Brazilian Journal of Analytical Chemistry
Pre-publication – Accepted after peer review
doi: 10.30744/brjac.2179-3425.TN-22-2023

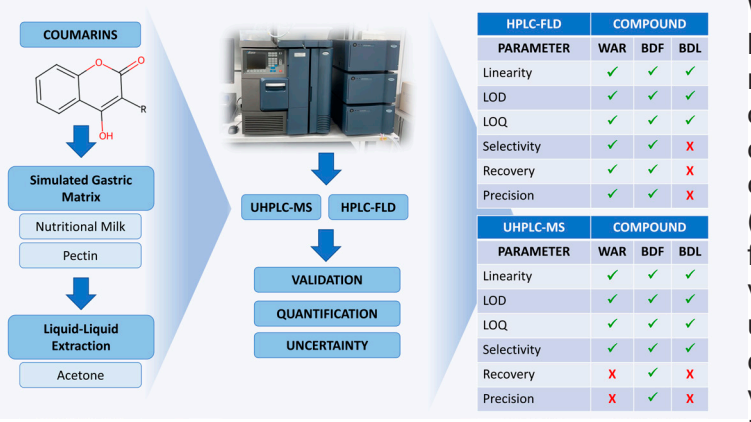
BrJAC

TECHNICAL NOTE

Validation and Uncertainty Calculation of Rodenticide Analysis Methods in a Simulated Gastric Content Matrix – *Uncertainty of Rodenticide Analysis Methods*

Flávio Augusto Ferreira Martins Bezerra, Youssef Bacila Sade, Jailton Carreteiro Damasceno, Renata Carvalho Silva*

Instituto Nacional de Metrologia, Qualidade e Tecnologia (Inmetro), Avenida Nossa Senhora das Graças, 50, Duque de Caxias-Xerém, Postal code 20250-020, Rio de Janeiro, Brazil



Warfarin (WAR), brodifacoum (BDF) and bromadiolone (BDL) are compounds present in rodenticides, highly toxic to rats, humans and other animals. These compounds can be detected in complex matrices, such as stomach contents, by liquid chromatography techniques (HPLC) with mass spectrometry (MS) or fluorescence detection (FLD). However, no validated method showed determination of uncertainty in the quantification of these compounds. In this study, we compare the validation parameters of two analytical methods, HPLC FLD and ultra high performance liquid chromatography (UHPLC – MS), with uncertainty estimation for the three cited compounds. The results showed that UHPLC-MS outperformed HPLC FLD, however both methods were considered adequate for detection of WAR, BDF or BDL in samples of simulated human stomach contents, especially in cases of suspected contamination.

Keywords: chromatography, complex matrix, coumarin, measurement uncertainty, quantification

INTRODUCTION

Rodenticide poisoning is a common global health problem. The groups most affected by rodenticide poisoning in Brazil are children (accidental exposure) and adults (homicide and suicide attempts). Rodenticide poisoning typically occurs via ingestion of coumarin-derived anticoagulant rodenticides, such as warfarin (WAR- Structure A), brodifacoum (BDF- Structure B), bromadiolone (BDL- Structure C) (Figure 1), and indandione.[1] Coumarins are metabolic derivatives of phenylalanine comprising a benzene

Cite: Bezerra, F. A. F. M.; Sade, Y. B.; Damasceno, J. C.; Silva, R. C. Validation and Uncertainty Calculation of Rodenticide Analysis Methods in a Simulated Gastric Content Matrix — *Uncertainty of Rodenticide Analysis Methods. Braz. J. Anal. Chem.* (Forthcoming). http://dx.doi.org/10.30744/brjac.2179-3425.TN-22-2023

Submitted 16 March 2023, Resubmitted 08 July 2023, Accepted 19 July 2023, Available online July 2023.

ring attached to a pyran ring. They can be isolated from plants, fungi, and bacteria and have potent anticoagulant properties.[2]

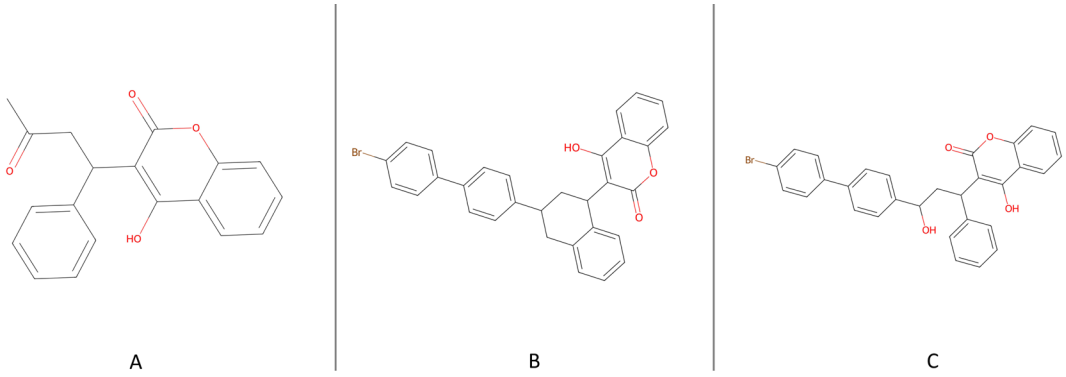


Figure 1. The structures of the coumarin compounds.

