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RESEARCH PAPER

Development of purity certified reference materials to establish metrological traceability for the measurement of nitroimidazoles in agricultural products

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Abstract

It is of significant importance to public health that reliable monitoring of nitroimidazoles be conducted, while certified reference materials (CRMs) are essential for accurate and reliable detection. A project has been initiated with the objective of developing nitroimidazole purity CRMs to ensure that results from nationwide monitoring laboratories for nitroimidazoles in antibiotic residues can be compared and traced. The candidates were successively characterized in terms of their structure by means of infrared (IR) spectroscopy and mass spectrometry (MS). The mass balance (MB) method and the quantitative nuclear magnetic resonance (qNMR) method were utilized to determine the purity of nitroimidazoles with remarkable accuracy. Furthermore, a methodical investigation was conducted on homogeneity, stability, and uncertainty. Six nitroimidazole purity CRMs, including tinidazole (GBW09252), secnidazole (GBW09286), ronidazole (GBW09288), metronidazole (GBW(E)090755), dimetridazole (GBW(E)090819), and ornidazole (GBW(E)090820), were finally manufactured following authorization from China's State Administration for Market Regulation (SAMR). By using these CRMs, it is possible to improve the traceability, accuracy, and comparability of nitroimidazole measurements in a range of agricultural products, protecting public health.

Keyword Nitroimidazole · Certified reference material · Metrological traceability · Uncertainty

Introduction

Nitroimidazoles constitute a group of organic compounds derived from 5-nitrosubstituted imidazole rings [1]. The most prominent members of this class include tinidazole (TNZ), secnidazole (SNZ), ronidazole (RNZ), metronidazole (MNZ), dimetridazole (DMZ), and ornidazole (ORZ) [2]. They represent a significant class of antibiotics employed for the management of infections triggered by protozoa and anaerobic bacteria in humans and animals [3, 4]. The application of nitroimidazoles extends to the prevention and treatment of several animal diseases, including genital trichomoniasis in cattle, hemorrhagic enteritis in pigs, coccidiosis in poultry, and nosemosis in bees [5]. Nevertheless, nitroimidazoles and their metabolites have been demonstrated to possess genotoxic, carcinogenic, and mutagenic properties due to the presence of the same original nitroimidazole ring, which is a recognized carcinogenic agent [6, 7]. These compounds can be ingested by humans through the consumption of animal products and have been demonstrated to have an impact on human health. Consequently, numerous countries have enacted legislation prohibiting their use in all food animals [8]. It is evident that there has been a continued prevalence of abuse and misuse of nitroimidazoles in recent times. The high solubility and limited biodegradability of these compounds have led to their ubiquitous presence in water and animal-based food [9]. For nitroimidazoles, the European Community Reference Laboratories have established a minimum requisite performance level (MRPL) of 1 μg/kg, without making any distinctions between matrices, in order to protect the public's health and the environment [10]. In accordance with the stipulations outlined in Chinese Ministry of Agriculture (MOA) Bulletin No. 193 and State Drug Administration (SDA) Bulletin No.

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227, the utilization of MNZ, DMZ, their derivatives, and preparations in food animals for the purposes of promoting growth has been prohibited since 2002. Moreover, the use of MNZ and DMZ for therapeutic purposes in animals is expressly permitted by the GB 31650–2019 National Food Safety Standard—Maximum Residue Limits for Veterinary Drugs in Foods, provided that they are not detected in animal food. Furthermore, the Chinese Ministry of Agricultural and Rural Affairs (MOARA), in Announcement No. 250, explicitly states that the use of TNZ and RNZ in food animals is prohibited as of 2019.

The ingestion of food derived from animals represents a pivotal aspect of the dietary regimen of Chinese citizens, and the quality and safety of these products are of paramount importance to public health. During routine supervision and sampling, it has been established that the illicit use and abuse of nitroimidazoles persist during the farming of aquaculture, livestock, and poultry. Furthermore, instances of nitroimidazoles being detected in agricultural products have been reported on occasion. It is of the utmost importance that all laboratories employ the highest standards of accuracy and metrological traceability in order to guarantee the dependability and comparability of permanent and baseline monitoring of data. Certified reference materials (CRMs), which are characterized by high level of reliability and measurement traceability, are widely used for the assessment of analytical methods, the instrument of equipment, the determination of material property values, and the application of quality control measures during the production process [11, 12]. Because purity CRMs are used to show the traceability and comparability of measurement results, they represent the highest level of metrological traceability [13, 14]. The World Health Organization (WHO), International Pharmacopoeia (Ph. Int.), and European Pharmacopoeia (Ph. Eur.) have long regarded the mass balance (MB) method as the optimal approach for the fundamental examination of organic compounds [15–17]. Over the past several years, there have been notable developments in analytical methods for the precise measurement of organic CRMs. The quantitative nuclear magnetic resonance (qNMR) method and the differential scanning calorimetry (DSC) method have emerged as particularly valuable techniques for this purpose [18–24]. These methods offer a number of advantages, including high sensitivity and accuracy, in addition to simplicity of operation and analysis [25–28]. The lack of purity CRMs for nitroimidazole can lead to various challenges concerning the precision, consistency, and verifiability of nitroimidazole measurements in agricultural products. Consequently, the Institute of Quality Standard and Testing Technology for Agro-Products (IQSTAP) within the Chinese Academy of Agricultural Sciences (CAAS) has produced several dependable CRMs for nitroimidazole purity to ensure that measurements have a clear traceability to the International System of Units (SI), thereby validly guarantees comparable and dependable measured results obtained in the mandatory routine monitoring laboratories.

This study delineates several pivotal steps for the fabrication of purity CRMs in accordance with ISO 17034:2016 [29] and ISO 33405:2024 [30], including testing for homogeneity, monitoring stability, characterization, and assessing uncertainty. It was successfully accomplished to prepare and approve six nitroimidazole purity CRMs by the State Administration for Market Regulation (SAMR) of China. These include tinidazole (GBW09252), secnidazole (GBW09286), ronidazole (GBW09288), metronidazole (GBW(E)090755), dimetridazole (GBW(E)090819), and ornidazole (GBW(E)090820), which together encompass the objectives of routine testing for livestock and poultry products as well as products with an aquatic origin. By using these nitroimidazole purity CRMs as sources, risk monitoring for the quality and safety of agricultural products would be improved by facilitating metrological traceability for nitroimidazole measurement in monitoring plans.

