	0.002500000
	2	4.13	4.54	4.335		0.002256250		0.042025000	0.042025000
5	1	3.99	4.07	4.030	4.2375	0.043056250	0.043014760	0.001600000	0.001600000
	2	4.42	4.47	4.445		0.043056250		0.000625000	0.000625000
6	1	4.12	4.35	4.235	4.4475	0.045156250	0.000006760	0.013225000	0.013225000
	2	4.79	4.53	4.660		0.045156250		0.016900000	0.016900000
7	1	4.25	4.28	4.265	4.4275	0.026406250	0.000302760	0.000225000	0.000225000
	2	4.59	4.59	4.590		0.026406250		0.000000000	0.000000000
8	1	4.56	4.52	4.540	4.6175	0.006006250	0.029790760	0.000400000	0.000400000
	2	4.68	4.71	4.695		0.006006250		0.000225000	0.000225000
9	1	4.38	4.3	4.340	4.3425	0.000006250	0.010485760	0.001600000	0.020277760
	2	4.43	4.26	4.345		0.000006250		0.007225000	0.007225000

(continued)

Table 3 (continued)

Measurement results				$\overline{x}_{ij*}$	$\overline{x}_{i**}$	$\widehat{w}_{ij}^2$	$\widehat{b}_i^2$	$\hat{e}_{ijn}^2$	
RM number	Analytical surface number	Measurement number						Measurement number	
		1	2					1	2
10	1	4.40	4.29	4.345	4.425	0.006400000	0.000396010	0.003025000	0.003025000
	2	4.55	4.46	4.505		0.006400000		0.002025000	0.002025000
11	1	4.25	4.51	4.380	4.455	0.005625000	0.000102010	0.016900000	0.016900000
	2	4.51	4.55	4.530		0.005625000		0.000400000	0.000400000
12	1	4.35	4.30	4.325	4.535	0.044100000	0.008118010	0.000625000	0.000625000
	2	4.71	4.78	4.745		0.044100000		0.001225000	0.001225000
13	1	4.41	4.39	4.400	4.4275	0.000756250	0.000302760	0.000100000	0.000100000
	2	4.48	4.43	4.455		0.000756250		0.000625000	0.000625000
14	1	4.08	4.15	4.115	4.385	0.072900000	0.003588010	0.001225000	0.001225000
	2	4.69	4.62	4.655		0.072900000		0.001225000	0.001225000
15	1	4.13	4.40	4.265	4.395	0.016900000	0.002490010	0.018225000	0.018225000
	2	4.44	4.61	4.525		0.016900000		0.007225000	0.007225000
16	1	4.80	4.67	4.735	4.6525	0.006806250	0.043097760	0.004225000	0.004225000
	2	4.54	4.60	4.570		0.006806250		0.000900000	0.000900000
17	1	4.23	4.36	4.295	4.4525	0.024806250	0.000057760	0.004225000	0.004225000
	2	4.53	4.69	4.610		0.024806250		0.006400000	0.006400000
18	1	4.55	4.64	4.595	4.6425	0.002256250	0.039045760	0.002025000	0.002025000
	2	4.82	4.56	4.690		0.002256250		0.016900000	0.016900000
19	1	4.20	4.45	4.325	4.54	0.046225000	0.009044010	0.015625000	0.015625000
	2	4.72	4.79	4.755		0.046225000		0.001225000	0.001225000

(continued)

Table 3 (continued)

Measurement results				$\overline{x}_{ij*}$	$\overline{x}_{i**}$	$\widehat{w}_{ij}^{2}$	$\widehat{b}_{i}^{2}$	$\hat{e}_{ijn}^{2}$	
RM number	Analytical surface number	Measurement number						Measurement number	
		1	2					1	2
20	1	4.62	4.32	4.470	4.525	0.003025000	0.006416010	0.022500000	0.022500000
	2	4.55	4.61	4.580		0.003025000		0.000900000	0.000900000
21	1	4.34	4.44	4.390	4.5025	0.012656250	0.003317760	0.002500000	0.002500000
	2	4.63	4.60	4.615		0.012656250		0.000225000	0.000225000
22	1	4.44	4.64	4.540	4.5825	0.001806250	0.018933760	0.010000000	0.010000000
	2	4.55	4.70	4.625		0.001806250		0.005625000	0.005625000
23	1	4.44	4.29	4.365	4.5375	0.029756250	0.008574760	0.005625000	0.005625000
	2	4.72	4.70	4.710		0.029756250		0.000100000	0.000100000
24	1	4.43	4.40	4.415	4.49	0.005625000	0.002034010	0.000225000	0.000225000
	2	4.53	4.60	4.565		0.005625000		0.001225000	0.001225000
25	1	4.51	4.32	4.415	4.5075	0.008556250	0.003918760	0.009025000	0.009025000
	2	4.61	4.59	4.600		0.008556250		0.000100000	0.000100000

