Determination of pentobarbital in ingredients of animal origin and in finished pet foods using liquid chromatography tandem mass spectrometry (LC-MS/MS)

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ABSTRACT

This bulletin is a matrix extension of LIB 4648 (determination of pentobarbital in tallow using LC-MS/MS) and describes the identification and quantitative determination of pentobarbital in numerous ingredients of animal origin, including: canned and dry dog and cat foods, rendering stream products such as meat and bone meal (MBM) and tallow, and various meats including: beef, bison, elk, goat, horse, lamb, pork, rabbit, venison, kidney (bison), and liver (beef). Following the methodology in LIB 4648, pentobarbital was determined by a shakeout extraction with acetonitrile, dilution, separation using an Agilent Eclipse Plus-C18 liquid chromatographic column and detection using negative mode electrospray ionization (ESI) on a SCIEX QTRAP 5500 hybrid linear ion trap mass spectrometer. Multiple reaction monitoring (MRM) was performed, fragmenting the [MH]- precursor ion into product ions. Recoveries were calculated using solvent calibration curves with deuterated internal standard correction for matrix effects. The method was validated at concentration levels of 10, 50, and 250 ng/g. Some matrices were validated at additional concentration levels. The average accuracy for pentobarbital spiked into all matrices at 50 ng/g was 97% with 3% RSD. The average calculated method detection limit (MDL) across all matrices was 1.6 ng/g and the average limit of quantitation (LOQ) for all matrices was 5.3 ng/g. The validation data for tallow from LIB 4648 is included in this document to provide a comprehensive record of method performance for all matrices studied.

INTRODUCTION

Pentobarbital is a short acting barbiturate used in animals for sedation, anesthesia, and euthanasia. If a pet consumes food containing pentobarbital, they may develop symptoms such as nausea, vomiting, dizziness, loss of balance, as well as a condition known as nystagmus (eyes darting side to side in an erratic fashion). This can lead to pets having difficulty to standing and walking in a straight manner[1]. In high doses pentobarbital can cause coma and death[1].

During the 1990s, the FDA Center for Veterinary Medicine (CVM) received reports from veterinarians that pentobarbital seemed to be losing effectiveness in dogs as an anesthetizing agent. CVM officials investigated the theory that the dogs were being exposed to pentobarbital in their food supply[2]. A 1998 CVM limited survey of retail dog food samples concluded that there appeared to be associations between rendered or hydrolyzed ingredients and the presence of pentobarbital in dog food. Common pet food ingredients meat and meat and bone meal (MBM) (MBM), beef and meat and bone meal (MBM) (BBM), animal fat (AF), and animal digest (AD) are rendered or hydrolyzed from animal sources that could include euthanatized animals[2]. From this data, it was thought that the pentobarbital residues were entering pet foods from euthanatized, rendered cattle or possibly horses[2]. In 2000, CVM tested a theory that euthanatized dogs and cats were subjected to the rendering process and used as an ingredient in pet food, however tests for dog and cat DNA in dog food disproved this suggestion[2]. More recently, canned dog foods were found to be contaminated with high concentrations of pentobarbital[3], which lead to the FDA canned dog food product alert in February 2018[1]. Subsequent investigations identified the presence of pentobarbital in tallow sources that were used to manufacture dog foods[4].

In 2018, FDA issued a national assignment to expand surveillance of fats/grease/tallow/oils of animal origin that could be used in pet food manufacturing. In 2019, the ORA Denver Laboratory (DENL) developed and validated LIB 4648[5] for the qualitative and quantitative analysis of pentobarbital in tallow. In the current study, the method performance was verified in additional pet food ingredients and finished pet foods. The matrices tested in this study were dry dog and cat food, canned dog and cat food, meat and bone meal (MBM), and numerous meats which may be used as pet food ingredients.