In Brazil, 25,892 cases of rodenticide poisoning were reported between 2017 and 2021, including 19,267 cases of attempted suicide, 4742 accidents, and 362 cases of attempted murder.[3] Biological samples can be collected and sent to forensic toxicology laboratories for analysis in cases of suspected rodenticide poisoning due to accidental ingestion or criminal intoxication.[4]

Rodenticides inhibit vitamin K epoxide reductase, causing active vitamin K deficiency, such that vitamin-K-dependent clotting factors are not activated and remain non-functional, causing massive bleeding. Coumarin rodenticides can be distributed in different tissues, and blood and liver are the primary matrices used in forensic analysis. Owing to the long half-life of coumarin rodenticides, blood and liver tissue samples are preferred for *ante-* and *post-mortem* analyses, because they contain the highest concentrations of active compounds.[1] However, in Brazil, stomach content samples have frequently been collected for the forensic analysis of coumarin compounds, and a validated method for the identification of coumarin compounds in animal stomach contents has recently been published.[5–7] However, the only validated methods for the analysis of coumarin rodenticides involve the use of other matrices such as blood and liver samples.[5–17]

The stomach content is a complex matrix; therefore, several researchers have used artificial media to mimic it. Simulated gastric fluids containing pepsin, small amounts of bile salts, lecithin, and synthetic surfactants have been widely used in in vitro drug dissolution studies. However, simulated gastric fluids cause numerous interferences during analysis and lead to overestimating the physiologically important conditions of the actual stomach content. Therefore, simulated gastric fluids have recently been replaced with media containing Ensure® Plus nutrition shake, which have been more efficient in simulating "fed stomachs" and have facilitated the analysis of different compounds.[18]

Liquid chromatography (LC) methods, such as high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC), and gas chromatography (GC) in conjunction with mass spectrometry (MS), ultraviolet (UV), and fluorescence detection (FLD), have been used for compound analysis. Although CG–MS and LC–UV are commonly used to analyse rodenticides in biological samples, the types of samples that can be analysed using these methods are limited. This is attributed to the detection limit of GC–MS being approximately 10 times higher than that of LC–MS. Conversely, methods based on UV detection have low sensitivity for rodenticide quantification, especially in the concentration range of 10–100 ng mL^{-1}.[1] Therefore, HPLC FLD,[8–10] HPLC–MS,[11] and UHPLC–MS [7,12–17] are the most commonly used methods for analysing rodenticides in biological samples.[1] A crucial step prior to chromatographic analysis is sample preparation using methods such as liquid–liquid extraction or solid-phase extraction (SPE), which can be used to isolate, purify, and concentrate analytes.[1,5–17] Once the extraction and chromatography methods have been selected, they must be validated, if necessary.[19] Furthermore, it is critical to evaluate the measurement uncertainty of quantitative methods.

According to the International Vocabulary of Metrology, measurement uncertainty is a non-negative parameter that characterises the dispersion of values assigned to a measurement.[20] The ISO/IEC 17025 standard states that testing laboratories should evaluate the uncertainty of measurements. If the measurement uncertainty of a method cannot be accurately estimated, an estimate should be made based on a theoretical understanding of the method or practical experience of the method performance. Furthermore, uncertainty evaluation should consider the contributions that are significant to the measurement results.[21]

Validation and evaluation of the uncertainty of a method are critical for forensic toxicology laboratories that quantify rodenticides in gastric content matrices. Established procedures are inadequate for yielding reliable results because they do not have measurement uncertainty. Materials should be collected and extracted prior to analysis, and these steps should be reproducible and allow for satisfactory analyte recovery. Therefore, in this study, we optimised and compared the performance of HPLC FLD and UHPLC–MS as methods for identifying rodenticidal coumarin compounds in simulated human stomach content samples. Furthermore, we determined the factors that contributed to measurement uncertainty.

MATERIALS AND METHODS

Chemicals and reagents

The WAR, BDF, and BDL standards were purchased from LGC-GmbH (Germany). Ultra-high-temperature–processed Ensure® Plus nutrition shakes were obtained from Abbott (Brazil). Acetone, acetonitrile, and methanol were acquired both from Sigma-Aldrich (Brazil) and SK Chemicals (South Korea). Apple pectin, acetic acid, ammonium acetate, triethylamine, and ammonium hydroxide were purchased from Sigma-Aldrich (Brazil).

Preparation of the stock solutions

Stock solutions of WAR, BDF, and BDL in acetonitrile were prepared in triplicate, and their concentrations were determined gravimetrically. The working solutions were stored at 2–8 °C according to Chalermchaikit et al.[22] The concentrations of the WAR, BDF, and BDL stock solutions were 597.4, 617.6, and 590.9 μg g^{-1}; 661.2, 546.5, and 646.8 μg g^{-1}; and 636.9, 589.2, and 625.2 μg g^{-1}, respectively.

Preparation of the working solutions

To construct the calibration curves of the WAR, BDF, and BDL solutions, working solutions with nominal concentrations of 300, 500, 700, 900, and 1100 ng g^{-1} were prepared in triplicate by diluting the stock solutions with a methanol–water mixed solvent (1:1 (v/v)).[23] The dilutions were performed in vials by adding predetermined volumes of the working solutions to the mixed solvent. The volumes of the working solutions were measured gravimetrically using an analytical balance, and the actual concentrations of the diluted solutions were calculated using the experimental data.

Sample preparation

Preparation of the simulated stomach content matrix and enriched matrices

A simulated stomach content matrix was used because of the complex and diverse chemical composition of the real stomach content matrix. The experimental medium simulated the initial composition of the postprandial stomach (’fed state’). Furthermore, it contained numerous interferents and mimicked the physiological conditions of a real stomach matrix, which could interfere with rodenticide analysis (e.g. pH, osmolarity, and analyte adsorption on the surfaces of the solid matrix components).[24]

Simulated stomach content samples were prepared as follows. The nutrient composition of the Ensure® Plus nutrition shake used in this study was comparable to that of the standard North American breakfast, according to the Food and Drug Administration. The viscosity of Ensure® Plus nutrition shake was increased using 0.45% pectin to obtain a medium simulating the initial composition of the postprandial stomach.[25]

WAR, BDF, and BDL stock solutions were added to simulated stomach content matrices to obtain enriched matrices with five analyte concentrations (in triplicate) at nominal concentrations of 300, 500,

700, 900, and 1100 ng g^{-1}. The concentrations of WAR, BDF, and BDL in the enriched matrices were determined gravimetrically, and the volumetric masses of the stock solutions and enriched matrices were measured in 50 mL conical tubes using an analytical balance to a final mass of 5 g.

Extraction of the enriched matrices

Both the preparation of the fortified matrix and the extraction process were carried out on the same day in the ambient temperature range of the test laboratory (20-25 °C).