Experimental

Chemicals and materials

Tinidazole and metronidazole were obtained from Jingnanjie Co. Ltd., Shanghai, China. Secnidazole, dimetridazole, and ornidazole were sourced from TCI (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan). Ronidazole was sourced from JK Chemical Co. Ltd., Beijing, China. HPLC-grade methanol and acetonitrile, acquired from Merck (Darmstadt, Germany), were used as the experiment's solvents and mobile phases. The CRMs of water content of solid (GBW13518, 10.05 ± 0.20 mg/g), ethyl paraben (GBW(E)100,064, $99.7\% \pm 0.2\%$), and benzoic acid (GBW06117, $99.995\% \pm 0.009\%$) were procured by NIM (National Institute of Metrology, China), while the creatinine (SMR 914a, $99.7\% \pm 0.3\%$) was purchased from the NIST (National Institute of Standards and Technology, USA) as the internal standard for qNMR. Deuterium-labeled reagents, including methanol (D4) and acetate acid (D4), were procured from Sigma-Aldrich (St. Louis, MO, USA). The multi-component calibration solution (standard 2A) utilized for inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES) calibration was sourced from Agilent Technologies (Santa Clara, CA, USA). All other reagents were sourced from Merck (Darmstadt, Germany).

Apparatus

The primary component and organic impurities were measured using a Waters 2695 high-pressure liquid chromatography (HPLC) system, which was fitted with a 2417 ultraviolet (UV) detector (Waters, USA). The moisture content of the nitroimidazole candidates was measured using a Karl Fischer titrator DL32 (Mettler-Toledo, Switzerland). The Nexis GC-2030 gas chromatography system, equipped with an HS-10 headspace injector (Shimadzu, Japan) and an ICP-MS X series 2 (Thermo Fisher, USA), was employed to measure volatile and non-volatile impurities, respectively. An NMR spectrometer featuring a cryoprobe (Bruker Advance III, Germany), operating at a frequency of 400 MHz, was employed in the context of qNMR analysis. Using an XS105 analytical balance (Mettler-Toledo, Switzerland), a weight measurement procedure was applied to the samples.

Preparation of the CRM candidates

Once the characterization and preliminary homogeneity testing procedures had been completed, the bulk material was sealed in brown glass vials of 100 mg each in accordance with the established protocol. A total of 400 vials of tinidazole, 170 of secnidazole, 200 of ronidazole, 500 of metronidazole, 219 of dimetridazole, and 236 of ornidazole were prepared for consideration as potential candidates for the CRMs. The samples were stored at − 20 °C in a dark environment to prevent any deterioration or instability over time, thus ensuring the integrity and quality of the samples for future analysis.

Identity of the CRM candidates

Infrared spectroscopy analysis

Approximately 20 mg of the candidate and 100 mg of KBr were weighed and placed in an agate mortar. They were ground and mixed for 5–10 min, and then pressed into translucent tablets using a tablet press pressurized to 20 MPa. The resulting tablets were subjected to infrared spectrometer analysis, and the results were subtracted from the KBr blank.

Mass spectrometry analysis

After dissolving the candidate in 0.2% formic acid-containing water, a solution with an approximate concentration of 0.1 mg/mL was produced. An electrospray ionization mass spectrometer was used to perform flow injection analysis in positive mode in order to determine the molecular weights of the whole molecule as well as its fragments. A 20 μL/min flow rate was used, the capillary temperature was kept at 400 ℃, the spray voltage was set at 5.5 kV, and the sheath gas flow and auxiliary gas flow were both controlled at 55 arb.

Test of homogeneity and stability

The homogeneity and stability test was conducted using HPLC–UV analysis, employing the optimized conditions outlined in Table S1. Each packaged unit was assigned a unique numerical identifier, with the number corresponding to the order in which the units were to be dispensed. Twenty-five bottles of metronidazole and fifteen bottles of other nitroimidazole were randomly selected and subjected to between-bottle homogeneity testing in accordance with a random number table. A total of three subsamples were prepared from each bottle with a concentration of 1 mg/mL, which were then subjected to testing for homogeneity within the bottle. An analysis of variance (ANOVA) in single factor was performed on the data in order to evaluate the results. Over the course of 6 months and a year, respectively, the long-term stability of metronidazole, dimetridazole, ornidazole, tinidazole, secnidazole, and ronidazole purity CRMs for storage at − 20 °C was assessed. Two vials were chosen at random from the batches of CRMs for each nitroimidazole, and triplicate measurements were made at predetermined intervals. The vials were kept for 1, 3, 5, 7, and 9 days at 20 °C, 40 °C, and 60 °C in order to evaluate the short-term stability. Each sample was subjected to HPLC–UV analysis, the purity value is calculated by subtracting the moisture content measured at a predetermined time from the area normalization result, as well as the non-volatile and volatile impurities measured during the purity determination, and then stability tests were evaluated using regression analysis.

Purity determination

Mass balance method

Prior to the identification of the purity of nitroimidazole, it was necessary to optimize the separation conditions and parameters of HPLC–UV in order to ensure the detection of a greater number of impurities. The structure-related impurities were quantified through the utilization of a Waters HPLC system, which was equipped with a UV detector and operated under optimized conditions, as detailed in Table S1.

The water content of the nitroimidazoles was determined through Karl Fischer titration. After the moisture analyzer was fully balanced, the experimental conditions were verified with CRMs of the water content of solids to check that the measured value basically agreed with the certificate value. We weighed about 5 mg of the sample, quickly opened the titration cell, added the sample, and controlled the addition process in about 5 s. To eliminate the effect of

air humidity on the results, we performed a blank experiment to simulate the sample addition process, but did not add any samples, and calculated the air humidity blank value.

Non-volatile impurities were screened and measured using inductively coupled plasma mass spectrometry (ICP-MS). A quantity of approximately 10 mg of the sample was weighed and then treated with 3 mL of nitric acid and 1 mL of hydrogen peroxide. The mixture was heated at 180 ℃ for 2 h, after which it was allowed to cool. The sample was then transferred to a volumetric flask and rinsed three times with high-purity water, and the washings were collected. The sample was then diluted to 25 mL with water and analyzed by ICP-MS.

Volatile impurities were quantified using headspace gas chromatography with a flame ionization detector (FID), targeting volatile organic compounds such as methanol, ethanol, acetonitrile, ethyl acetate, acetone, n-hexane, isopropanol, xylene, and dichloromethane. About 20 mg of the sample was weighed and dissolved in 2 mL of DMSO for GC–MS analysis. Instrumental conditions: chromatographic column DB-624 (0.32 mm × 1.8 μm, 30 m), hydrogen flow rate 40 mL/min, air flow rate 400 mL/min, detector temperature 260 ℃; programmed heating conditions: initial 45 ℃ held for 5 min, heating to 60 ℃ at 7 ℃/min, and then 15 ℃/min to 190 ℃, injection volume 1 mL, split ratio 5:1, thermostat temperature 90 ℃, sample flow path temperature 115 ℃, transfer line temperature 120 ℃.