METHODS AND MATERIALS

Equipment

a) LC-MS/MS instrument. – 5500 QTRAP hybrid quadrupole linear ion trap mass spectrometer (SCIEX, Foster City, CA) utilizing a TurboV™ ion source with the TurboIonSpray® (i.e., electrospray ionization) probe installed and coupled to an Agilent 1200 Series binary pump, degasser, thermostated column compartment (Agilent Technologies, Santa Clara, CA) and HTC PAL autosampler (CTC Analytics, LEAP Technologies, Carrboro, NC, USA). Analyst 1.6.2 software was used to acquire and analyze the data (SCIEX). MultiQuant software was used for quantitative data analysis and reporting.
b) LC column - Zorbax Eclipse Plus-C18 2.1 x 50 mm, 1.8 µm, (P/N: 959757-902, Agilent Technologies).
c) Pipettors – variable volume (5 µL to 1000 µL) (Eppendorf, Hauppage, NY), or equivalent.
d) Centrifuge – Sorvall LYNX 4000, refrigerated to 4 ºC, capable of accelerating 50 mL tubes to 6000 rpm (ThermoFisher Scientific, Waltham, MA), or equivalent.
e) Shaker – 2010 Geno/Grinder (Spex Sample Prep, Metuchen, NJ, USA), or equivalent.
f) Vortex mixer – Vortex Genie 2 (Scientific Industries, Bohemia, NY), or equivalent.
g) Sonicating bath – Branson 2510 or 8510 (Cole-Palmer, Vernon Hills, IL, USA), or equivalent.
h) Centrifuge tubes – 50 mL disposable, conical, graduated polypropylene tubes with cap (Falcon® Blue Max™, P/N:352070, Becton Dickinson, Franklin Lakes, NJ), or equivalent.

i) Syringe filters –Acrodisc 13 mm 0.2 µm nylon syringe filters, P/N 4427T (Pall Life Sciences, Port Washington, NY)
j) Syringe - 1-mL, disposable (P/N 309602, Becton Dickinson, Franklin Lakes, NJ).
k) Microcentrifuge tubes – 1.7 mL snap cap tubes, polypropylene, non-sterile (P/N: CLS3622-500EA) Sigma Aldrich, St. Louis, IL, USA)
l) LC vials – 2 mL glass amber HPLC autosampler vials (Thermo Scientific P/N: C4011-6W
m) pre-slit snap caps for vials (Thermo Scientific P/N: C4011-6W and C4011-55)
n) Appropriate mixers, blenders, food processors, (i.e. Robot Coupe) *etc.* used to homogenize sample matrix if necessary

REAGENTS AND SOLUTIONS

Note: Equivalent reagent or solution sources may be substituted. The expiration time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever is earlier.

Reagents and Standards

a) Solvents
    1. Water, Fisher, LC-MS grade
    2. Acetonitrile, Fisher, LC-MS grade
b) Reagents
    1. Diluent for standards: 50/50 water/acetonitrile (v/v)
c) LC systems mobile phases
    1. Mobile Phase A – 100% water
        (Note: Store mobile phase A in an amber bottle and protect from light)
    2. Mobile Phase B – 100 % acetonitrile.
d) Analytical standards
    1. Pentobarbital, 1.000 ± 0.005 mg/mL in methanol, 1 mL/ampoule, part # P-010 (Cerriliant, Round Rock, TX)
    2. Pentobarbital-D_5, 1.000 ± 0.005 mg/mL in methanol, 1 mL/ampoule, part # P-013 (Cerriliant, Round Rock, TX)
e) Negative control – All pet food and meat control samples were acquired from local grocery stores with the exception of horsemeat which was obtained from the FDA Forensic Chemistry Center (FCC). Tallow controls were purchased from an online retailer. Meat and bone meal (MBM) controls were provided by CVM and/or were samples previously received in the DENL. All control sources were tested to determine that pentobarbital was not present above the stated method detection level (MDL) prior to use as validation/verification sources.