Approximately 5 g of acetone was added to each enriched matrix under shaking. The mixtures were then centrifuged at 17,000 g at 4 °C for 15 min to obtain two-phase systems. The supernatants were collected, filtered into 5 mL borosilicate glass flasks using 0.22 µm polyvinylidene fluoride membranes (Analítica, Brazil), and allowed to rest overnight in a refrigerator (2-8 °C) to decant any suspended particles that could interfere with the analysis. Thereafter, 1 mL of each supernatant sample was collected and transferred to a 2 mL HPLC glass vial (Waters, USA).

Instrumentation

Chromatographic separation was performed using an Alliance HPLC system (Waters, USA) equipped with a separation module (e2695) and an analytical column (Symmetry C-18, 4.6 mm × 75 mm, 3.5 µm particle size; Waters, USA). The temperature of the column was maintained at 50 °C during separation, and the sample injection volume was 10 µL. Gradient elution was performed using a mobile phase comprising 40 mM ammonium acetate, 0.2% acetic acid, and 0.2% triethylamine in ultrapure water (mobile phase A) or methanol (mobile phase B) at a flow rate of 0.5 mL min^{-1}. The gradient used for BDF and BDL was as follows: 0–2 min, 52% B; 2–13 min, linear gradient to 82% B; 13–16 min, linear gradient to 87% B; and 16–30 min, linear gradient to 52% B. The gradient used for WAR was as follows: 0–2 min, 20% B; 2–10 min, linear gradient to 52% B; 10–16min, linear gradient to 70% B; 16–23 min, linear gradient to 82% B; 23–27 min, linear gradient to 52% B; and 27–30 min, linear gradient to 20% B. The analytes were detected using a Waters 2475 multiwavelength fluorescence detector (FLD) with emission and excitation wavelengths of 390 and 318 nm, respectively.[22] The Empower software (Waters, USA) was used to process and analyse the chromatograms.

UHPLC analysis was performed using an Acquity H-Class system (Waters, USA) equipped with an Acquity UPLC® BEH C-18 column (2.1 mm × 50 mm, 1.7 µm particle size; Waters, USA). The column temperature during separation was maintained at 50 °C, and the injection volume was 1 µL. Gradient elution was performed using a mobile phase comprising water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.3 mL min^{-1}. The gradient used was as follows: 0–2 min, 5% B; 2–6 min, linear gradient to 90% B; 6–8 min, 90% B; and 8–10 min, linear gradient to 5% B. The analytes were detected using a Xevo® TQ-S triple quadrupole mass spectrometer (Waters, USA) equipped with an electrospray ionisation (ESI) source. Data were collected under the following experimental conditions: nebulisation pressure of 69 kPa; source temperature of 150 °C; and collision flux, desolvation, and gas cone of 0.15 mL min^{-1}, 1000 L h^{-1}, and 150 L h^{-1}, respectively. The capillary voltage was optimised to 2.35 kV in the negative ESI mode. Dwell times ranging between 10 and 110 ms per transition were selected for each analyte. The multiple reaction monitoring (MRM) mode was used to monitor ion transitions. For each compound, the most and second-most intense product ions were selected for quantitative and qualitative analyses, respectively. The monitored transitions were as follows: 307.1 → 161.0 and 307.1 → 250.1 for the identification and quantification of WAR, respectively, 523.1 → 143.0 and 523.1 → 80.0 for the quantification and identification of BDF, respectively, and 527.0 → 181.0 and 527.1 → 275.0 for the quantification and identification of BDL, respectively.[17] The MassLynx software with the TargetLynx add-on (Waters) was used to process and analyse the experimental data.

Validation

Calibration curves of WAR, BDF, and BDL in solutions and enriched matrices

Linearity was investigated by analysing the calibration curves of WAR, BDF, and BDL in solutions and enriched matrices in the concentration range of 300–1100 ng g^{-1}. Five points were selected in this concentration range, and experiments were performed in triplicate for a total sample space of 15. Calibration curves were constructed by plotting the instrument response signal (peak area) against the analyte concentration, which was determined gravimetrically. Linearity was evaluated by analysing the results of the linear regression curves.[23] Prior to performing linear regression, the raw data were analysed using the Grubbs and Cochran tests to identify outlier values and determine the homoscedasticity of the data. A linear regression model was then applied to the experimental data using the Excel Data Analysis tool to evaluate the statistical significance of the regressions (at a confidence level of 95%), obtain the standardised residual plots, and determine the correlation coefficients (r^2). The acceptance criteria for the linearity parameter were $r^2 \geq 0.98$ (7), a *p*-value of the F-test for regression of < 0.05, and a residual plot with a random distribution around the Y-axis (no trends), confirming linearity.[19] The limits of detection and quantification (*LOD* and *LOQ*, respectively) were defined as the lowest concentrations with signal-to-noise ratios of 3 and 10, respectively, and were calculated according to Equations 1 and 2:[19]

$$LOD = 3.3\,\frac{s}{b} \qquad \text{Equation 1}$$

$$LOQ = 10.0\,\frac{s}{b} \qquad \text{Equation 2}$$

where *s* and *b* are the standard deviation of the blank solution and slope of the calibration curve of the matrix, respectively.

Considering previously reported data on coumarin rodenticides in the stomach contents of animals, we established that the *LOQ* acceptance criterion was *LOQ* ≤ 1 μg g^{-1}.[6] As *LOD* was three times smaller than *LOQ*, the *LOD* acceptance criterion was defined as *LOD* ≤ 0.33 μg g^{-1}.

