Title

Extensive evaluation of a new LC-MS/MS method to quantify monofluoroacetate toxin in the kidney

Authors

Langston, James

Stump, Samuel

Filigenzi, Michael

et al.

Publication Date

2024-04-06

DOI

10.1093/jat/bkae032

Peer reviewed

Title: Extensive evaluation of a new LC-MS/MS method to quantify monofluoroacetate toxin in the kidney

Authors: James Langston[1], Samuel Stump[1], Michael Filigenzi[1], Andriy Tkachenko[3], Jake Guag[3], Robert Poppenga[1], and Wilson K Rumbeiha[2]

[1] University of California Davis, California Animal Health and Food Safety Laboratory, Davis, CA, USA

[2] University of California Davis, Department of molecular Biosciences, Davis, CA, USA

[3] Center for Veterinary Medicine, US Food and Drug Administration, Laurel, MD, USA

There are no potential conflicts of interest to disclose.

Corresponding author: Wilson K Rumbeiha, Professor in Department of Molecular Biosciences, University of California, Davis, Davis, CA, USA., Email: wkrumbeiha@ucdavis.edu, Phone: (530) 752-6285

Funding source: This study was funded (FOA PAR-18-604) and performed in collaboration with the United States Food and Drug Administration's Veterinary Laboratory Investigation and Response Network (FDA Vet-LIRN) under grant U18 FD006443.

Word count: 6,406 (text+ref: 4906 + 3 tables:1500 = 6406; max allowed = 7000)

Acknowledgement: We would like to acknowledge the technical advice of Scott Radke and Dwayne Schrunk from Iowa State University in the early stages of this project. We also would like to thank Gregory Tyson and Sarah Nemser for support during this project.

Abstract

Monofluoroacetate (MFA) is a highly lethal toxin which causes death by inhibiting cellular ATP production. The heart and brain are the primary target organs. Acute death is attributed to cardiac fibrillation and/or convulsions. Although it occurs naturally in some plants, a major source of animal intoxication is access to sodium monofluoroacetate (NaMFA) pesticide which continues to be a concern in the US and around the world despite restricted use in some countries including the US. There are also concerns about misuse of this pesticide for malicious poisoning. Currently, a tissue-based diagnostic method for NaMFA intoxication in animals is lacking. There is a critical need by the veterinary diagnostic community for a simple, sensitive, and reliable tissue-based diagnostic test to confirm NaMFA poisoning in animals. We have developed and extensively evaluated a sensitive novel LC-MS/MS method suitable for this purpose. The limits of detection (LOD) and limits of quantitation (LOQ) are 1.7 ng/g and 5.0 ng/g, respectively. The accuracy and precision met or exceeded expectations. The method performance was verified using incurred kidney obtained from animal diagnostic cases. This novel kidney-based method is now available for clinical use and can help with diagnostic purposes, including detecting potential issues related to animal foods.

Keywords

Compound 1080, Monofluoroacetate, LC-MS/MS method, analytical method

Introduction

Sodium monofluoroacetate (NaMFA; pesticide Compound 1080) is one of the most highly toxic compounds [1-3]. It is produced naturally in plants and is also manufactured and used as a pesticide for killing rodents and livestock predators around the world [1,2,4,5]. It is a highly lethal compound with broad spectrum activity, including for humans and a wide variety of animal species [1]. The basic mechanism of acute toxicity is that *in vivo* it is metabolized to fluorocitrate, which then interferes with ATP formation in the Kreb's cycle [1,6]. The brain and the heart which depend highly on aerobic respiration are the most vulnerable organs although it is a systemic toxicant affecting all tissues in the body. Death from ventricular fibrillation and/or respiratory failure occurs acutely following exposure to this toxin either through plant or pesticide ingestion [7]. Survivors of acute exposures develop long-term lingering effects. In humans, these include persistent neurological signs arising from cerebellar degeneration [8]. Secondary intoxication of nontarget species is also possible, especially for scavengers of acutely dead animals. Because of its high toxicity and also given there is no specific antidote, it has been banned in the US, but it remains popular in many other countries around the world. Another cause of concern about Compound 1080 is the potential misuse of this chemical as a terrorist agent [9]. Despite the restricted use of this pesticide in some countries including the US, incidents of NaMFA intoxication continue to occur in this country and around the world. As a result, it is important for the veterinary diagnostic community to have diagnostic tests available to quickly confirm a diagnosis of NaMFA intoxication. This is not only vital for livestock health, but also for public health protection.