The limit of detection (LOD) for each technique (HPLC–UV, ICP-MS, GC–MS) is listed in Table S2. Consequently, the purity of the CRMs can be estimated indirectly by employing the following Formula (1):

$$P_{\mathrm{MB}} = P_0 \times \left(100\% - X_{\mathrm{W}} - X_{\mathrm{V}} - X_{\mathrm{NV}}\right) \times 100\% \quad (1)$$

The purity of the primary component is quantified by high-performance liquid chromatography with ultraviolet detection (HPLC–UV), and is designated as P_0. The mass fractions of water, volatile impurities, and non-volatile inorganic impurities, respectively, are designated as X_{W}, X_{V}, and X_{NV}.

Quantitative nuclear magnetic resonance (qNMR) method

The candidate and internal standard were weighed accurately into a glass vial at a molar ratio of 1:1, which was then fully dissolved in 1 mL of deuterium reagent. Subsequently, the mixture was transferred to an NMR tube with a 5-mm diameter for analysis. To ensure accurate and detailed NMR analysis, a 0.3-Hz line widening and an exponential multiplication window function were employed. Baseline-peak coincidence was employed to ensure the accurate execution of integration within a restricted range of ± 0.1 Hz. The data were gathered and processed using Topspin 2.1 software. This process included baseline calibration, chemical shift correction using spectral data from deuterated solvents, and both manual and automatic phase adjustment. The specific experimental conditions are listed in Table S3. The purity of the CRMs was calculated using the Formula (2) below:

$$P_{\mathrm{NMR}} = \frac{I_{\mathrm{x}}}{I_{\mathrm{std}}} \bullet \frac{n_{\mathrm{std}}}{n_{\mathrm{x}}} \bullet \frac{M_{\mathrm{x}}}{M_{\mathrm{std}}} \bullet \frac{m_{\mathrm{std}}}{m_{\mathrm{x}}} \bullet P_{\mathrm{std}} \quad (2)$$

The peak area integration of the analyte and internal standard is designated by I_{x} and I_{std}, respectively. The variables n_{std} and n_{x} denote the spin numbers of the internal standard and the analyte alike. Analyte and internal standard molecular weights are denoted by the letters M_{x} and M_{std}, respectively. The mass of the internal standard and that of the analyte are respectively denoted by m_{std} and m_{x}. P_{std} represents the purity of the internal standard.

Uncertainties

The homogeneity test (u_{bb}), long-term stability (u_{lts}), short-term stability (u_{sts}), and characterization (u_{char}) were found to be the main sources of uncertainty, as stated in ISO 33405:2024. To estimate u_{char}, the uncertainties from qNMR (u_{qNMR}) and MB (u_{MB}) were used.

Results and discussion

Qualitative analysis

Fourier transform infrared (FT-IR) spectra showed that the aromatic C–H telescoping vibration was responsible for the distinctive absorption bands at 3000–3100 cm^{-1}, and the aromatic C = C telescoping vibration was responsible for the bands at 1500–1600 cm^{-1}. The O = N–O telescoping vibration was identified by peak observations within the range of 1350–1450 cm^{-1}, and the C–H bending vibration was identified by peak observations within the 700–800 cm^{-1} range. It can therefore be posited that the aforementioned absorption peaks may be utilized as infrared fingerprints with a view to ascertaining the structure of nitroimidazoles. A comparative analysis of the experimentally determined FT-IR spectra with the reference data revealed a high degree of correspondence between the experimentally observed characteristic absorption peaks and the standard data, indicating that with a high degree of confidence, the six nitroimidazole CRM candidates can be identified (Fig. 1). Furthermore, the mass spectra (Fig. 2) of the nitroimidazole CRM candidates were obtained using electrospray ionization mass spectrometry with flow injection in the positive

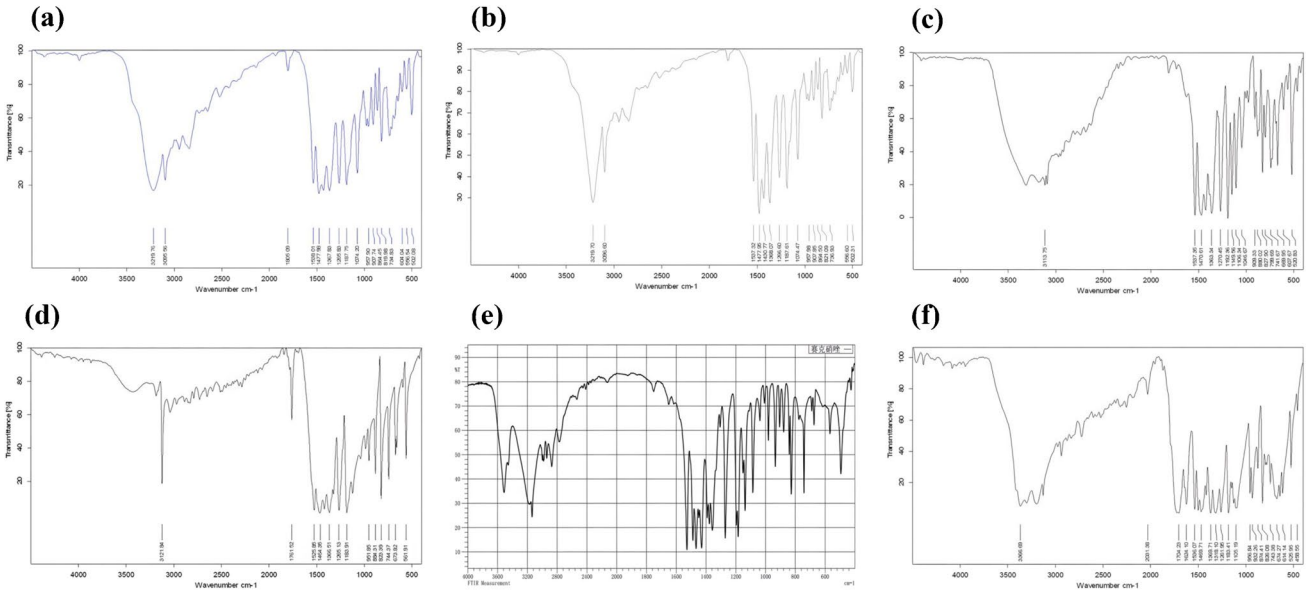

Fig. 1 FT-IR spectra for the nitroimidazole candidates: **a** metronidazole, **b** tinidazole, **c** ornidazole, **d** dimetridazole, **e** secnidazole, **f** ronidazole