Selectivity

To determine the selectivity of the quantitative analysis methods, a *t*-test was performed to compare the slopes of the curves of the WAR, BDF, and BDL solutions and matrices enriched with these compounds. Before performing the t-test, an F-test was conducted to check the variance homogeneity between the curves for the solutions and matrices. Subsequently, a *t*-test was performed with a confidence level of 95% to compare the values of slopes (from the straight lines of the compounds in solutions and matrices) within the minimum and maximum slope values of the linear regressions.[19] To assess the selectivity of the qualitative analysis methods, the response (peak area) of the blank (rodenticide-free matrix) was compared with those of the first points of the matrix curves, and the results were used to evaluate the degree of interference of the matrix with the analyte signal.[26] The acceptance criterion for quantitative methods was a *p*-value of the *t*-test > 0.05.[19] For qualitative analysis methods, the signal of the blank matrix should not exceed 20% of the response at a concentration of 300 ng g^{-1}.[26]

Recovery

Recovery was determined by selecting two concentrations from the curves of the WAR, BDF, and BDL solutions and enriched matrices (300 and 700 ng g^{-1}). Using the matrix peak areas and equations describing the curves of the WAR, BDF, and BDL solutions, we determined the actual concentrations of WAR, BDF, and BDL in the enriched matrices at preselected concentrations. Recovery was determined by calculating the recovery rate (*EP* (%) according to Equation 3:

$$EP\ (\%) = R \times 100 = \frac{Xmr}{Xmt} \times 100 \qquad \text{Equation 3}$$

where *R* is the ratio between the mean real concentration of the enriched matrix (*Xmr*) and mean gravimetric concentration of the enriched matrix (*Xmt*).[19]

The acceptance criterion for the EP was determined to be EP ≥ 62% using a method for analysing coumarin rodenticides in matrices extracted with acetone.1

Precision

The precision of the analytical methods was determined by preparing enriched matrices with concentrations of 300 and 700 ng g^{-1} in septuplicate. After the samples were extracted and analysed using LC, we determined the actual concentrations of WAR, BDF, and BDL in the enriched matrices at preselected concentrations using matrix signal data and the equations describing the curves of the WAR, BDF, and BDL solutions. The precision of each method was determined by calculating the coefficient of variation (*CV*), according to Equation 4:[19]

$$CV(\%) = \frac{s}{Xv} \times 100 \qquad \text{Equation 4}$$

where *Xv* is the actual concentration of the enriched matrix.

To determine the intra-day precision of the analytical methods, chromatographic analyses were performed on the day that the enriched matrices were prepared and extracted with acetone (day 1). To determine the inter-day precision of the analytical methods, chromatographic analyses were performed using the same samples, equipment, and analysts as those used for the intra-day precision experiments, and measurements were performed on two consecutive days (days 2 and 3). The *CV* value of the inter-day precision was calculated as the mean of the *CV* values of the data collected during the three days.[19] The acceptance criterion for precision was CV < 12.2%, which was established based on a method used to analyse coumarin rodenticides in matrices extracted with acetone.[1]

Uncertainty of concentration measurements

The concentrations of WAR, BDF, and BDL (x_R) were predicted using the calibration curves of the corresponding solutions and by the dilution factor (F_d), according to Equation 5:

$$x_R = \frac{y_d - a}{b}\frac{1}{F_d} \qquad \text{Equation 5}$$

where y_R is the average signal value (peak area) of the sample and *a* and *b* are the coefficients of the fitted line.[27]

The dilution factor was calculated by the ratio between the matrix mass and the total mass after addition of acetone and its uncertainty (u_{Fd}) was determined considering the repeatability of matrix masses and the calibration uncertainty of the analytical balance as sources. In addition, the uncertainty of the average x_R values was determined using the law of propagation of the uncertainty to the prediction equation, according to Equation 6:

$$u_{x_R}^2 = \left(\frac{\partial x_R}{\partial F_d}\right)^2 u_{F_d}^2 + \left(\frac{\partial x_R}{\partial y_R}\right)^2 u_{y_d}^2 + \left(\frac{\partial x_R}{\partial a}\right)^2 u_a^2 + \left(\frac{\partial x_R}{\partial b}\right)^2 u_b^2 + 2\left(\frac{\partial x_R}{\partial a}\right)\left(\frac{\partial x_R}{\partial b}\right) u_a u_b r_{a,b} \qquad \text{Equation 6}$$

where u_{yd} is the standard uncertainty of the reproducibility of the measurements of the sample signal, u_a is the standard uncertainty of *a*, u_b is the standard uncertainty of *b* and $r_{a,b}$ is the correlation coefficient between the coefficients of the fitted line.[27]

These data allowed us to build a cause–effect diagram for the established mathematical model (Figure 2). The inputs of the uncertainty model were the repeatability of the responses or signals (Y_R), the coefficients of the fitted line (a and b) as well as the dilution factor (F_d), and the output was X_R.

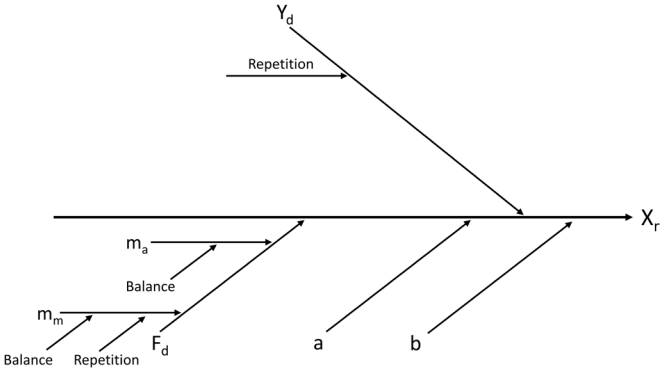


Figure 2. Sources of uncertainty considered in the Ishikawa diagram.

RESULTS AND DISCUSSION

Optimization

Acetonitrile[6,16] was used to prepare the working solutions, and acetone was used as the extraction solvent[5,6,15] because of the high solubilities of WAR, BDF, and BDL in these solvents. Acetonitrile, which is commonly used for rodenticide extraction,[14,16] was also tested; however, the average recoveries of WAR, BDF, and BDL in acetonitrile were lower than the acceptance criteria. Once the optimal solvents were selected for the preparation of the stock, working, and extraction solutions, we determined the method best suited for LC.