Whereas there are analytical methods to detect and quantify Compound 1080 in environmental samples like bait, feed, and water [5,10-12] there is a critical lack of tissue-based diagnostic methods in veterinary medicine. Ideally, it would be useful to use blood, urine and tissues from affected animals to confirm active clinical cases or confirm the cause of death in deceased animals, including related to potential consumption of contaminated animal foods. Such tests are extremely limited for NaMFA. The goal of this study was to develop and validate a fast and simple tissue-based method to detect monofluoroacetate (MFA) toxin for diagnosis of pesticide Compound 1080 (i.e., NaMFA) intoxication in animals.

Following a thorough literature review, the kidney was chosen as a sample of choice as it was consistently found to contain the highest amount of MFA residues [1,2,13,14]. This is understandable because NaMFA is very water soluble. Whereas about 70% is metabolized *in vivo*, the remaining 30% is excreted through urine *via* the kidneys hence the reason this tissue was selected as a diagnostic sample of choice in deceased animals.

Liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) is a commonly used analytical toxicology approach to achieve high sensitivity and selectivity in biological matrices [15,16]. Here we report a new method suitable for quantitation of MFA in kidney tissue. To ensure that the newly developed method will perform as expected in the future, its performance was extensively evaluated in three phases: (i) in-house validation using non-blinded fortified kidney samples according to FDA guidelines [17], (ii) verification using incurred kidney samples from animal diagnostic cases by applying the exhaustive extraction approach and (iii) by an independent laboratory using blinded (i.e. unbiased) fortified kidney samples according to AOAC guidelines designed

for collaborative studies [18].

Materials and Methods

Chemicals and materials

Water, methanol, and acetonitrile were of HPLC grade or higher (Fisher). Formic acid and trifluoroacetic acid were of Optima LCMS grade (Fisher). Ammonium formate was MS grade (Sigma). Ammonium hydroxide in water (28% - 30% w/w) was certified ACS plus grade (Fisher). Sodium monofluoroacetate (MFA; 1.0 mg/mL solution in water) and $^{13}C_2$, d_2-sodium monofluoroacetate ($^{13}C_2$, d_2-NaMFA at 1.0 mg/mL in water (internal standard)) were obtained from Cambridge Isotope Laboratories.

Samples

Blank samples of bovine kidney were obtained from a local supermarket. Diagnostic (incurred) kidney samples were obtained from samples submitted for testing to the California Animal Health and Food Safety Laboratory (CAHFS lab), Davis, California.

Preparation of Analytical Standards

In vivo, NaMFA transforms to monofluoroacetate anion (MFA) which is the molecule detected analytically. Working analytical standard solutions in 0.01% formic acid and 1% methanol in acetonitrile were prepared at 1.0, 0.10, and 0.010 µg/mL levels of monofluoroacetate ion (MFA) from a commercially available 1.0 mg/mL NaMFA solution in water, diluting with 0.01% formic acid and 1% methanol in acetonitrile, adjusting for the sodium cation. Working labeled internal standard solutions in water were prepared at 0.50 and 0.25 µg/mL levels of internal standard from a commercially available 1.0 mg/mL internal standard solution in water, diluting with

acetonitrile or 0.01% formic acid and 1% methanol in acetonitrile respectively, adjusting for the sodium cation (see Supplementary Materials for additional details). Working standard and internal standard solutions were stored for up to 1 year at 4 °C. Calibration standards were prepared fresh in 0.5% trifluoroacetic acid in acetonitrile from working standards at 0.10 - 50 ng/mL levels of MFA ion, and also contained 1.25 ng/mL internal standard.

Sample Preparation and Clean-Up

A 1.00 g sample of chopped bovine kidney was weighed into a 50 mL plastic centrifuge tube, and internal standard was added at 10 ng/g. To this was added 4 mL of water and two stainless steel grinding balls (9.5 mm), and it was homogenized for 5 min at 750 rpm using a Geno/Grinder SPEX. The grinding balls were removed, and it was centrifuged at 10 °C for 10 min at 4,000 x g. The resulting supernatant was filtered through a 1 µm GMF-150 syringe filter (Whatman). The filtrate was passed through a Pierce Protein Concentrator (3 kDa MWCO; PES) at 10 °C for 60 min at 4,000 x g. The flow-through was further cleaned up using Waters Oasis MAX Solid Phase Extraction (SPE) cartridges (500 mg; 6 cc). Cartridges were first conditioned with 4 mL methanol followed by equilibration with 2 x 3 mL water. Next, 2 mL of flow-through from the protein concentrator was passed through. The cartridge was washed successively with 4 mL of 5% ammonium hydroxide and 4 mL of methanol, pulling dry after the methanol and discarding the wash flow-through. It was next washed with 3 mL of 0.5% trifluoroacetic acid in acetonitrile, pulling dry after, and this wash was repeated once again, discarding the wash flow-throughs. The cartridge was then eluted with 2 x 2 mL of the same solvent (0.5% trifluoroacetic acid in acetonitrile), pulling dry after each elution, and collecting the eluant flow-through which contained the analyte. The resulting eluant was pooled, filtered through a 0.22 µm Millex syringe

filter (PES; Millipore), and analyzed by LC-MS/MS. The complete protocol will be published on www.protocols.io – open access repository platform.