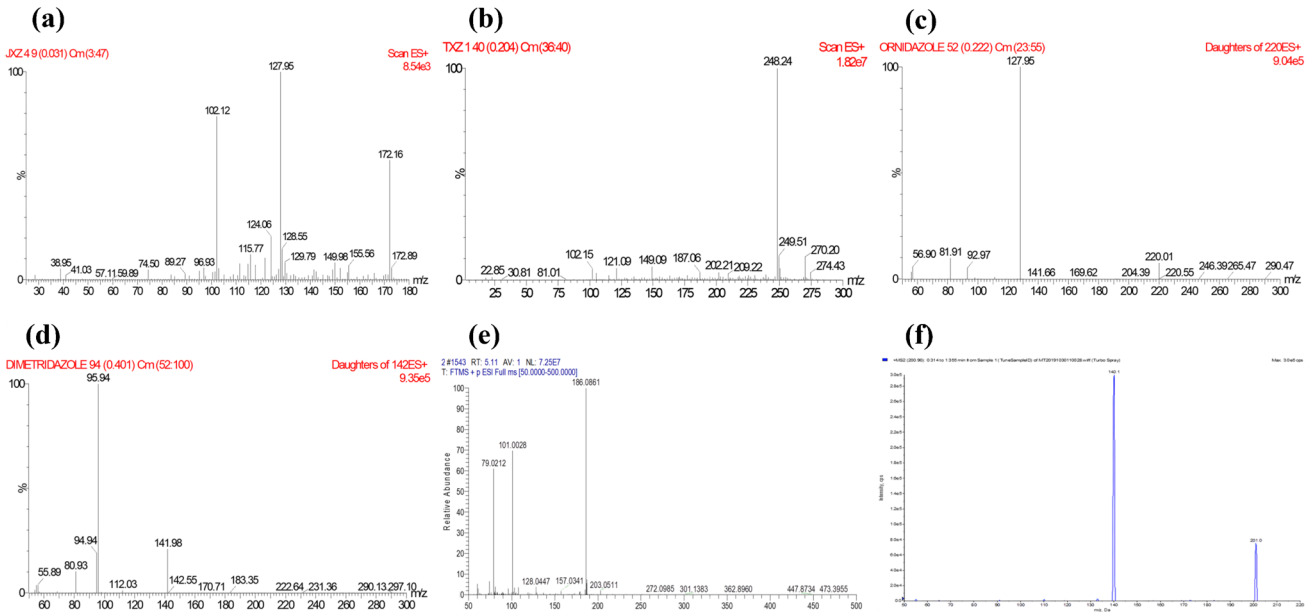

Fig. 2 MS spectra for the nitroimidazole candidates: **a** metronidazole, **b** tinidazole, **c** ornidazole, **d** dimetridazole, **e** secnidazole, **f** ronidazole

mode. The most prominent peaks of $[M+H]^+$ were identified as matching the molecular ions, which is in accordance with the theoretical m/z values calculated using the International Union of Pure and Applied Chemistry (IUPAC) International Atomic Scale. Furthermore, the major ion fragments also exhibited a high degree of concordance, thereby providing compelling evidence that the six nitroimidazole CRM candidates can be qualified and deemed suitable for further evaluation.

Homogeneity

The results of the conducted homogeneity test on each nitroimidazole compound are presented in Fig. 3. According to the *F*-test for analysis of variance, no statistically significant differences were found between or within bottles, as evidenced by the calculated *F* being smaller than the critical *F* (Table S4). In consideration of the aforementioned observations, it can be concluded that the candidate nitroimidazole

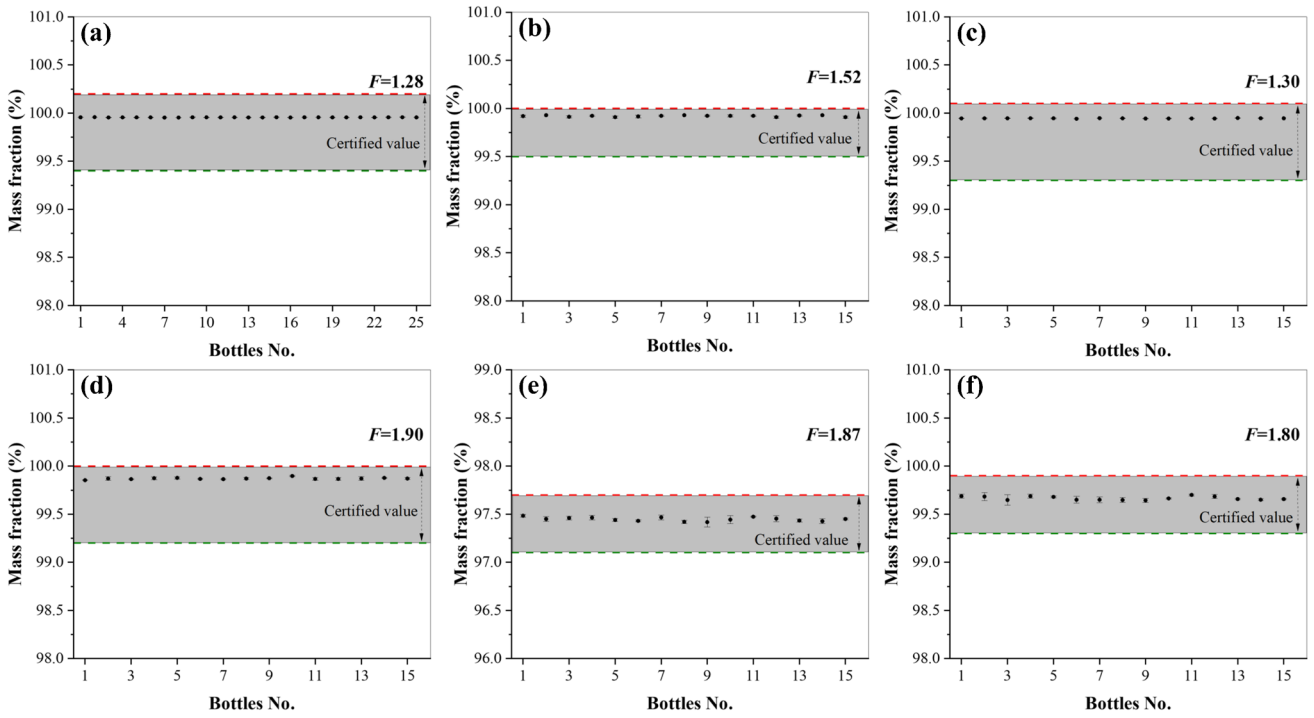

Fig. 3 The results of the homogeneity testing for the nitroimidazole CRM candidates: **a** metronidazole, **b** tinidazole, **c** ornidazole, **d** dimetridazole, **e** secnidazole, **f** ronidazole

CRMs prepared within the framework of this study exhibited sufficient homogeneity.