which is 4.16% of the mean value of the component being certified. When performing calculations in accordance with the current edition of GOST 8.531–2002, the standard uncertainty from heterogeneity for this example was 3.9%. Thus, since a sufficiently large number of measurements for monolithic materials are factored into this edition, the estimates of standard uncertainties evaluated according to the algorithm proposed in this article and in the current edition of GOST 8.531–2002 are almost the same.

Conclusion

The article analyzed algorithms for evaluating standard uncertainty from heterogeneity in accordance with the interstate standard GOST 8.531–2002 and the international standard ISO Guide 35:2017. Based on these standards, we have developed new algorithms for estimating the homogeneity of dispersed and monolithic materials. These algorithms employ the analysis of variance (ANOVA) incorporated in both ISO Guide 35:2017 and GOST 8.531–2002. The main distinction between the developed algorithms and ISO Guide 35:2017 is the elaboration of specific algorithms suitable for calculation and automation. The major difference from GOST 8.531–2002 is the updated calculation of the standard uncertainty from heterogeneity when it is comparable to the type A evaluation of standard uncertainty. The other dissimilarities are the presence of data gaps and consideration of general cases when selecting an arbitrary number of RM samples and an arbitrary number of repeated measurements in each RM sample. The developed algorithms were tested on various real-world examples, including simulated data, and their applicability was proved.

The research results demonstrate that the approach outlined in ISO Guide 35:2017 is a more effective way of evaluating standard uncertainty from heterogeneity. It is modernized and considers the minimum representative sample mass. Moreover, the algorithm is applicable to studying composition indicators as well as properties of solid and liquid substances and materials.

Thus, to harmonize GOST 8.531–2002 and ISO Guide 35:2017, as well as to increase confidence in the results of determining RM metrological characteristics in Russia at the international level, the updated algorithms will be used to revise GOST 8.531–2002.

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Conflict of Interest The article was prepared on the basis of a report presented at the V International Scientific Conference “Reference Materials in Measurement and Technology” (Yekaterinburg, September 13–16, 2022). The article was admitted for publication after the abstract was revised, the article was formalized, and the review procedure was carried out.

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A Comparative Analysis of OIML Documents and Russian Rules on the Use of Reference Materials

Narine G. Oganyan and Vladimir I. Dobrovolskiy

Abstract The article analyzes the compliance of Russian legal documents with the Recommendations of the International Organization of Legal Metrology regarding the use of reference materials in the fields covered by the state control and supervision. The problem of ensuring the quality of measurement results is relevant for each laboratory, performing measurements in any fields of activity. In addition to a reliable and proven measurement method, a decisive role in obtaining comparable results is played by reference materials. In order to ensure the traceability of measurements, a reference material must be certified. Certified reference materials are obligatory for the state metrological control and supervision of the quality and safety of food and pharmaceutical products, quality of measurements in clinical laboratories, environmental safety, etc. Considering the important role of reference materials in the process of ensuring the uniformity of measurements in analytical laboratories, this study can contribute to the promotion of ideas for improving the Russian legislative framework in the relevant area.