Chromatographic Conditions

Liquid chromatography (LC) was performed on an Agilent 1290 Infinity II system with binary gradient pump and temperature-controlled autosampler. Conditions were based on those previously reported for analysis of NaMFA from infant formula [19]. Chromatographic separation used an Acquity UPLC BEH Amide column, 1.7 µm, 2.1 x 100 mm (Waters) at ambient room temperature (~25 °C). Mobile phase A consisted of 5 mM ammonium formate and 0.01% formic acid in water, and Mobile phase B was 100% acetonitrile. Gradient elution was done with a flow rate of 0.45 mL/min starting at 10% A for 3 min, increasing to 60% A over 1 min, holding at 60% A for 1.5 min, and then re-equilibrating at 10% A for 10.5 min. Samples were held at 10 °C and were injected at a 5 µL volume.

Mass Spectrometry Conditions

Mass spectrometry was performed using a SciEx Triple Quad 7500 QTRAP mass spectrometer which was connected to the chromatography system described above. Eluate from the chromatographic column was ionized by electrospray ionization in negative ion mode with a spray voltage of 4,000 volts. Ion source temperature was 500 °C with curtain gas and ion source gas 2 at 40 psi, and ion source gas 1 at 90 psi. Multiple reaction monitoring (MRM) was used, monitoring the $[M-H]^-$ transitions 77 *m/z* -> 57 *m/z* (quantifier ion) and 77 *m/z* -> 33 *m/z* (qualifier ion) for the unlabeled MFA analyte, and 81 *m/z* -> 60 *m/z* for the $^{13}C_2$, d_2-MFA labeled

internal standard. Collision energies for the indicated transitions were 14, 17, and 16 volts respectively. Sciex OS software was used for data acquisition and processing.

Data Processing and Analysis

Extracted ion chromatograms (EICs) were prepared for each of the three MRM transitions and used for compound identification and quantitation. MFA in unknown samples was positively identified by comparison to reference standards. Criteria for identification included: (1) the quantifier and qualifier ions eluting within 0.1 min of each other and with signal-to-noise (S/N) ratio ≥3 based on peak areas (2) the retention time within ±0.25 min of the mean retention time of MFA in all calibration standards analyzed in the same batch, and (3) the ratio of the qualifier ion and quantifier ion peak areas (ion ratio) being within ±20% of the mean ion ratio of MFA in all calibration standards analyzed in the same batch. External standards containing labeled internal standard at 10 ng/ml were used for calibration, at 0.10 - 50 ng/mL levels of MFA with 1.25 ng/mL $^{13}C_2$, d_2-MFA labeled internal standard in 0.01% formic acid and 1% methanol in acetonitrile (not in matrix-matched calibration curve). Calibration curves were constructed by plotting area ratios of the quantifier ion divided by the internal standard ion versus the corresponding standard concentration. Curves were fit to a linear regression with 1/x weighting. Unknown sample concentrations were determined from the calibration curves using the resulting curve fit equation and accounting for the 8-fold dilution factor.

In-house method validation of the method using non-blinded fortified kidney

The method was fully validated by originators in-house according to the FDA guidelines [20] by establishing parameters such as matrix effect, recovery, selectivity, sensitivity (LOD and LOQ), accuracy and precision using non-blinded fortified samples in kidney.

Matrix effect and recovery of fortified (spiked) samples

The matrix effect in bovine kidney was evaluated by post-extraction addition [21]. Unfortified control kidney was prepared and extracted, and the resulting pooled extract after SPE clean-up was spiked with MFA at concentrations of 5.0 ng/mL and 100.0 ng/mL ("post-spike samples"). Additional samples were prepared in solvent only (0.5% TFA in acetonitrile) at the same 5.0 ng/mL and 100 ng/mL MFA concentrations ("solvent-spike samples"). The matrix effect was then calculated by dividing the quantifier ion peak areas of post-spike samples by the corresponding solvent-spike samples and multiplying by 100.

For determination of recovery in bovine kidney, control kidney was spiked with MFA at concentrations of 5.0 ng/g and 100 ng/g , and then prepared, extracted, and analyzed as described ("pre-spike samples"). The recovery was then calculated by dividing the quantifier ion peak areas of pre-spike samples by the corresponding post-spike samples (same as used for matrix effect) and multiplying by 100 [21].