Stability

The observed stability may indicate a tendency for the characteristic values associated with the CRM to change in relation to storage and transport conditions. The short-term stability at 20, 40, and 60 °C as well as the long-term stability at − 20 ℃ were assessed in order to look into the stability of nitroimidazoles at different temperatures (Figs. 4 and 5). This was achieved by utilizing regression lines that were fitted to a plot of the responses of the compounds against time. The slope of the line, designated by the symbol β_1, and the standard deviation of β_1, designated by the symbol $s(\beta_1)$, were calculated using a t-test, a statistical procedure that enables the estimation of population means and the calculation of confidence intervals. Absolute slope value was considered statistically insignificant at the 95% confidence level when the value of $|\beta_1|$ fell below the threshold value $t_{0.95,n-2} \times s(\beta_1)$ at the 95% confidence level and with 2 degrees of freedom. As illustrated in Tables S5 and S6, in each instance, the magnitude of the slope was found to be below the product of $t_{0.95,n-2} \times s(\beta_1)$, thereby substantiating the remarkable stability of the developed nitroimidazole candidates.

The MB method for evaluating purity

Measurements of the primary component and impurities

In order to determine the separation of the primary component from the impurities and between the impurities, the conditions for the high-performance liquid chromatography-ultraviolet separation were tested prior to the measurement of the nitroimidazoles. This entailed the composition of the mobile phase, the characteristics of the column, and the wavelength for each nitroimidazole. The optimized conditions outlined in Table S1 were employed to obtain chromatograms of six nitroimidazoles in methanol. The typical chromatogram for every nitroimidazole and impurity dissolved in methanol is displayed in Fig. 6. The data presented in Table S7 provides the peak area and percentage of impurities at varying wavelengths. Seven bottles of each nitroimidazole were subjected to HPLC–UV analysis, and area normalization was used to determine the mass fractions of each nitroimidazole's main constituents.

Measurement of water content

It is of the utmost importance to acknowledge the role of water as an impurity in the development of purity CRMs. In order to guarantee the veracity and dependability of the outcomes attained, the titrator was situated within an

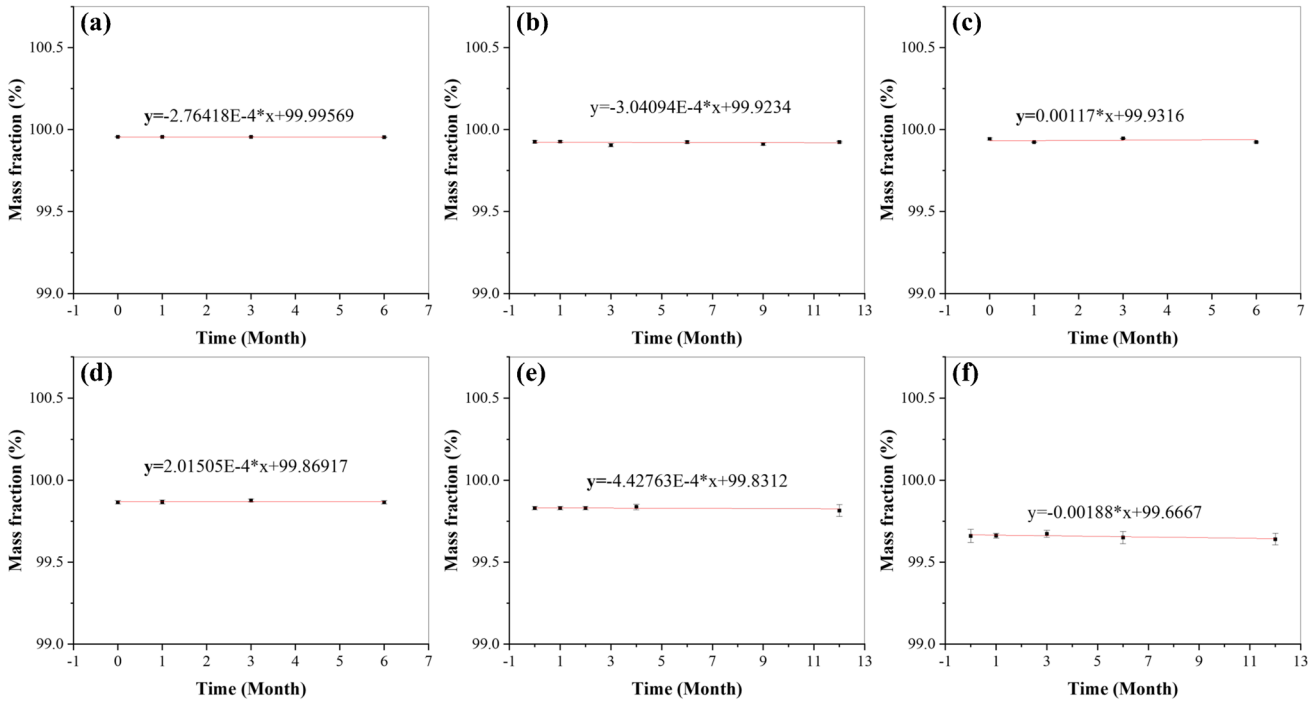

Fig. 4 The results of the long-term stability testing for the nitroimidazole CRM candidates: **a** metronidazole, **b** tinidazole, **c** ornidazole, **d** dimetridazole, **e** secnidazole, **f** ronidazole

environment that was both drying and subjected to a consistent temperature. Prior to initiating the titration process, the system underwent a validation test using a water standard. The water content of each nitroimidazole was quantified to be 0.038% ± 0.007%, 2.410% ± 0.080%, 0.004% ± 0.001%, 0.019% ± 0.005%, 0.220% ± 0.095%, and 0.131% ± 0.007%, respectively, for tinidazole, secnidazole, ronidazole, metronidazole, dimetridazole, and ornidazole.