Keywords Certified reference material · CRM production · CRM type approval · CRM expertise · State metrological control and supervision · Legal requirements

Abbreviations

GSO	Approved type of reference material
OIML	International Organization of Legal Metrology
RF	Russian Federation
RM	Reference material

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CRM	Certified reference material
BIPM	International Bureau of Weights and Measures

Introduction

A decision on the conformity of products to their quality requirements is primarily based on the sufficient accuracy of measurement results provided by analytical laboratories [1–3]. Therefore, every laboratory, regardless of the field of its activity, should be aware of the need to ensure the quality of its measurement results. Over the past decades, an agreement has been reached between experts from various countries on how the required quality of measurements can be achieved. The starting point undoubtedly involves the choice of a reliable and proven method. However, this cannot be considered a sufficient basis for comparing the results obtained in various laboratories. In order to ensure the comparability of the results, it is necessary to ensure the metrological traceability of these measurements [1, 4]. In analytical laboratories, a decisive role in this issue is played by references in the form of chemicals, which are called reference materials (RMs). RMs are used for the verification, calibration, and validation of methods, assessment of measurement uncertainties, and quality control. In addition, it should be kept in mind that a particular RM can be used only for one measurement purpose, for example, for calibration or product quality control [5].

According to the international metrological vocabularies JCGM 200:2012, ISO/IEC Guide 99:2007, and RMG 29–2013 [6–8], a certified reference material (CRM) is a "reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures". According to the metrological vocabulary RMG 29–2013, operating within the Russian Federation (RF): "a certified reference material is a reference material, accompanied with the documentation issued by an authoritative body, which indicates one or more values of a certain property with the appropriate measurement accuracy characteristics (uncertainties) and traceability, which are established using reasonable procedures." Therefore, the CRM definition is fully consistent with the foreign analogues.

As can be seen from the RM and CRM definitions, in order to ensure the traceability of measurements, without which it is impossible to pass the accreditation procedure by any measuring laboratory for a compliance with the requirements of ISO/IEC17025:2017 [9, 10], laboratories need to use CRMs.

It is obvious that laboratories have to use CRMs in such areas, as the quality and safety of food and pharmaceutical products, quality of measurements in clinical laboratories, environmental safety, etc., where ensuring the uniformity of measurements is regulated by the State in accordance with the requirements of the Federal Law of June 26, 2008 No. 102-FZ [11]. In addition, laboratory specialists should know and understand how to correctly select RMs for their measurements and that not all samples, offered on the market, are produced with the same quality level. It is important to ensure that any used CRM is developed and characterized in a technically sound manner. Generally, detailed information about homogeneity and stability studies, certification methods, as well as uncertainties and deviations in the declared RM values is available from a reliable producer and this information may be used to assess their reliability. As indicated above, a CRM must be accompanied with a certificate (or other identical document) that includes the evaluated uncertainty associated with the assigned value. The criteria for the competence of CRM producers, recognized at the interstate level, are determined by ISO 17034:2016[1] [12, 13].

It is clear that the practice of RM production and use differs from country to country. Therefore, it is extremely risky to leave this issue without appropriate state regulation. For this purpose, within the framework of the International Organization of Legal Metrology (OIML), the Document OIML D18 [14] was developed, formulating legal requirements for RMs, used in metrological activities in accordance with the national legislation. OIML D18 is based on the General principles regarding the use of RMs as measuring standards, which are provided in both ISO/REMCO and OIML publications. The document defines the minimum content of technical procedures that form the elements of the state metrological control and supervision applied to RMs. If necessary, procedures, depending on the specifics and practical experience in a particular country, may be changed and extended in national regulatory documents.

As we have already defined, the traceability of measurements in the field covered by the state control and supervision should be ensured by using CRMs.

How to Determine Whether the RMs Used Are Within the Scope of State Control and Supervision

In practice, this is often a difficult problem. According to OIML D18 [14], when considering whether the used RM is within the scope of state control and supervision or not, it is advisable to use the information specified in the CRM Certificate on the intended metrological use, such as:

- using the CRM as a measuring standard for verification, calibration and testing of measuring instruments;
- using the CRM to standardize measurement procedures and/or to determine their uncertainties while measuring;
- using the CRM for calibration of measuring instruments in measurement procedures.

Information on whether relevant measuring instruments or measurement procedures are in the field covered by the state metrological control and supervision or

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[1] *Note*: In the RF, GOST R ISO 17034-2021 [12] State Standard, forced in December 2021, has not yet commonly used by RM producers.

not may be used to determine whether this CRM is covered by the state metrological control and supervision. A useful guidance in resolving this issue may be a list of the fields covered by the state regulation, whose legal documents provide specific details. The Document OIML D 12[2] [16] may be a useful guidance.