Method limit of detection (MLOD) and Method limit of quantitation (MLOQ)

The method limit of detection (MLOD) and method limit of quantitation (MLOQ) for bovine kidney were determined by analysis of seven replicates of control kidney spiked with MFA at 5.0 ng/g and internal standard at 10 ng/g, prepared and analyzed over three separate days. The MLOD was determined by multiplying the standard deviation from the analyses by 3.143 (Student's t-value, single-tailed, n-1, 1 - $\alpha = 0.99$) [22]. The MLOQ was determined by multiplying the MLOD by 3.

Method Accuracy and Precision

Intra-day accuracy and precision were determined for bovine kidney by triplicate analysis of control kidney spiked with MFA at 5.0, 10, 20, and 100 ng/g and internal standard at 10 ng/g, with samples at a given spike level prepared and extracted on the same day. Inter-day accuracy and precision were determined by analysis of control kidney spiked at 5.0, 10, and 20 ng/g and internal standard at 10 ng/g. At 5.0 ng/g, analysis was done of 7 replicates over three separate analysis batches. At 10 and 20 ng/g, analysis was done of 6 and 5 replicates respectively over 2 separate batches.

Stability

Stability of MFA in bovine kidney was demonstrated by spiking into control kidney at 20 ng/g and 100 ng/g levels, and comparing five different conditions: (i) prepared freshly with no storage, (ii) stored 1 day at ambient room temperature (~25 °C), (iii) stored 1 day at 8 °C, (iv) stored 1 day at –20 °C, and (v) stored 35 days at –20 °C. Additionally, freeze/thaw stability was evaluated at the same levels by fortifying, freezing 2h at –20 °C, thawing at ambient room temperature for 30 min, repeating this freeze/thaw cycle a second time, freezing a third time overnight (~16h), and then thawing a third time. Samples were analyzed as described above. Labeled internal standard was added to each just prior to the extraction but was not present during the sample treatments.

Analysis of incurred kidney

Canine and ovine kidneys were obtained from animals exhibiting clinical signs consistent with Compound 1080 toxicosis and submitted for diagnostic testing. Canine and ovine kidneys were

analyzed as described above under the sample preparation and clean-up section. The extraction efficiency [23,24] was evaluated for one canine sample (1.00 g) using the exhaustive extraction approach [25,26] by applying three rounds of extraction to the same kidney sample. In the first round, a sample of incurred canine kidney was extracted as described above. After extraction, the supernatant was removed. The pelleted kidney material was re-extracted in the same manner as in the first round, by adding an additional 4 mL of water and the supernatant was again collected. This was repeated an additional time, for a total of three successive extractions. Each of the three extracts was then separately cleaned-up by SPE and analyzed by LC-MS/MS as described above.

Collaborative method evaluation using blinded (i.e., unbiased) fortified kidney

Design of the Blinded Method Test (BMT)

The BMT is an exercise in which an independent laboratory (i.e., organizers) prepares and ships test samples to method originators (i.e., participants) who analyze them in an unbiased (i.e., blinded) manner [27]. In the BMT, an emphasis is made on providing an adequate blinding of study samples to those who are involved in the analysis and the assessment of results. The purpose of the blinding is to eliminate any possibility of sharing information regarding study samples with the participant. This includes information about the fortification levels (i.e., spiking concentrations), the number of fortified levels, and the number of replicates at each fortification level. Knowing either the number of replicates or the number of analyte levels would mean that the lab personnel are only partially blinded. Partial blinding allows participants to group similar results and identify possible outliers even without knowing the specific analyte concentrations.

Preparation and analysis of BMT samples

Bovine kidney was purchased from a local supermarket, chopped into 1 cm x 1 cm chunks, and ~300.00 g of this was pre-homogenized in a Waring Commercial Blender at high speed for 3 min. Pre-homogenized kidney was aliquoted (1.00 g per tube) and fortified by adding standard spiking solution.

Each BMT sample was a single-use unit [28] and subsampling was not possible because each was supplied in an aliquot (1.00 g per tube) only sufficient for the extraction and analysis once, not allowing for any sample to be analyzed two or more times and submitting an average value by the participant lab. Such an averaging, which was not possible to occur in this BMT, would compromise estimation of accuracy and precision (i.e., results would look better than they actually were).

All samples were then shipped overnight on ice packs to the originators and kept at –20 °C until analyzed. An aliquot of 1 µg/mL MFA solution in 0.01% formic acid and 1% methanol in acetonitrile, which was used to prepare BMT samples, was also shipped for preparation of calibration standards. Participants were requested to analyze 34 samples on two separate days (see Supplemental Material Table S1) and complete prepopulated analyst worksheets capturing details on steps performed, materials used and data processing applied (e.g., extracted ion chromatogram peak integration, ion ratio calculations and others) for review by organizers. Results were evaluated according to AOAC guidelines [18] designed for collaborative studies (i.e., samples prepared in one lab and analyzed in a different lab) using unbiased (i.e., blinded) samples.