Preparation of Standards

Pentobarbital and pentobarbital-D_5 were ordered as prepared solutions with concentration of 1,000 µg/mL (1 mg/mL).

a) Pentobarbital intermediate standard was prepared at a concentration of 2,500 ng/mL by combining 25 µL of the 1,000 µg/mL stock standard with acetonitrile for a total volume of 10.0 mL.
b) Pentobarbital ICV intermediate standard was prepared at a concentration of 2,500 ng/mL by combining 25 µL of the 1,000 µg/mL stock standard with acetonitrile for a total volume of 10.0 mL.
c) An internal standard (ISTD) intermediate of pentobarbital-D_5 was prepared at a concentration of 2,500 ng/mL by combining 25 µL of the 1000 µg/mL deuterated stock standard with acetonitrile for a total volume of 10.0 mL.

Note: All solutions expire 1 year from the preparation date when stored at 4°C.

Tables 1 and 2 are examples of the intermediate standards and the solvent calibrants. Table 3 demonstrates the concentrations of the calibrants in-vial and the equivalent concentration in-sample for use in the processing method generated by the extraction procedure.

Typically, six calibration standards (Cal 1 to Cal 6) are analyzed with every batch of samples as shown in Table 2. The Cal-1 standard at a concentration of 1.0 ng/mL, which is equivalent to an in-sample concentration of 10.0 ng/g (Table 3), is used as the lower limit of quantitation (LLOQ). The "MDL Cal" prepared at a concentration of 0.5 ng/mL (Table 2) was only included in calibration curves for validation batches to quantitate 5 ng/g or 10 ng/g spiked samples. In addition, initial validation batches included additional calibrators at 12.5 ng/mL and 100 ng/mL to test higher concentrations of pentobarbital, but these calibrators are not generally used for routine analysis.

Table 1: Intermediate Standard Preparation (in acetonitrile)

Standard Name	Stock Pentobarbital Concentration (µg/mL)	Volume Pentobarbital Stock Standard Added (mL)	Final Volume (mL)	Final Pentobarbital Concentration Intermediate Standard (ng/mL)
Pentobarbital-2,500	1,000	0.025	10.0	2,500
Pentobarbital ICV	1,000	0.025	10.0	2,500
Pent-D_5 (ISTD)	1,000	0.025	10.0	2,500

Table 2: Calibrants Prepared for Calibration Curve (in 50:50 acetonitrile:water)

Calibrants	Initial Concentration Pentobarbital Intermediate Standard (ng/mL)	Volume of Pentobarbital Intermediate Standard Added (mL)	Volume D5-ISTD Intermediate Standard (2,500 ng/mL) Added (mL)	Final Volume (mL)	Final Concentration Pentobarbital (ng/mL)	Final Concentration D_5 ISTD (ng/mL)
MDL Cal	2,500	0.010	0.100	50.0	0.50	5.00
Cal-1		0.020	0.100	50.0	1.00	
Cal-2		0.010	0.020	10.0	2.50	
Cal-3		0.020	0.020	10.0	5.00	
Cal-4		0.040	0.020	10.0	10.0	
Cal-5		0.100	0.020	10.0	25.0	
Cal-6		0.200	0.020	10.0	50.0	
Cal-ICV	2,500	0.040	0.020	10.0	10.0	

Table 3: Calibrants In-Vial Concentration Compared to In-Sample Concentration Equivalency

Calibrants	In-Vial Final concentration (ng/mL)	Sample Weight (g)	Volume ACN used to extract (mL)	Dilution Factor	Equivalent In-Sample Concentration (ng/g)	In-Sample Final Concentration D_5 ISTD (ng/g)
MDL Cal	0.50	2.00	10	2.00	5.00	50.0
Cal-1	1.00				10.0	
Cal-2	2.50				25.0	
Cal-3	5.00				50.0	
Cal-4	10.0				100	
Cal-5	25.0				250	
Cal-6	50.0				500	
Cal-ICV	10.0				100	