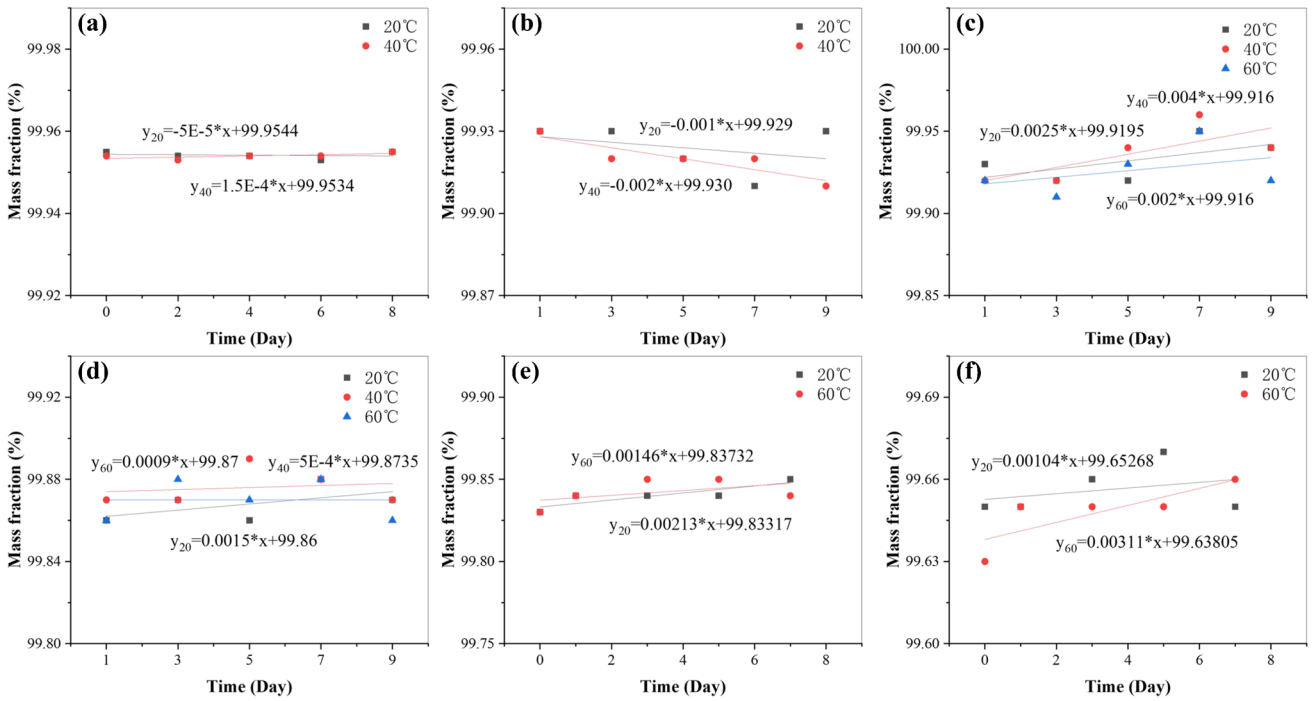

Fig. 5 The results of the short-term stability testing for the nitroimidazole CRM candidates: **a** metronidazole, **b** tinidazole, **c** ornidazole, **d** dimetridazole, **e** secnidazole, **f** ronidazole

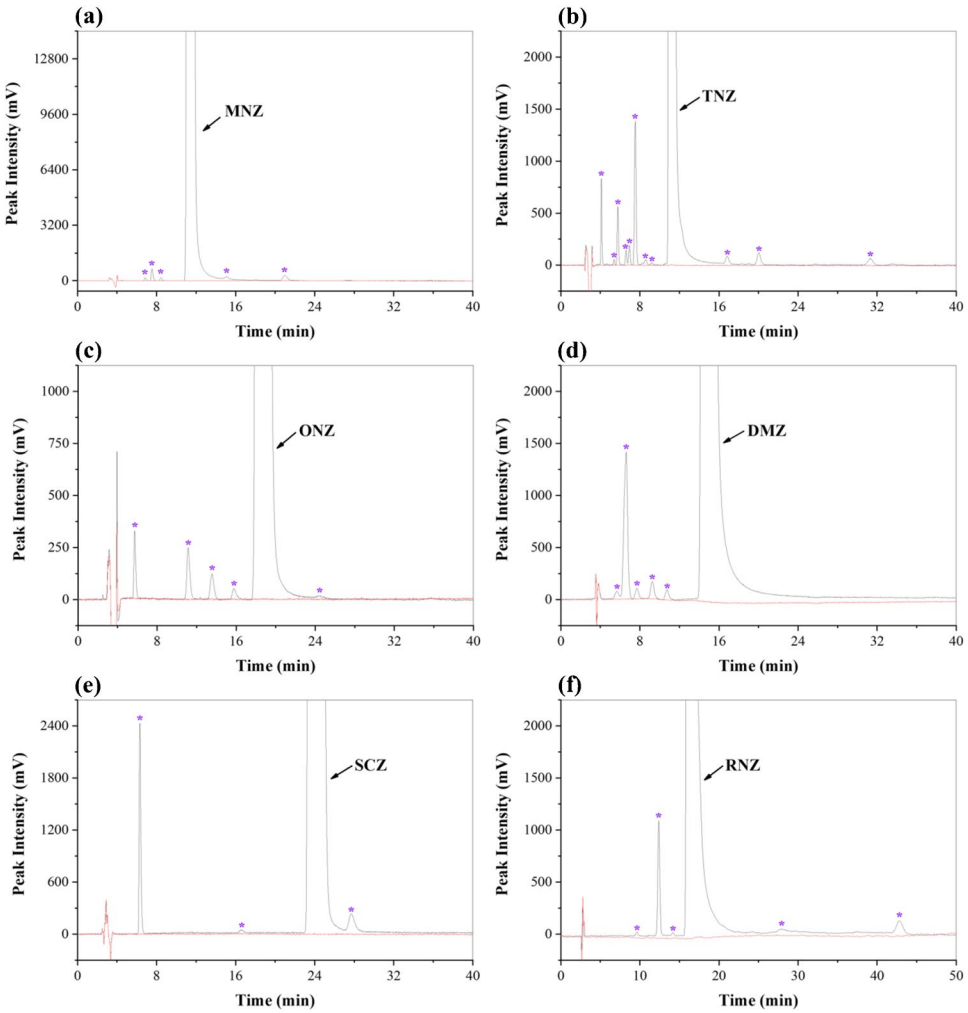

Fig. 6 Chromatograms of nitroimidazoles and impurities in solution (red, blank solvent): **a** metronidazole, **b** tinidazole, **c** ornidazole, **d** dimetridazole, **e** secnidazole, **f** ronidazole

Determination of other impurities

Headspace gas chromatography was coupled with a flame ionization detector (FID) and inductively coupled plasma mass spectrometry (ICP-MS) to quantify the remaining impurities in the six nitroimidazole CRMs. The former method was employed to ascertain the volatile organic impurities, while the latter was utilized to identify the non-volatile inorganic impurities. The content of volatile impurities in secnidazole was found to be 0.016%, while that of ronidazole was determined to be 0.025%. It was demonstrated that the content of volatile impurities in the remaining nitroimidazoles was negligible. The content of non-volatile inorganic impurities in each nitroimidazoles was determined to be 0.222% for tinidazole, 0.014% for secnidazole, 0.017% for ronidazole, 0.092% for metronidazole, 0.0102% for dimetridazole, and 0.0094% for ornidazole, respectively. Formula (1) can be used to determine the percentage by mass of each nitroimidazole as determined by the MB method. Table 1 contains a list of each nitroimidazole's purity.