Results and Discussion

In-house method validation using fortified non-blinded samples.

Ion ratios of the fragments for standards and fortified kidney material were all within ± 20% (Figure S1A, D). There were no false positive signals for blanks (Figure S1B, C). Stability of MFA in fortified kidney samples was demonstrated under conditions typical of those for diagnostic samples, including shipment and storage, as well as over freeze/thaw cycles, with comparisons to freshly fortified material (Table S2).

Matrix effects were assessed (Table 1) by MFA added at 5.0 and 100 ng/g to unfortified kidney extract (post-spiked) and to standards prepared in solvent at the same levels (post-extraction spike method) [21]. Ratios of responses for post-spiked sample to standards were determined to be 65% and 58% for 5.0 and 100 ng/g levels respectively. These values indicate a negative matrix effect. Negative matrix effects for LC-MS/MS methods are commonly observed for biological matrices, resulting from ion suppression by the matrix components [15]. These effects were fully compensated for in the method by employing a stable isotope-labeled internal standard, which is a well-established approach [15,21,29].

Recovery was determined for kidney fortified with MFA at 5.0 and 100 ng/g levels (pre-spiked) to unfortified kidney extract post-spiked at those levels [21]. Recoveries were determined to be 61 and 68% respectively for the 5.0 and 100 ng/g levels (Table 1). The observed recovery effect may be attributed to extraction and SPE inefficiencies. The recovery effect was fully compensated for by including the internal standard.

Accuracy and precision were determined for the method from both intra- and inter-day results, at fortified MFA levels from 5.0 – 100 ng/g for intra-day, and from 5.0 – 20 ng/g for inter-day

(Table 2). Intra-day accuracies ranged from 100 – 112%, and inter-day from 100 – 110%. Intra-day precision ranged from 1.1 – 10 %RSD, and inter-day from 3.2 - 12 %RSD. These values indicate high levels of accuracy and precision, well within established AOAC and FDA guidelines [18,17].

Calibration standards were shown to be linear over a wide range, from 0.10 - 50 ng/mL levels of MFA, with corresponding coefficient of determination (R^2) values of ≥0.99. Average back-calculated values for calibrators, run before and after the samples, were within the FDA guidelines of 80 – 110% accuracy [17]. Injection carry-over was determined to be negligible (≤0.1%), as demonstrated by injecting a solvent blank after a 25 ng/mL standard.

The method limit of detection (MLOD) and method limit of quantitation (MLOQ) were determined from analysis of seven replicates of kidney fortified with 5.0 ng/g MFA, analyzed over three separate days [22]. The MLOQ was determined to be 5.0 ng/g, and the MLOD was 1.7 ng/g.

Method verification using incurred kidney

Method performance parameters, such as sensitivity (e.g., LOD and LOQ) and accuracy (i.e., closeness of a result to the true value), may or may not be the same for fortified versus incurred analytes. This is because there could be differences in the analyte extraction efficiency [23,24], which refers to the rate at which an analyte transfers from the matrix into the extraction solution. To ensure that a validated method is fit for intended use, the extraction efficiency was evaluated using the exhaustive extraction approach [25,26] by subjecting the same sample to three successive rounds of extraction. The majority of the total amount of MFA was obtained in the

first extraction (86%, 69 ng/g), with significant decreases with each successive extraction, 13% (11 ng/g) and 1% (1.1 ng/g) respectively for the second and third extractions. The amount of MFA present in the second and third extractions may be largely attributed to extract remaining in the surrounding of the pelleted material after the first round.

Analysis of a sample from another area of the same canine kidney revealed MFA at 124 ng/g suggesting some spatial variability in the distribution of MFA deposits within the kidney. Analysis of kidney samples from two ovine cases revealed presence of MFA at 19 and 24 ng/g which matched with results (i.e., 20 and 16 ng/g respectively) obtained by an independent laboratory using an alternative GC-MS method requiring complex radioactive ^{14}C-labeling approach (unpublished results).

Overall, the results indicate suitability of the method for analysis of incurred residues of MFA in kidney of various animal species.

Method performance evaluation in the Blinded Method Test (BMT)

The FDA guidelines [17] were followed during in-house validation of the method using non-blinded fortified samples (see above). However, the FDA guidelines are too stringent (especially at concentrations <100 ng/g) for evaluation of collaborative studies such as this BMT in which samples are prepared in one laboratory and analyzed in a different one (i.e., additional sources of results variability are introduced in BMT vs. in-house validation experiments). AOAC guidelines [18], which were designed for collaborative studies, were used for evaluation of BMT results (Table 3).