We used a gradient chromatography method because it yielded better separation of analytes from matrices than the isocratic method, as described in several recent papers on the analysis of rodenticides.[16,17] An HPLC-FLD gradient method (the chromatographic run method most indicated in articles for analysis in complex matrices)[22] was used, and it yielded satisfactory results for the analysis of WAR, BDF, and BDL in the gastric content matrix, as the analyte peaks were separate from the matrix interference peaks. The gradient UHPLC–MS method was based on a previously described protocol.[17] For these methods, the signals of the compounds were detected only at concentrations higher than 200 ng g^{-1}; therefore, a working concentration range of 300–1100 ng g^{-1} was selected to compare the validation parameters of the HPLC FLD and UHPLC–MS methods. This concentration range was similar to that used for the analysis of raticides in animal gastric matrices (100–1000 ng g^{-1}).[7]

The acquisition mode of the MS instrument was set to MRM after selecting the gradient UHPLC method and working concentration range. These settings have been widely used in recent studies, as they allow the monitoring of different reactions and selection of fragments with good signal-to-noise ratios.[14–16] In contrast, the selected reaction monitoring (SRM) mode is used in rodenticide analysis only when bulk analyte data are available.[16] SRM is often used when researchers have already collected analyte data and would like to monitor the product ions produced by specific reactions of the *m/z* precursor ions selected during a previous MS stage instead of acquiring the entire mass spectra of the product ions.[28] However, the MRM mode yields lower *LOD* values; hence, the MRM mode is the most suitable mass acquisition mode for the analysis of coumarins, as it enables monitoring several transitions and selection of high signal-to-noise responses.[14] After optimising the methods and generating the calibration curves, the methods were evaluated by comparing the results considering the validation parameters and previously established acceptance criteria.

Validation parameters used to evaluate method performance

Table I summarises the results of the chromatographic validation methods for the analysis of rodenticides in the simulated stomach content matrices. The compounds that yielded unsatisfactory results were not used for subsequent parameter evaluation. The linearity values were analysed first.

Table I. Performance parameters for high-performance liquid chromatography–fluorescence detection (HPLC FLD) and ultra-high-performance liquid chromatography–mass spectrometry (UPLC–MS). Here, WAR, BDF, BDL, *LOD, LOQ, EP*, and *CV* denote warfarin, brodifacoum, bromadiolone, limit of detection, limit of quantification, recovery rate, and coefficient of variation, respectively.

Parameter	Acceptance criteria	Results for HPLC FLD	Satisfactory?	Results for UHPLC–MS	Satisfactory?
Linearity (matrix)	$r^2 \geq 0.98$, p-value < 0.05, and residual plot with random distribution	WAR: $r^2 = 0.99$ and p-value < 0.0001 BDF: $r^2 = 0.98$ and p-value < 0.0001 BDL: $r^2 = 0.98$ and p-value < 0.0001	Yes, for all coumarins.	WAR: $r^2 = 0.98$ and p-value < 0.0001 BDF: $r^2 = 0.99$ and p-value < 0.0001 BDL: $r^2 = 0.99$ and p-value < 0.0001	Yes, for all coumarins.
LOD (matrix)	*LOD* ≤ 330 ng g^{-1}	*LOD* of WAR = 24.4 ng g^{-1} *LOD* of BDF = 77.8 ng g^{-1} and *LOD* of BDL = 138.2 ng g^{-1}	Yes, for all coumarins.	*LOD* of WAR = 0.1 ng g^{-1} *LOD* of BDF = 3.2 ng g^{-1} and *LOD* of BDL= 11.2 ng g^{-1}	Yes, for all coumarins.
LOQ (matrix)	*LOQ* ≤ 1000 ng g^{-1}	*LOQ* of WAR = 74.1 ng g^{-1} *LOQ* of BDF = 235.7 ng g^{-1} and *LOQ* of BDL = 418.6 ng g^{-1} WAR: white sign = 0 u.a. and smallest point sign = 2 245 254 u.a.	Yes, for all coumarins.	*LOQ* of WAR = 0.3 ng g^{-1} *LOQ* of BDF = 9.8 ng g^{-1} and *LOQ* of BDL = 33.9 ng g^{-1} WAR: white sign = 39 u.a. and smallest point sign = 695 413 u.a.	Yes, for all coumarins.
Selectivity	response should be lower than 20% of the response for a concentration of 300 ng g^{-1}	BDF: white sign = 0 u.a. and smallest point sign = 15 782 643 u.a. BDL white sign = 3 286 426 u.a. and smallest point sign = 8930648 u.a.	Only for WAR and BDF.	BDF: white sign = 743 u.a. and smallest point sign = 60 945 u.a. BDL white sign = 98 u.a. and smallest point sign = 3560 u.a.	Yes, for all coumarins.
Recovery	*EP* ≥ 62%	*EP* of WAR = 67.2%	Yes	*EP* of WAR = 49.5% and *EP* of BDF = 64.5%	BDF only.
Precision	*CV* ≤ 12%	*CV* of WAR: intra-day precision = 1.0% and inter-day precision = 1.2%	Yes	*CV* of BDF: intra-day precision = 2.1% and inter-day precision = 2.0%	Yes
Relative uncertainty	N.A.[a]	u WAR = 6.4%	N.A.[a]	u BDF = 11.5%	N.A.[a]

[a] N.A. = Not Applicable

Linearity, LOD, and LOQ values of the chromatography methods

The linearity of the curves for the WAR, BDF, and BDL solutions and enriched matrices was evaluated in the working concentration range of 300–1100 ng g^{-1}. In recent studies on the validation of analytical methods for rat poison, only r^2 values were used to evaluate linearity, with $r^2 > 0.99$.[14,16,17] The r^2 values in this study were lower than those previously reported. However, the r^2 values of the curves were close to the r^2 value considered satisfactory in the linearity acceptance criterion in a recent study on rodenticides in an animal gastric content matrix.[7] In addition, the results met the criteria for evaluating linearity in terms of the *p*-values of the F-test and inspection of the residual plots.[19]

According to the acceptance criteria, the curves of the WAR, BDF, and BDL solutions and enriched matrices were linear. However, the curves of BDF and BDL obtained using HPLC FLD exhibited wider residue scattering at higher concentrations. This was ascribed to the low solubilities of BDF and BDL in methanol and water, which were the solvents used to prepare the working solutions and mobile phases. The residue plots of the HPLC curves of WAR, BDF, and BDL in the enriched matrices did not show the same patterns of residue scattering (WAR, BDF, and BDL were present in the acetone-containing supernatant). For the UHPLC–MS data, we encountered no problems during the visual evaluation of the residue graphs for all samples, except for the BDL solutions. This further emphasised the usefulness of UHPLC–MS for the quantitative analysis of rodenticides and justified its use in recent studies.[16,17]