Pentobarbital-D5 (2,500 ng/mL): 40 µL is added to all samples = 0.04 mL/2 grams x 2,500 ng/mL= 50 ng/g	
Example Calculation:	=5.0 ng/mL in vial x (10 mL/2.00 g) x (1 mL/0.500 mL) = 50 ng/g in sample
	=Conc. Std (ng/mL)*Extraction Volume (mL) /Sample Weight (g) x Dilution Factor

Sample Preparation

Several different approaches to sample preparation were used as determined by the type of matrix. All meat matrices were cut into small pieces and the tissue was homogenized in a Robot Coupe food processor with dry ice until homogeneous consistency was achieved. Samples were stored frozen (< -10°C) prior to analysis. Retail pet foods were ground in the Robot Coupe food processor until a homogeneous consistency was achieved. Dry pet foods containing chewy bits were prepared in a similar fashion as other dry pet foods, with the addition of dry ice. Semi-solid samples such as tallow, and powdery samples such as meat and bone meal (MBM) were manually mixed or mixed via a blender to ensure a homogenous sample prior to removing a portion for analysis.

Extraction Procedure

The sample weight was recorded to at least three significant figures and calibrated pipettes/class A volumetric glassware was used. A 2.00 ± 0.05 g portion of each homogenized sample was weighed into a 50 mL centrifuge tube. An empty tube served as Reagent Blank (RB), portions of negative control material were weighed out to serve as negative control (NC) and matrix spikes. For batches that contained samples of different matrices analyzed together in, a negative control, spike, and duplicate spike was analyzed for each matrix type. To all samples in the batch, including RB, NC, and positive controls, 40 µL of 2,500 ng/mL pentobarbital-D_5 was added. Fortification level may be adjusted as necessary, as long as the concentration falls within the calibration curve. For validation and verification batches, samples were spiked at concentrations indicated in Table 4. To each tube 10 mL of acetonitrile was added. The tubes were capped and shaken to mix on the Geno/Grinder @ 500 rpm for 5 minutes and then sonicated for 30 minutes. After sonication, the tubes were vortexed for 30 seconds and then centrifuged at 6000 rpm at 4°C for 10 minutes. After centrifugation, 500 µL of sample supernatant was combined with 500 µL of water in a microcentrifuge tube, vortexed to mix, then filtered using a 0.2 µm nylon syringe filter into a LC vial and finally analyzed via LC-MS/MS.

Table 4: Fortified Concentration Levels Used for Method Validation

Fortification Level (ng/g)	Initial Concentration of Pentobarbital Intermediate Standard (ng/mL)	Volume of Intermediate Standard Used (mL)	Sample Weight (g)	Final Concentration In-Sample (ng/g)
10 ng/g	2,500	0.008	2.00	10.0
50 ng/g	2,500	0.040	2.00	50.0
250 ng/g	2,500	0.200	2.00	250

Instrumentation

a) LC-MS/MS system – The SCIEX 5500 Q TRAP was operated in triple quadrupole mode and calibrated per the manufacturer's instructions. The analyses were performed using electrospray ionization in negative mode. The instrument conditions were as follows: ion spray voltage, -3500 V; curtain gas, 30 (arbitrary units); GS1 and GS2, 50 and 60, respectively; probe temperature, 400 ºC. The entrance potential (EP) was -10, the declustering potential (DP) was set to -100 V, and the dwell time was 50 msec. Nitrogen served as sheath gas and collision gas with a CAD gas setting of medium. MRM experiments allowed the maximum sensitivity to be obtained for the detection of the target molecules. The optimization of MS parameters (declustering potential (DP), collision cell entrance potential (CEP) for precursor ions and collision energy (CE), collision cell exit potential (CXP) for product ions) was performed by compound optimization. Table 5 shows the values of the optimized parameters and the MRM product ion transitions used for the confirmation and quantification of pentobarbital.

Table 5: Optimized Pentobarbital MS Parameters.