Intra- and inter-day accuracies were determined to be within ±20% for the kidney samples fortified at 7.0 ng/g MFA (very close to the MLOQ of 5 ng/g), and within ±15% for samples fortified at 25 and 70 ng/g levels (Table 3). Intra- and inter-day estimates of the method precision (e.g., RSD(r), HorRat(r) and RSD(i)) matched well the AOAC requirements. All RSD(r) values were ≤12% and all RSD(i) values were ≤10%. HorRat(r) values, for Day-1 samples fortified at 7.0 and 25 ng/g levels were found to be 0.4 (Table 3), within the range of 0.3 – 1.3 for expected values [18]. Values for all of the others ranged from 0.05 – 0.20, below the expected range. Low HorRat(r) values are associated with better-than-expected results. These values were determined not to be of concern for this BMT as no sample averaging was possible given the BMT samples design (i.e., each sample was a single-use unit; see above), and the originating laboratory was properly blinded. Overall, BMT results satisfied not only AOAC, but also more stringent FDA guidelines written for in-house experiments using non-blinded samples.

Conclusions

We have developed and extensively evaluated a novel LC-MS/MS method suitable for quantitation of Compound 1080 (sodium monofluoroacetate) at low (1.7-5.0) ng/g levels in animal kidney. Accuracy and precision met or exceeded expectations for fortified samples during unblinded (i.e., in-house) and blinded (i.e., collaborative BMT) analyses. The method performance was also verified using incurred kidney obtained from animal diagnostic cases. Such extensive evaluation provides a high degree of confidence that the newly developed method will perform as expected when used for animal diagnostics or research purposes in the future.

Reference

1. Goncharov NV, Jenkins RO, Radilov AS (2006) Toxicology of fluoroacetate: a review, with possible directions for therapy research. Journal of applied toxicology : JAT 26 (2):148-161. doi:10.1002/jat.1118
2. Gooneratne SR, Eason CT, Milne L, Arthur DG, Cook C, Wickstrom M (2008) Acute and long-term effects of exposure to sodium monofluoroacetate (1080) in sheep. The Onderstepoort journal of veterinary research 75 (2):127-139. doi:10.4102/ojvr.v75i2.11
3. DeLey Cox VE, Hartog MA, Pueblo E, Racine M, Jennings L, Tressler J, Tuet WY, Stone S, Pierce SA, Thompson L, Dukes A, Hoard-Fruchey H, Wong B, McCranor BJ (2020) Methylene blue and monosodium glutamate improve neurologic signs after fluoroacetate poisoning. Ann N Y Acad Sci 1479 (1):196-209. doi:10.1111/nyas.14347
4. Giannitti F, Anderson M, Caspe SG, Mete A, East NE, Mostrom M, Poppenga R (2013) An outbreak of sodium fluoroacetate (1080) intoxication in selenium- and copper-deficient sheep in California. Veterinary pathology 50 (6):1022-1027. doi:10.1177/0300985813486813
5. Minnaar PP, McCrindle RI, Naude TW, Botha CJ (2000) Investigation of biological samples for monofluoroacetate and Dichapetalum cymosum poisoning in southern Africa. The Onderstepoort journal of veterinary research 67 (1):27-30
6. Arroyave Hoyos CL, Canas Galvis MA, Ospina Estrada D, Henao Solarte MC, Lopera Cardona S (2018) Intravenous Lipid Emulsion and Ethanol for Sodium Fluoroacetate Poisoning. American journal of therapeutics 25 (6):e756-e758. doi:10.1097/MJT.0000000000000765
7. Egekeze JO, Oehme FW (1979) Sodium monofluoroacetate (SMFA, compound 1080): a literature review. Veterinary and human toxicology 21 (6):411-416
8. Kim JM, Jeon BS (2009) Survivors from beta-fluoroethyl acetate poisoning show a selective cerebellar syndrome. J Neurol Neurosurg Psychiatry 80 (5):528-532. doi:10.1136/jnnp.2008.151605
9. Holstege CP, Bechtel LK, Reilly TH, Wispelwey BP, Dobmeier SG (2007) Unusual but potential agents of terrorists. Emergency medicine clinics of North America 25 (2):549-566; abstract xi. doi:10.1016/j.emc.2007.02.006
10. Bessaire T, Tarres A, Goyon A, Mottier P, Dubois M, Tan WP, Delatour T (2015) Quantitative determination of sodium monofluoroacetate "1080" in infant formulas and dairy products by isotope dilution LC-MS/MS. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 32 (11):1885-1892. doi:10.1080/19440049.2015.1087057
11. Method AO (2015) AOAC Official Method 2015.02 Sodium Fluoroacetate in Dairy Powder. AOAC Official Method. http://www.aoacofficialmethod.org/index.php?main_page=product_info&cPath=1&products_id=2928. Accessed 10/15 2023
12. AOAC Official Method (2015) AOAC 2015.04-2015 Monofluoroacetate in powdered nutritional prod. AOAC Official Method. http://www.aoacofficialmethod.org/index.php?main_page=product_info&cPath=1&products_id=2968. Accessed 10/26 2023