The *LOD* and *LOQ* values were determined using the curves of WAR, BDF, and BDL in the enriched matrices. The results were considered satisfactory, as they were comparable to those reported recently by researchers who analysed WAR, BDF, and BDL in stomach content matrices of animals[7] and other matrices using acetone extraction followed by LC FLD[8,9] and LC–MS.[11,13] The *LOD* values obtained in this study were lower than the critical *LOD* (260 ng g^{-1}). Therefore, our methods are suitable for the detection of coumarin compounds at concentrations below the critical *LOD*. The critical *LOD* was calculated considering the theoretical poisoning of a two-year-old child (the age group with the highest number of poisoning cases in Brazil,[29] the gastric volume after 1 h of fasting[30] and the toxic rodenticide dose for children of 0.014 mg kg^{-1}.[31] After confirming the linearity of the curves in solutions and the enriched matrices, the curves were compared to determine the selectivities of the methods and confirm whether the methods were quantitative.

Comparison of method selectivity and evaluation of intended use

Considering the acceptance criteria for qualitative methods, our results indicated that HPLC FLD and UHPLC–MS were selective for all the coumarins evaluated, except for HPLC FLD for BDL. This was attributed to the presence of a diastereomeric pair at different retention times in the chromatograms of all solutions and matrices using different mobile phases and isocratic and gradient methods, except for the BDF chromatograms, which presented only one peak, although BDF consisted of a diastereomeric pair. Therefore, the selectivity of the HPLC FLD method for BDL was unsatisfactory because two well-separated analyte peaks were present in the chromatograms of BDL. The integration method used for the enriched matrices was the same as that used for the blank matrices. Therefore, the two peaks corresponding to the diastereoisomer pair were integrated and processed together. The mean signal obtained from the blanks was 20% stronger than the mean signal for the lowest concentration point on the curve, an unsatisfactory result for selectivity. We hypothesised that the physical and chemical properties of the cis and trans diastereoisomers of BDL were different,[17] causing their different distributions in organic solvents and different retention times.

The diastereomers of BDL can be separated using achiral columns. Some researchers have reported the presence of two peaks in the chromatograms of BDL using C-8 and C-18 reversed-phase columns under acidic conditions and acetate or ammonium formate ions in the mobile phase and used the first and primary peaks to quantify BDL.[15] The use of only the primary peak of BDL for integration was not considered because the two peaks were not completely separated and commercial raticides typically contain two diastereoisomers.[17] Therefore, in this study, chromatographic analysis of BDL was performed by integrating the peaks of both diastereoisomers.

Selectivity analysis was performed to determine whether the matrix components interfered with the signals of the rodenticide isomers, and the methods were determined to be selective for detecting coumarins.

Comparison of method recovery and matrix complexity

The recovery values of HPLC FLD and UHPLC–MS were satisfactory according to the acceptance criteria. The recovery values obtained herein were similar to those reported in recent publications on the analysis of WAR, BDF, and BDL in animal gastric content matrices[7] and other matrices using acetone extraction followed by LC FLD analysis;[8,9] In contrast, the recovery values obtained herein were lower than those reported in publications on the use of LC–MS in other matrices[13,15] and the values recommended by the Association of Official Analytical Chemists (AOAC).[19] The matrix used in this study was complex, as the Ensure® Plus nutrition shake contains many macromolecules, such as carbohydrates, lipids, proteins, and vitamin K,[32] which can interact with rodenticides and hinder their recovery. Therefore, the composition of the extraction solvent can be changed to increase its affinity for the analytes. Nevertheless, the HPLC FLD and UHPLC–MS methods were classified as qualitative, such that recovery values were not required for validation. The recovery values of HPLC FLD for BDF and BDL and those of UHPLC–MS for BDL were not calculated because of the challenges encountered in constructing the curves of the compounds in solution. This is one of the reasons why rat toxin analysis via LC–MS has been used more frequently because it is considered more reliable and selective according to the reported validation parameters.[1]

Comparison of method precision values

The precision values of the HPLC FLD and UHPLC–MS methods were considered satisfactory based on the acceptance criteria. The intra- and inter-day precision values were consistent with those recently reported in papers on the analysis of WAR and BDF in an animal gastric content matrix[7] and other matrices using acetone extraction followed by LC FLD[8,9] and LC–MS analyses.[11,13,15] Moreover, the values were within the range recommended by the AOAC.[19] The performances of the HPLC FLD and UHPLC–MS methods were comparable, demonstrating the accuracy of the methods. The precision of the UHPLC–MS method for WAR was not calculated because the recovery of the method was unsatisfactory. The recovery values of UHPLC–MS for WAR, BDF, and BDL were lower than those of HPLC FLD, probably because of ionic suppression, a common shortcoming of methods using MS detectors.[10]

Ionic suppression is defined as the loss of signal from the analyte of interest owing to the co-elution and ionisation of an interfering compound in the matrix. To avoid false recovery values, the degree of ion suppression must be determined by analysing the degree of matrix interference in the analyte signal, which is evaluated using the selectivity parameter. If the blank matrix signal is too high, it is likely to be noise and should be corrected accordingly. To diminish the effect of ion suppression, the signal processing method (noise reduction) or extraction technique (e.g. using SPE) can be refined to diminish matrix interference.[33] The uncertainty of each method was calculated after all validation steps were performed.

Comparison of the uncertainty values of the HPLC–FLD and UHPLC–MS methods

Uncertainty (%) was calculated as the ratio between the uncertainties and averages of the measured values. The uncertainty of UHPLC–MS was lower than that of HPLC FLD (Table II). The expanded uncertainty was calculated using the contributions of the solution curve uncertainty at the concentration midpoint and repeatability uncertainty. Although the ABNT NBR 17025:2017 standard can be used to estimate the uncertainty of testing methods, measurement uncertainty in rodenticide analysis studies using LC has not yet been reported. Although not mandatory for qualitative methods, uncertainty is critical for guaranteeing result validity and ensuring accurate and reliable rodenticide concentration measurements. The uncertainties of the concentration values determined using rodenticide quantification assays can significantly help forensic experts identify the cause of poisoning, providing a method for conducting conformity assessment analyses with specified confidence levels. A well-executed analysis can help resolve many cases of rodenticide poisoning that have not yet been elucidated.