Analyte	RT (min)	Transition (m/z)			ISTD	CE (V)	CXP (V)	Average ion ratio, qual/quant %
Pentobarbital	4.20	225	→	182 85 138 42*	Pent-D_5	-19 -18 -21 -46	-13 -9 -10 -7	100 15 7 275*
Pentobarbital-D_5	4.20	230	→	187		-17	-10	N/A

*Alternate transition, m/z 225→42 was added for meat and bone meal (MBM). See results and discussion section.

b) HPLC system – Agilent 1260 HPLC system was equipped with pump, solvent degasser, autosampler, and column oven. An Agilent Zorbax Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm column was used and kept at 40°C oven temperature. The pump was operated at a flow rate of 0.350 mL/min. A binary gradient system was used to separate analytes comprising mobile phase A (water) and mobile phase B (acetonitrile); refer to Table 6 for the mobile phase gradient. The autosampler injection volume was 5 µL. Autosampler temperature was set to 15°C. The Combi Pal injector wash protocol was used with wash solvent 1 (95% water:5% acetonitrile) and wash solvent 2 (5% water:95% acetonitrile) to minimize injection carryover.

Table 6: LC Gradient for Pentobarbital Analysis

@Step	Time (min)	Flow Rate (µl/min)	A (%) (Water)	B (%) (Acetonitrile)
0	0.00	350	95.0	5.0
1	3.50	350	5.0	95.0
2	4.50	350	95.0	5.0
4	8.50	350	95.0	5.0

Some LC systems may require a longer hold of step 1 until after the analyte elutes.

RESULTS AND DISCUSSION

Method Validation

The purpose of this publication is to describe the validation and method performance for the analysis of pentobarbital in feed ingredients of animal origin and finished pet food. The initial validation for pentobarbital in tallow was published in LIB 4648[5]. The extraction procedure and analytical determination used in LIB 4648 remain unchanged from the current LIB with one necessary modification to acquire a 4th product ion transition for to confirm the identity of pentobarbital in the meat and bone meal (MBM) matrix (discussed below). Table 7 shows the results of the validation and Figures 1-9 show chromatograms for representative matrices.

Validation data was collected from fortified samples with pentobarbital concentrations of 10, 50, and 250 ng/g to cover the lower concentrations observed in the 1998-2000 dog food surveys[2] as well as higher contamination levels determined in recent events[3]. The initial level of interest was a pentobarbital concentration of 50 ng/g; therefore, initial MDL spikes were analyzed at 12.5 ng/g. Later in the method development process, the level of interest was lowered to 10 ng/g, requiring the analysis of additional samples fortified at 5 ng/g or 10 ng/g to evaluate the MDL and LOQ. The lower fortification levels were used to establish the method detection and quantitation limits, and to support a target testing level of 10 ng/g.

The method validation process for the tested matrices was subdivided into groups based on matrix similarities: meat and bone meal (MBM); retail dry and canned pet foods (dog and cat foods); horsemeat and other meat muscle matrices (bovine, bison, elk, goat, lamb, pork, rabbit, venison); offal meat including bison kidney, and beef liver. Due to the comprehensiveness of the matrix extension, the verification was performed in stages. Subsequently some of the parameters such as concentration or number of replicates varied, but validations for all the matrices met the FDA guidelines[6].

As documented in LIB 4648, tallow was validated by analyzing fortified samples from three tallow sources, with three replicates tested in each source at three concentrations, 12.5, 50 and 250 ng/g. Additionally, seven replicates were analyzed at 5 ng/g and 10 ng/g in one tallow source, and that data was used to determine MDL and LOQ, respectively.

The other sample matrices were validated according to the same FDA Level 2 validation procedure using three sources of the matrix, or as a matrix extension using one source[6]. The validation parameters for the different matrices are summarized in Table 7 below.