13. Adaska JM, Rimoldi G, Blanchard PC, Champagne J, Poppenga RH, Mostrom M (2018) Multiple episodes of 1080 (sodium monofluoroacetate) intoxication in a California calf-raising operation. J Vet Diagn Invest 30 (5):747-751. doi:10.1177/1040638718781435
14. Goh CS, Hodgson DR, Fearnside SM, Heller J, Malikides N (2005) Sodium monofluoroacetate (Compound 1080) poisoning in dogs. Australian veterinary journal 83 (8):474-479. doi:10.1111/j.1751-0813.2005.tb13296.x
15. Panuwet P, Hunter RE, Jr., D'Souza PE, Chen X, Radford SA, Cohen JR, Marder ME, Kartavenka K, Ryan PB, Barr DB (2016) Biological matrix effects in quantitative tandem mass spectrometry-based analytical methods: advancing biomonitoring. Crit Rev Anal Chem 46 (2):93-105. doi:10.1080/10408347.2014.980775
16. Kushnir MM, Rockwood AL, Bergquist J (2010) Liquid chromatography-tandem mass spectrometry applications in endocrinology. Mass spectrometry reviews 29 (3):480-502. doi:10.1002/mas.20264
17. Biopharmaceutics (2018) Bioanalytical method validation guidance for industry. U. S. Food and Drug Administration. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry. Accessed 10/25 2023
18. INTERNATIONAL A (2019) Appendix F: Guidelines for Standard Method Performance Requirements. In: Latimer GW (ed) AOAC Official Methods of Analysis, vol 1. AOAC International, 2019, AOAC International, 2019,
19. Mottier P (2019) Determination of Sodium Fluoroacetate in Infant Formula by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS): First Action 2015.03. Journal of AOAC International 98 (4):1127-1134
20. Chemical Method Validation Guidelines for the FDA FVM Program, 2nd ed (2015). U.S. Food and Drug Administration,
21. Cortese M, Gigliobianco MR, Magnoni F, Censi R, Di Martino PD (2020) Compensate for or Minimize Matrix Effects? Strategies for Overcoming Matrix Effects in Liquid Chromatography-Mass Spectrometry Technique: A Tutorial Review. Molecules 25 (13). doi:10.3390/molecules25133047
22. Code of Federal Regulations (2015) Title 40 CFR Appendix B to Part 136 - Definition and procedure for the determination of the method detection limit-revision 1.11. Code of Federal Regulations. https://www.govinfo.gov/app/details/CFR-2015-title40-vol23/CFR-2015-title40-vol23-part136-appB. Accessed 10/25 2023
23. The Organization for Economic Cooperation and Development (2007) Guidance document on pesticide residue analytical methods. The Organization for Economic Cooperation and Development. https://one.oecd.org/document/env/jm/mono%282007%2917/en/pdf. Accessed 10/26 2023
24. European commission directorate-general for health and food safety (2023) Technical guideline on the evaluation of extraction efficiency of residue analytical methods. European commission. https://food.ec.europa.eu/system/files/2023-05/pesticides_mrl_guidelines_wrkdoc_2017-10632.pdf. Accessed 10/26 2023
25. Environmental Protection Agency (2023) Guidance for addressing unextracted pesticide residues in laboratory studies. Environmental protection agency. https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/guidance-addressing-unextracted-pesticide-residues. Accessed 10/26 2023

26. International Standard (2012) ISO 10993-12:2012 Biological evaluation of medical devices - Part 12: Sample preparation and reference materials. International Standard. https://nhiso.com/wp-content/uploads/2018/05/ISO-10993-12-2012.pdf. Accessed 10/26 2023
27. Tkachenko A, Benson K, Mostrom M, Guag J, Reimschuessel R, Webb B (2021) Extensive Evaluation via Blinded Testing of an UHPLC-MS/MS Method for Quantitation of Ten Ergot Alkaloids in Rye and Wheat Grains. Journal of AOAC International 104 (3):546-554. doi:10.1093/jaoacint/qsaa173
28. the International Organization for Standardization (2017) ISO/Guide 35:2017 Reference materials — Guidance for characterization and assessment of homogeneity and stability. the International Organization for Standardization. https://www.iso.org/standard/60281.html. Accessed 10/25 2023
29. Zhou W, Yang S, Wang PG (2017) Matrix effects and application of matrix effect factor. Bioanalysis 9 (23):1839-1844. doi:10.4155/bio-2017-0214