Table II. Concentrations of warfarin (WAR) and brodifacoum (BDF) solutions determined using high-performance liquid chromatography–fluorescence detection (HPLC FLD) and ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS) and relative measurement uncertainties.

Compound	Chromatographic method	Concentration (ng g^{-1})	Relative uncertainty (%)
WAR	HPLC FLD	802 ± 51	6.4
BDF	UHPLC–MS	842 ± 97	11.5

CONCLUSION

According to the validation parameters, the HPLC FLD and UHPLC–MS methods were satisfactory for the detection of rodenticides in human gastric content matrices and forensic toxicology applications. For analyses that require quantitative tests, the UHPLC–MS method should be used, and the extraction step should be optimised to obtain adequate selectivity and recovery values.

Although in this study we did not use a real matrix, the performance of the artificial medium during the validation steps was similar to that reported for animal gastric content samples.[7] Therefore, HPLC FLD and UHPLC–MS can be used as references for the validation of real human gastric content matrices.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing and the financial funding from Faperj - Research Support Foundation of the State of Rio de Janeiro, Brazil. Grant from E_12/2019.

REFERENCES

(1) Imram, M.; Shafi, H.; Wattoo, S. A.; Chaudhary, M. T.; Usman, H. F. Analytical methods for determination of anticoagulant rodenticides in biological samples. *Forensic Sci. Int.* **2015**, *253*, 94–102. https://doi.org/10.1016/j.forsciint.2015.06.008

(2) Guerra, F. Q. S. *Avaliação da atividade antifúngica dos compostos cumarínicos frente às cepas do gênero Aspergillus*. Doctoral thesis, 2016, Universidade Federal da Paraíba, João Pessoa, PB, Brazil. https://repositorio.ufpb.br/jspui/handle/tede/9517

(3) Departamento de informática do Sistema Único de Saúde (DATASUS) do Ministério da Saúde, Governo Federal, Brasil. http://tabnet.datasus.gov.br/cgi/tabcgi.exe?sinannet/cnv/Intoxbr.def (accessed 2021-05-03).

(4) Ferreira, A. G. Química forense e técnicas utilizadas em resoluções de crimes. *Revista Acta de Ciência & Saúde* **2016**, *2*, 32–44. https://doi.org/10.17921/1890-1793.2021v16n16p16-23

(5) Hernandez-Moreno, D.; de la Casa-Resino, I.; Lopez-Beceiro, A.; Fidalgo, L. E.; Soler, F.; Perez-Lopez, M. Secondary poisoning of non-target animals in an Ornithological Zoo in Galicia (NW Spain) with anticoagulant rodenticides: a case report. *Veterinarni Medicina* **2013**, *58*, 553–559. https://doi.org/10.17221/7087-VETMED

(6) Gallocchio, F.; Basilicata, L.; Benetti, C.; Angeletti, R.; Binato, G. Multi-residue determination of eleven anticoagulant rodenticides by high-performance liquid chromatography with diode array/fluorimetric detection: investigation of suspected animal poisoning in the period 2012-2013 in north-eastern Italy. *Forensic Sci. Int.* **2014**, *244*, 63–69. https://doi.org/10.1016/j.forsciint.2014.08.012

(7) Gallocchio, F.; Moressa, A.; Stella, R.; Rosin, R.; Basilicata, L.; Bille, L.; Toson, M.; Biancotto, G.; Lega, F.; Angeletti, R.; Binato, G. Fast and simultaneous analysis of carbamate pesticides and anticoagulant rodenticides used in suspected cases of animal poisoning. *Forensic Sci. Int.* **2021**, *323*, 110810. https://doi.org/10.1016/j.forsciint.2021.110810
(8) Meiser, H. Detection of anticoagulant residues by a new HPLC method in specimens of poisoned animals and a poison control case study. *J. Anal. Toxicol.* **2005**, *29*, 556–563. https://doi.org/10.1093/jat/29.6.556
(9) Armentano, A.; Iammarino, M.; Lo Magro, S.; Muscarella, M. Validation and application of multi-residue analysis of eight anticoagulant rodenticides by high-performance liquid chromatography with fluorimetric detection. *J. Vet. Diagn. Invest.* **2012**, *24*, 307–311. https://doi.org/10.1177/1040638711433354
(10) Hernández, A. M.; Bernal, J.; Bernal, J. L.; Martín, M. T.; Caminero, C.; Nozal, M. T. Analysis of anticoagulant rodenticide residues in *Microtus arvalis* tissues by liquid chromatography with diode array, fluorescence and mass spectrometry detection. *J. Chromatogr. B* **2013**, *925*, 76–85. https://doi.org/10.1016/j.jchromb.2013.02.032
(11) Vandenbrouckev, V.; Desmet, N.; De Backer, P.; Croubels, S. Multi-residue analysis of eight anticoagulant rodenticides in animal plasma and liver using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry. *J. Chromatogr. B* **2008**, *869*, 101–110. https://doi.org/10.1016/j.jchromb.2008.05.011
(12) Fourel, I.; Hugnet, C.; Goy-Thollot, I.; Berny, P. Validation of a new liquid chromatography–tandem mass spectrometry ion-trap technique for the simultaneous determination of thirteen anticoagulant rodenticides, drugs, or natural products. *J. Anal. Toxicol.* **2010**, *34*, 95–102. https://doi.org/10.1093/jat/34.2.95
(13) Dong, X.; Lianga, S.; Sun, S. Determination of seven anticoagulant rodenticides in human serum by ultra-performance liquid chromatography-mass spectrometry. *Anal. Methods* **2015**, *7*, 1884–1889. https://doi.org/10.1039/C4AY02536A
(14) Cao, X.; Yang, X.; Liu, Z.; Jiao, H.; Liu, S.; Liu, L.; Meng, Q. Rapid simultaneous screening and detection of 12 anticoagulant rodenticides in food by ultra-performance liquid chromatography-triple quadrupole/linear ion trap tandem mass spectrometry. *Food Analytical Methods* **2017**, *10*, 3538–3547. https://doi.org/10.1007/s12161-017-0922-2
(15) Fourel, I.; Damin-Pernik, M.; Benoit, E.; Lattard, V. Core-shell LC–MS/MS method for quantification of second generation anticoagulant rodenticides diastereoisomers in rat liver in relationship with exposure of wild rats. *J. Chromatogr. B* **2017**, *1041–1042*, 120–132. https://doi.org/10.1016/j.jchromb.2016.12.028
(16) Seljetun, K. O.; Eliassen, E.; Karinen, R.; Moe, L.; Vindenes, V. Quantitative method for analysis of six anticoagulant rodenticides in faeces, applied in a case with repeated samples from a dog. *Acta Vet. Scand.* **2018**, *60*. https://doi.org/10.1186/s13028-018-0357-9
(17) Nosal, D.; Feinstein, D. L.; Chen, L.; van Breemen, R. B. Separation and quantification of superwarfarin rodenticide diastereomers—bromadiolone, difenacoum, flocoumafen, brodifacoum, and difethialone—in human plasma. *Journal of AOAC International* **2020**, *103*, 770–778. https://doi.org/10.1093/jaoacint/qsaa007
(18) Jantratid, E.; Janssen, N.; Reppas, C.; Dressman, J. B. Dissolution media simulating conditions in the proximal human gastrointestinal tract: An update. *Pharm. Res.* **2008**, *25*, 1663–1676. https://doi.org/10.1007/s11095-008-9569-4.
(19) Instituto Nacional de Metrologia, Normalização e Qualidade Industrial – Inmetro, Brasil. http://www.inmetro.gov.br/Sidoq/Arquivos/Cgcre/DOQ/DOQ-Cgcre-8_08.pdf (accessed 2021-05-03).
(20) Organisation Internationale De Métrologie Légale. https://www.oiml.org/en/files/pdf_v/v002-200-e07.pdf (accessed 2021-05-03).
(21) International Organization for Standardization. *General requirements for the competence of testing and calibration laboratories*. ISO/IEC 17025:2017. Geneva, 2017.