Thus, according to the international documents, in the RF, relevant legal documents determine both fields covered by the metrological control and supervision and a list of measurements related to these fields, according to which it is possible to determine whether the CRM is included in the legislative fields.

Necessary Criteria for the Compliance of a CRM with the Requirements

The necessary conditions for the use of CRMs in the fields covered by the state metrological control and supervision are their compliance with the specified metrological, technical, and administrative requirements, established in the country. Three types of requirements developed on the basis of OIML D3 [17] recommended and adapted for CRMs are provided below.

Metrological requirements include:

- list of CRM metrological characteristics to be determined;
- format of their presentation;
- requirements for measuring instruments and methods used in determining the metrological characteristics of CRMs, requirements for the content of CRM certification programs and procedures, etc.

Technical requirements include:

- CRM product form;
- list of accompanying technical documents for the initial and subsequent CRM production;
- requirements for packing and marking of the CRM delivered to the user, etc.

Administrative (legal) requirements for CRMs include:

- list of metrological activities, where the CRM is intended to be used;
- requirements for the type and format of documents accompanying the CRM, when delivered to the user;
- if necessary, designation of types and methods to control CRM metrological characteristics in the process of its use;
- details of the state registration in order to identify CRM or CRM batches, etc.

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[2] *Note*: In the RF, the fields covered by the state metrological control and supervision are laid in the Federal Law of June 26, 2008 No. 102-FZ [9], and the list of measurements related to the scope of the State Control for Measurement Uniformity Assurance are laid in the Government Decree dated November 16, 2020 No. 1847 [15].

During the development of some CRMs, metrological, technical, and administrative requirements may be preliminary included in the project prepared at the initial stage of works.

In addition, the requirements to CRMs, authorized for the use in the field of the state metrological control and supervision, may be set out in the relevant regulatory document on the legislative metrology or a state standard.[3]

It is evident that, similar to any measuring standard or instrument, CRMs, approved for the use in the fields covered by the state metrological control and supervision, are also subject to the state metrological control and supervision [14].

CRM Metrological Control

The main purpose of metrological control is to ensure the compliance of CRMs with metrological, technical, and administrative (legal) requirements specified above.

A reasonable question is "What should be taken into account for metrological control to make sure the compliance of a CRM with the legal requirements?"

According to the relevant OIML provisions, the metrological control of measuring instruments normally includes the evaluation or testing of samples of a measuring instrument type and its approval, the verification (initial, periodic and other) or calibration of measuring instruments, the metrological supervision of the issue and use of measuring instruments and the conditions for their use, etc. In the case with CRM, all these operations are not always justified and may be limited [14] to the following operations, which can be represented schematically (Fig. 1):

At the same time, it is advisable to indicate in the regulatory document of the National Service of Legal Metrology, which ways (form, content, and procedures) the metrological control of a CRM shall be implemented.

According to the foregoing, type approval is one of the ways for the metrological control of used CRMs, in which the compliance of a CRM to legislative requirements is assessed (to make an examination). Before making a decision about the approval of the CRM type, it is necessary to make sure that the legal requirements are fully taken into account and reflected in the technical documentation of the CRM:

- performance or target specification for the CRM production;
- CRM certification program or procedure, CRM certificate model or another identical document;
- documents, accompanying the CRM when delivered to the consumer, or other documents specified by administrative requirements.

To conduct the expertise, it is also important to assess whether measuring instruments used to establish the certified CRM values are covered by the metrological control and whether the measuring standards used are higher in the traceability

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[3] *Note*: In the RF, on the basis of the Federal Law dated June 26, 2008 No. 102-FZ [9], only approved type of RMs are allowed to use in the field covered by the State metrological control and supervision.