Tables

Table 1. Matrix effects and recovery for MFA in fortified bovine kidney

Fortified Concentration (ng/g)	Matrix Effect[a] (%)	RSD (%)	Recovery[a] (%)	RSD (%)
100	58	22	68	22
5	65	8.9	61	18

[a]Based on average of three replicates each, using peak areas for quantifier ions only (no compensation made for labeled internal standard)

Table 2. Accuracy and precision obtained during in-house validation using non-blinded fortified bovine kidney according to FDA guidelines [17]

	Intra-day Results				Inter-day Results		
MFA Fortified (ng/g):	5.0	10	20	100	5.0	10	20
MFA Found[a] (ng/g):	5.6	11	20	103	5.5	11	20
RSD (%):	10	6.2	4.7	1.1	12	4.5	3.2
Accuracy (%):	112	110	100	103	110	110	100

[a]For intra-day results, based on average of three replicates at each level. For inter-day results, based on average of seven replicates at 5 ng/g, six replicates at 10 ng/g, and five replicates at 20

ng/g, all done over two or three separate analysis batches. Matrix and recovery effects were compensated for by the labeled internal standard ($^{13}C_2$, D_2-MFA).

Table 3. Summary of results[e] reported by the participating laboratory for blinded (i.e., unbiased) fortified bovine kidney samples prepared by an independent laboratory (i.e., BMT organizers)

	Intra-day						Inter-day					
	MFA (ng/g) fortified	MFA (ng/g) found[a]	Accuracy (%) found	RSD(r)% expected	RSD(r)% found	HorRat(r) found[c]		Conc. (ng/g) fortified	Conc. (ng/g) found[a]	Accuracy (%) found	RSD(i)% found	PRSD(R)% expected[d]
Day-1	7 (n=4)	6.3	90	17	12	0.4	Combo (Day-1 & Day-2)	7 (n=9)	6.1	87	9.9	34
	25 (n=4)	25	100	14	9.9	0.4						
	70 (n=4)	69	99	12	1.1	0.05						
	10[b] (n=1)	8.7	87	16	n/a	n/a		25 (n=8)	24	96	10	28
	60[b] (n=1)	58	97	12	n/a	n/a						
Day-2	7 (n=5)	5.9	84	17	7.2	0.2		70 (n=7)	67	96	4.4	24
	25 (n=4)	22	88	14	4.4	0.2						
	70 (n=3)	65	93	12	5.3	0.2						
	10[b] (n=1)	8.2	82	16	n/a	n/a						
	60[b] (n=1)	58	97	12	n/a	n/a						

n/a = not applicable

[a]Matrix and recovery effects were compensated for by the labeled internal standard ($^{13}C_2$, D_2-MFA).

[b]"Mystery" test samples were included by BMT organizers to minimize analyst's biases in possible clustering (or grouping) close (or similar) values for replicated samples during the sample analysis or data processing steps. See Table S1 in the Supplemental Material section for details.

[c]HorRat(r) values of 0.3-1.3 are expected according to AOAC guidelines [18] and values <0.3 are considered as "too good" and, therefore, results must be reviewed for possible averaging by

participant (i.e., an extra amount of sample is provided so participant analyzes the same sample several times and the reports the average) or possible intentional/unintentional unblinding of analysts.

RSD(r)% - precision in collaborative studies under repeatability conditions (i.e., samples were prepared in a different laboratory vs. analyzed, all test samples were analyzed in the same laboratory on the same day using the same equipment and reagents by the same analyst)

RSD(R)% - precision in collaborative studies under reproducibility conditions (e.g., samples were prepared in a different laboratory vs. analyzed, test samples were analyzed in different laboratories by different analysts using different equipment and reagents). [d]This is expected value according to AOAC guidelines [18] for collaborative studies when test samples are analyzed in multiple laboratories, which was not performed in our study and indicated here for comparison purposes.

RSD(i)% – precision in collaborative studies under intermediate conditions (e.g., samples were prepared in a different laboratory vs. analyzed; all test samples were analyzed in the same laboratory but on different days using different calibration curves and reagents). The intermediate precision (RSD(i)%) value is expected to be between expected RSD(r)% and expected RSD(R)% [30,31].

[e]This is a summary of results. See Table S1 in the Supplemental Material section for raw results reported by the analyst and not modified by BMT organizers (i.e., not trimmed by any type of averaging) allowing readers to apply alternative statistical approaches (e.g., non-AOAC based) to decide if the method performance satisfies their needs.