(22) Chalermchaikit, T.; Felice, L. J.; Murphy, M. J. Simultaneous determination of eight anticoagulant rodenticides in blood serum and liver. *J. Anal. Toxicol.* **1993**, *17*, 56–61. https://doi.org/10.1093/jat/17.1.56
(23) Bertoldi, F. C.; Deschamps, F. C.; Silva Junior, A. A.; Correa, A. F.; Franco, M. F.; Eberlin, M. N. Validação de um método analítico rápido por CLAE-UV para determinação de cumarina em guaco (*Mikania glomerata* Sprengel) confirmado com espectrometria de massas. *Revista Brasileira de Plantas Medicinais* **2016**, *18*, 316–325. https://doi.org/10.1590/1983-084X/15_160
(24) Marques, M. R. C.; Loebenberg, R.; Almukainzi, M. Simulated biological fluids with possible application in dissolution testing. *Dissolution Technol.* **2011**, *18*, 15–28. https://doi.org/10.14227/DT180311P15
(25) Klein, S.; Butler, J.; Hempenstall, J. M.; Reppas, C.; Dressman, J. B. Media to simulate the postprandial stomach I. Matching the physicochemical characteristics of standard breakfast. *J. Pharm. Pharmacol.* **2004**, *56*, 605–610. https://doi.org/10.1211/0022357023367
(26) Verbic, T.; Dorkó, Z.; Horvai, G. Selectivity in analytical chemistry. *Revue Roumaine de Chimie* **2013**, *58*, 569–575. https://www.researchgate.net/publication/285481211
(27) Ramsey, M. H.; Ellison, S. L. R.; Rostron, P. (Eds.) *Measurement uncertainty arising from sampling – A guide to methods and approaches*. Eurachem / CITAC Guide, 2nd edition, 2019. https://www.eurachem.org/images/stories/Guides/pdf/UfS_2019_EN_P2.pdf (accessed 2021-05-03).
(28) Vessecchi, R.; Lopes, N. P.; Gozzo, F. C.; Dörr, F. A.; Murgu, M.; Lebre, D. T.; Abreu, R.; Bustillos, O. V.; Riveros, J. M. Nomenclaturas de espectrometria de massas em língua portuguesa. *Quím. Nova* **2011**, *34*, 1875–1887. https://doi.org/10.1590/S0100-40422011001000025
(29) Vilaça, L.; Volpea, F. M.; Ladeira, R. M. Intoxicações exógenas em crianças e adolescentes atendidos em um serviço de toxicologia de referência de um hospital de emergência brasileiro. *Revista Paulista de Pediatria* **2020**, *38*, e2018096. https://doi.org/10.1590/1984-0462/2020/38/2018096
(30) Carmona, B. M.; Almeida, C. C. A.; Vieira, W. B.; Fascio, M. N. C.; de Carvalho, L. R.; Vane, L. A.; Barbosa, F. T.; Nascimento Junior, P.; Módolo, N. S. P. Dinâmica ultrassonográfica dos volumes do conteúdo gástrico após a ingestão de água de coco ou sanduíche de carne. Um estudo cruzado controlado e randômico com voluntários saudáveis. *Revista Brasileira de Anestesiologia* **2018**, *68*, 584–590. https://doi.org/10.1016/j.bjan.2018.06.008
(31) Roberts, J. R.; Reigart, J. R. Environmental Protection Agency (EPA). *Recognition and Management of Pesticide Poisonings*. https://www.epa.gov/pesticide-worker-safety/recognition-and-management-pesticide-poisonings (accessed 2021-05-03).
(32) Abbott. https://www.ensure.abbott/br/nossos-produtos/ensure-plus-advance.html (accessed 2021-03-18).
(33) Lanetwork. https://www.labnetwork.com.br/noticias/decodificando-a-espectrometria-de-massas-supressao-ionica/ (accessed 2021-05-03).