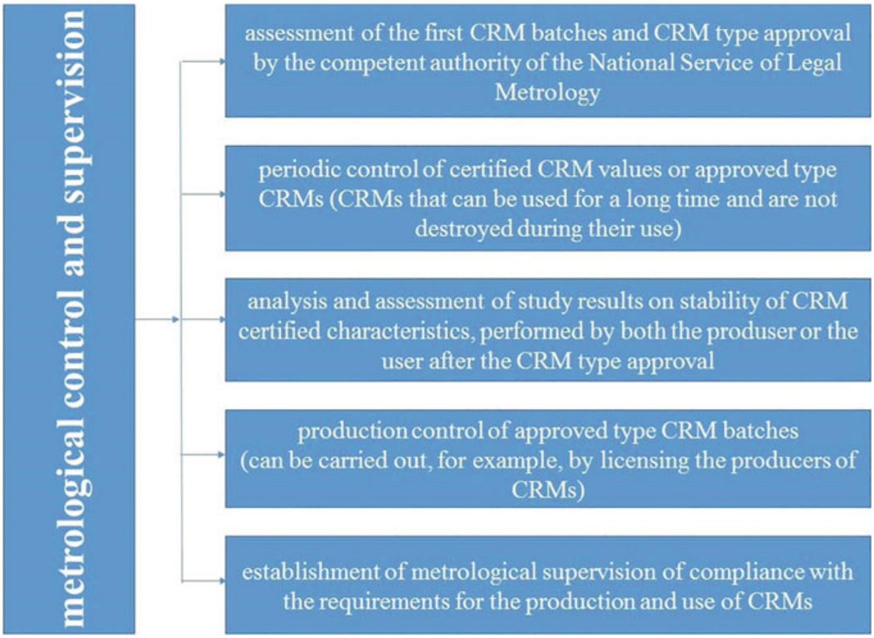

Fig. 1 Set of operations on the CRM metrological control and supervision, according to the provisions of OIML

chain [18]. The expertise may be general or detailed, including introduction to the CRM preparing procedure and production supervision of the compliance with the requirements of technical documents. In the latter case, it is possible to confirm the competence of the CRM producer by the accreditation procedure in accordance with the requirements of ISO 17034:2016 [12], provided that the national authority has established this possibility.[4]

Based on the positive results of assessing the compliance of a CRM with the established requirements, the type of CRM is approved by the competent authority of the National Service of Legal Metrology. When approving the CRM type, rules for verification and certification of the necessary standards should be established. The type approval may be accompanied by both the issuance of a “CRM type approval certificate” and the authorization of the CRM producer to stamp a type approval mark on the accompanying CRM documents.[5] The model of the certificate and/or mark

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[4] *Note*: In the RF, on the basis of the Order of the Ministry of Industry and Trade dated August 28, 2020 No. 2905 [19], in terms of a CRM compliance assessment, the test procedure of RM have been established, including the examination of documentation.

[5] *Note*: In the RF, based on the Order of the Ministry of Industry and Trade dated February 03, 2015 No. 164 [20], the approved type of the CRM gets the status of the State Reference Material (SRM/GSO), is issued the “RM type approval Certificate,” the relevant information is included in the State Register of Approved Types of Reference Materials of the Federal Information Fund of Measurement Uniformity Assurance.

are established by the authority that approves the CRM type. The validity of the type approval certificate may be limited with the possibility to extend. The extending is possible provided that during the validation period no new requirements to use the CRM in accordance with its purpose have been established (such restrictions may be the changes in the rules for calibration of measuring instruments or in the regulatory documents on test and control methods determining the use of the CRM).

The use of CRMs may be metrologically controlled by the relevant authorized metrological bodies by means of periodic verification or certification. The subsequent batches of CRMs of approved type, which, in particular, are not subject to periodic inspection, may be metrology controlled through licensing, which usually includes periodic monitoring by the producer of CRM compliance with the technical documentation when producing new batches. When developing regulatory documents of the national legal metrology service, it is important to take into account the recommendations of ISO Guide 33:2015 [21] to establish administrative (legal) requirements and the recommendations of ISO Guide 30:2015, ISO Guide 31:2015, and ISO Guide 35:2015 [22–24], for metrological and technical requirements. It should be noted that all these Guides are translated into Russian and approved for the use in the Russian Federation [25–27].