Purity determination by qNMR method

Another frequently employed methodology for the determination of purity in CRMs is qNMR. This is accomplished by contrasting the candidate's absorption peak intensities with the internal standard's absorption peak intensities. Purity CRMs may be employed in the assessment of the purity of nitroimidazole CRM candidates, with a view to establishing their traceability to the SI unit in terms of metrology. There must be no overlap between the two quantitative signal peaks that are derived from the internal standard and the analyte, respectively. Ideally, the peaks should remain as closely spaced as possible. A typical qNMR spectrum used for nitroimidazole quantitative analysis is shown in Fig. 7. Quantitative peaks were observed corresponding to the internal standard and nitroimidazoles, designated I_{int} and I_{sam}, respectively. The identified protons were deemed appropriate for quantitative analysis due to their distinctiveness and the lack of overlap with other peaks, thus allowing for accurate and precise measurements. Seven vials of each

Table 1 Purity assignment results from MB and qNMR for nitroimidazoles (%)

No	Tinidazole		Secnidazole		Ronidazole		Metronidazole		Dimetridazole		Ornidazole	
	MB	qNMR	MB	qNMR	MB	qNMR	MB	qNMR	MB	qNMR	MB	qNMR
1	99.58	99.75	97.53	97.45	99.67	99.59	99.89	99.58	99.88	99.73	99.92	99.86
2	99.64	99.76	97.34	97.41	99.64	99.57	99.81	99.67	99.86	99.75	99.97	99.81
3	99.64	99.73	97.46	97.36	99.57	99.59	99.86	99.71	99.85	99.76	99.91	99.78
4	99.69	99.64	97.34	97.45	99.58	99.49	99.76	99.63	99.83	99.81	99.98	99.83
5	99.73	99.78	97.48	97.26	99.61	99.53	99.84	99.68	99.83	99.79	99.91	99.84
6	99.65	99.68	97.56	97.45	99.61	99.47	99.85	99.65	99.79	99.75	99.95	99.81
7	99.62	99.63	97.48	97.21	99.65	99.45	99.92	99.65	99.87	99.73	99.89	99.84
Mean	99.65	99.71	97.46	97.37	99.62	99.53	99.85	99.65	99.84	99.76	99.93	99.82
SD	0.05	0.06	0.09	0.10	0.04	0.06	0.05	0.04	0.03	0.03	0.03	0.03
F	0.6481		0.7572		0.3975		1.6113		1.0317		1.6644	
Overall	99.7		97.4		99.6		99.7		99.8		99.9	

of the nitroimidazoles were subjected to qNMR analysis, and the resulting purities are presented in Table 1.

Purity certification

The *F*-test was employed to statistically analyze the MB and qNMR measurement results. The aim of the analysis was to identify any statistically significant differences between the two methods concerning the outlying variances, with a significance level of $\alpha = 0.05$. The calculated F value is less than the critical F value, $F_{0.05(6,6)}$, which equals to 4.28, as shown in Table 1. Consequently, it was found that the results of the two separate methods demonstrated similar accuracy. The purity of each nitroimidazole was ascertained by taking the mean of the measurement results that were obtained.

Uncertainty

Uncertainty of homogeneity

A calculation was performed in accordance with Formula (3) to determine the uncertainty of homogeneity.

$$u_{\mathrm{bb}} = \sqrt{\frac{M_{\mathrm{between}} - M_{\mathrm{within}}}{n}} \tag{3}$$

The uncertainty related to the homogeneity of the data is represented by the symbol u_{bb}. The symbols M_{between} and M_{within} represent the inter-bottle and intra-bottle mean squares, respectively. The number of measurements is represented by the value of the variable n.

Uncertainty of stability

According to Formula (4), the uncertainty of stability $u_{\mathrm{stability}}$ was calculated, which includes the uncertainties of both short-term stability u_{sts} and long-term stability u_{lts}.

$$u_{\mathrm{stability}} = s(\beta_1) \times t \tag{4}$$

The uncertainty associated with the slope, designated as $s(\beta_1)$, is a function of the monitoring period, t.

Uncertainty surrounding the MB method

The fundamental component, moisture content, and non-volatile impurity determinations are the main sources of uncertainty in the MB method. The calculation of these values can be achieved by employing the following Formula (5).

$$u(P_{MB}) = P_{MB} \times \sqrt{\left[\frac{u(P_0)}{P_0}\right]^2 + \frac{\left[u(X_W)\right]^2 + \left[u(X_V)\right]^2 + \left[u(X_{NV})\right]^2}{(1 - X_w - X_V - X_{NV})^2}} \tag{5}$$

P_{MB} stands for the purity as determined by the MB method in this context. The percentage of nitroimidazoles and the uncertainty related to the area normalization method are denoted by the variables P_0 and $u(P_0)$, respectively. The content and uncertainties of moisture, volatile impurities, and non-volatile impurities are represented by the symbols X_{w}, $u(X_{\mathrm{W}})$, X_{V}, $u(X_{\mathrm{V}})$, X_{NV}, and $u(X_{\mathrm{NV}})$, respectively.

The uncertainty associated with the moisture content, $u(X_{\mathrm{W}})$, was calculated in accordance with the specified Formula (6).

$$u(X_{\mathrm{W}}) = X_{\mathrm{W}} \sqrt{{u_{\mathrm{rel},1}}^2 + \left[\frac{u(m)}{m}\right]^2 + \left[\frac{u(W)}{W}\right]^2 + \left[\frac{u(f)}{f}\right]^2} \tag{6}$$