In addition, the National Service of Legal Metrology should clearly define the conditions (the same or different) applicable to imported CRMs, as well as for their admission to use in the fields covered by the state metrological control and supervision.[6]

Thus, the conditions for admission to the use of domestic and imported CRMs in the fields covered by the state metrological control and supervision are clearly established in the Russian Federation.

As might be expected, it is impossible to be limited only to the metrological control of CRMs used in the field of the state metrological control and supervision. Along with the metrological control described above, it is necessary to provide for the metrological supervision of compliance with the legislation requirements for the production and use of CRMs, which is entrusted to the metrological supervision bodies of the National Service of Legal Metrology.

The content and procedure of the metrological supervision shall be specified in the relevant national regulatory document. Here, it is recommended to take into account the provisions of the International Document OIML D 9[7] [29].

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[6] *Note*: In the RF, on the basis of the Federal Law dated June 26, 2008 No. 102-FZ [9], in the field covered by the state technical regulation, regardless of the RM producing country, only approved type reference materials are allowed to be used.

[7] *Note*: In the RF, the content and procedure for the metrological control/supervision is established in the Decree of the Government of the Russian Federation dated June 29, 2021 No. 1053 [28].

It should also be noted that the full information on legal documents, including related to use of RMs in the Russian Federation, may be find on the official website of the Federal Information Fund for Measurement Uniformity Assurance.

According to the document OIML D1 [30], us a rule, when detecting violations of the rules and misfits during the control of the market and production process by the metrological supervision, to take coercive action, fixed at the legislative level.[8]

In order to confirm the reliability of the obtained results, national legal metrology services, together with CRM manufacturers, are recommended to organize and/or participate in international comparisons of CRMs used in areas covered by the scope of the state metrological control and supervision. National Services of Legal Metrology in the respective countries are encouraged to sign agreements on the mutual recognition of CRM type approval certificates for removing technical barriers. For example, the list of CRMs used in the field covered by the state metrological control and supervision, may include the corresponding CRMs from the database of the International Bureau of Weights and Measures (BIPM), in accordance with the Agreement CIPM MRA [31] on the mutual recognition of standards and calibration certificates, signed by national metrological institutes.[9]

Conclusion

Thus, as shown by the conducted comparative analysis, the Russian legal framework regarding the use of reference materials in the fields covered by the state technical regulation was developed and approved in accordance with the OIML documents.

Nevertheless, some issues remain to be finalized.

1. No clear procedure is available for rules and regulations on the confirmation of the CRM producer competence. The absence of the "RM competent producer" status leads to the absence of such an important RM term in the country as a CRM. That is, the producer is not authorized to issue the legitimate RM Certificate to assign to the RM the status of the CRM. At the bottom of this, an additional stage of testing for the purpose of the type approval by an authorized body arises. In fact, if the decision about type approval is positive, the RM is given the status of a GSO (State Reference Material), bypassing the status of a CRM, and entered in the Register of Approved Types of Reference Materials. This approach complicates and greatly increases the duration of RM type approval procedure for their admission to the scope of the state technical regulation. The development of rules and regulations on the confirmation of the RM producer competence will let the level of RM production and simplify the RM type approval procedure to raise in a couple of years.

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[8] *Note*: In the RF, the coercive actions have not been developed and are not laid legally in relation to violations and inconsistencies of the CRM with the data specified in the Type Approval Certificate.

[9] *Note*: The Russian Federation regularly participates and conducts such international comparisons within the framework of the Agreements between the Interstate Council on Standardization, Metrology and Certification (ISC) of the Commonwealth of Independent States (CIS) and the Euro-Asian Cooperation of State Metrological Institutions regional metrological organization (COOMET). It should be noted that this area is in a constant process of development and improvement.

2. Moreover, no coercive actions are applied to producers and suppliers of CRMs, established at the legislative level. It is suggested that, in the case of detecting a significant non-compliance of CCO/GSO with the established requirements, which is caused by the systematic negligence of the CRM producer/supplier, an extreme coercive action be applied as leverages on the CRM producer/suppliers. In addition, the "GSO" status should be disqualified and the CRM producer and/or supplier should receive the status of "unfair" or "incompetent", with the inclusion of this information in the relevant state information database (for example, add a special column in the register of GSO approved types).

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