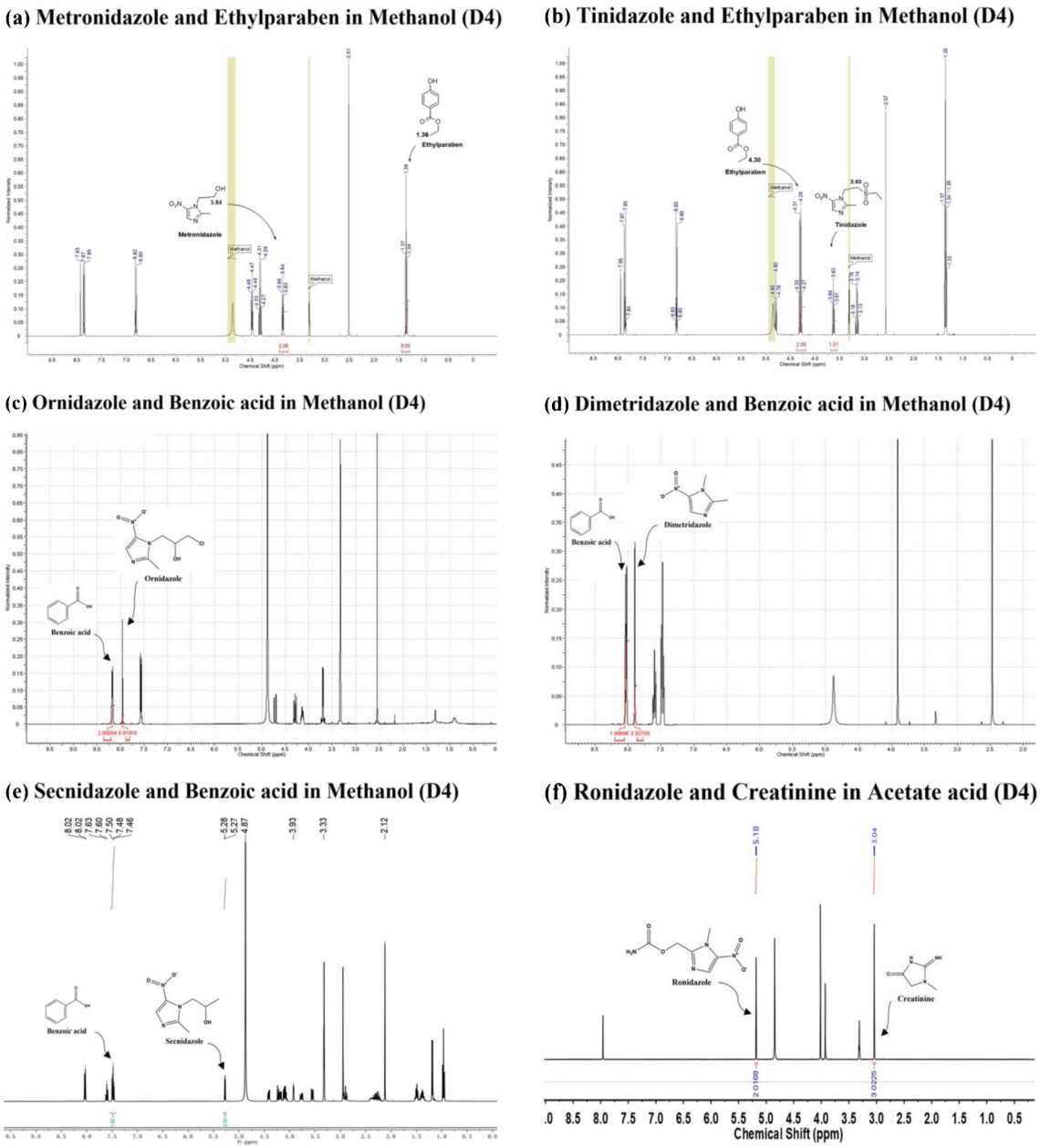

Fig. 7 NMR spectra of six nitroimidazoles; labeled peaks were used for quantitative analysis: **a** metronidazole, **b** tinidazole, **c** ornidazole, **d** dimetridazole, **e** secnidazole, **f** ronidazole

The moisture content of the CRM candidate is designated as X_W. The relative standard deviation of the measurements is designated as $u_{rel,1}$. The mass of water and the potential nitroimidazole are denoted as m and W, respectively. The correction factor, denoted as f, is computed using the moisture reference material's standard uncertainty as a basis for calculation.

Uncertainty of the qNMR method

Variabilities in measurement repeatability are the main cause of uncertainty in the qNMR technique, the precision and accuracy of the weighing procedure, the stability and reliability of the internal standard, and the accuracy and precision of the molecular weight determination. These quantities can be calculated using the following Formula (7).

Table 2 Uncertainty of the nitroimidazole purity CRMs (%)

Nitroimidazoles	u_{MB}	u_{qMNR}	u_{char}	u_{bb}	u_{lts}	u_{sts}	u_{CRM}	U
Tinidazole	0.0330	0.0710	0.0360	0.0035	0.0468	0.0135	0.07	0.20
Secnidazole	0.1338	0.1477	0.1008	0.0140	0.0052	0.0190	0.10	0.30
Ronidazole	0.0377	0.1787	0.1037	0.0132	0.0102	0.0114	0.11	0.30
Metronidazole	0.0940	0.1200	0.1600	0.0010	0.0020	0.0020	0.20	0.40
Dimetridazole	0.0790	0.1450	0.1700	0.0064	0.0020	0.0190	0.20	0.40
Ornidazole	0.0180	0.1220	0.1300	0.0009	0.0040	0.0051	0.20	0.40

$$u\left(P_{qNMR}\right) = P_{qNMR}\sqrt{\left[\frac{u\left(I_x/I_{std}\right)}{I_x/I_{std}}\right]^2 + \left[\frac{u\left(M_x\right)}{M_x}\right]^2 + \left[\frac{u\left(M_{std}\right)}{M_{std}}\right]^2 + \left[\frac{u\left(m_x\right)}{m_x}\right]^2 + \left[\frac{u\left(m_{std}\right)}{m_{std}}\right]^2 + \left[\frac{u\left(P_{std}\right)}{P_{std}}\right]^2} \quad (7)$$

The formula for standard uncertainty of the area ratio between the internal standard and the quantitative peak of the nitroimidazole candidate is $\frac{u\left(I_x/I_{std}\right)}{I_x/I_{std}}$. The relative standard uncertainties derived from the molar mass of the internal standard and the nitroimidazole candidate are represented, respectively, by the expressions $\frac{u\left(M_x\right)}{M_x}$ and $\frac{u\left(M_{std}\right)}{M_{std}}$. The $\frac{u\left(m_{std}\right)}{m_{std}}$ and $\frac{u\left(m_x\right)}{m_x}$, respectively, represent the standard uncertainty related to the weighing mass of the nitroimidazole candidate and the internal standard. The standard deviation of the relative purity of the internal standard is $\frac{u\left(P_{std}\right)}{P_{std}}$. A comprehensive overview of uncertainties is presented in Table 2.

The uncertainty associated with the characterization of u_{char} should be calculated in accordance with the specifications outlined in Formula (8).

$$u_{char} = \sqrt{\left(\frac{u_{MB}}{2}\right)^2 + \left(\frac{u_{qMNR}}{2}\right)^2 + \left(\frac{P_{MB} - P_{qMNR}}{2}\right)^2} \quad (8)$$

Expanded uncertainty

Using the suggested Formulas (9) and (10), the expanded uncertainty U and the combined standard uncertainty u_{CRM} were calculated.

$$u_{CRM} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2 + u_{sts}^2} \quad (9)$$

$$U = k \times u_{CRM} \quad (10)$$

The expanded uncertainty, represented by the parameter $k = 2$, falls within the 95% confidence interval.

Conclusion

The present work describes a detailed process for the development of six nitroimidazole purity CRMs. Initially, a series of structural analyses were conducted on the high-purity candidates using IR spectroscopy and MS. Subsequently, the purity of each nitroimidazole compound was accurately quantified by two distinct, traceable methodologies: MB and qNMR. In addition, the homogeneity, stability, and uncertainties associated with these nitroimidazoles have been sufficiently examined. Following a series of rigorous analytical and quality control procedures, the reliable purity CRMs of tinidazole, secnidazole, ronidazole, metronidazole, dimetridazole, and ornidazole have been successfully prepared and have obtained the requisite approvals from the SAMR of China. Through an unbreakable traceability chain, the above CRMs can be traced back to the SI unit, thereby offering technical support and the assurance of value traceability in the analysis of agricultural products and related disciplines. Furthermore, the resulting measurement is guaranteed to be reliable and accurate, thereby ensuring the protection of public health.

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Declarations

Conflict of interest The authors declare no competing interests.

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