Calibration Curve and Linearity

Calibration curves were established from the solvent calibrant standards with concentrations 1, 2.5, 5, 10, 25, 50 ng/mL in vial (corresponding to 10, 25, 50,100, 250, 500 ng/g in sample). The x-axis corresponded to analyte concentration and the internal standard corrected peak area response was plotted on the y-axis. All calibration curves were generated with the SCIEX MultiQuant software, and a linear fit (not forced through zero) was used. In cases where a larger dynamic range was required (for example, initial testing of tallow, horsemeat, and wet dog food where the calibration curve ranged from 5 ng/g-1000 ng/g), a linear fit with 1/x weighting was used. When a

smaller dynamic range was used, no weighting was used. Correlation coefficients (r^2) were typically greater than 0.998, with the average r^2 shown in Table 7.

Accuracy and Precision

Method accuracy (trueness) was determined by calculating the percent recovery of pentobarbital based on a solvent calibration curve where the peak area for the pentobarbital peak was corrected with the response of the deuterated pentobarbital internal standard. The FDA guidelines specify that analyte recovery should be within the range 80%-110% for analytes with concentrations ranging from 100 to 1000 µg/kg (ng/g, ppb) and 60%-115% corresponding to concentrations ranging from 10-100 µg/kg with an RSD of ≤22%[6]. Average recoveries for all matrices spiked at 50 ng/g ranged from 89-110%, meeting the FDA criteria. Dry dog food at the 250 ng/g level showed recoveries slightly higher than the FDA criteria (116%). Recoveries for the 10 ng/g level for elk (123%) and beef liver (121%) were also slightly elevated. Average recoveries for all other matrices and levels met the FDA criteria (ranging from 85-112%). The precision in all matrices at all concentration levels met the FDA criteria with RSD <17%. Accuracy, precision, method detection limit (MDL), limit of quantitation (LOQ) and typical linearity (r^2) for each matrix and fortification level are shown in Table 7.

Table 7: Accuracy, precision, MDL, LOQ, and typical linearity (r^2) for each matrix and fortification level

Matrix	Accuracy (as % Recovery) ± % RSD			MDL (ng/g) n = 7	LOQ (ng/g) n ≥ 7	Average Linearity (r^2)
	10 ng/g n ≥ 6	50 ng/g n ≥ 6	250 ng/g n ≥ 6			
Finished Pet Food Products						
Dry Dog Food	98 ± 16	110 ± 6	116 ± 6	2.9	9.3	0.9989
Wet Dog Food	98 ± 8	108 ± 5	107 ± 4	2.5	7.8	0.9994
Dry Cat Food	100 ± 6	90 ± 5	85 ± 5	1.8	5.8	0.9999
Wet Cat Food	106 ± 3	97 ± 0.3	94 ± 5	1.0	3.2	1.0000
Rendering Stream Products						
Tallow	98 ± 12	105 ± 4	110 ± 3	3.6	11.6	0.9991
Meat and bone meal (MBM)	91 ± 9	92 ± 2	92 ± 3	2.3	8.0	0.9991
Various Meat Products						
Beef	102 ± 5	97 ± 4	95 ± 4	1.7	5.2	0.9997
Bison	108 ± 3	95 ± 4	94 ± 6	1.0	3.2	0.9998
Elk	123 ± 3	100 ± 2	92 ± 3	1.2	3.9	0.9994
Goat	109 ± 4	96 ± 3	94 ± 2	1.3	4.2	0.9998
Horse	101 ± 3	102 ± 3	106 ± 1	0.9	2.9	0.9991
Lamb	112 ± 3	89 ± 1	88 ± 2	0.9	3.0	0.9998
Pork	104 ± 3	91 ± 2	88 ± 1	0.9	3.0	1.0000
Rabbit	112 ± 5	95 ± 2	92 ± 3	1.6	5.2	0.9999
Venison	107 ± 2	96 ± 2	104 ± 2	0.8	2.6	1.0000
Kidney (Bison)	96 ± 6	90 ± 2	93 ± 2	1.8	5.7	0.9999
Liver (Beef)	121 ± 3	97 ± 3	94 ± 5	1.6	5.0	0.9997
Average for all matrices	105 ± 5	97 ± 3	97 ± 3	1.6	5.3	0.9996

Method Detection Level

The method detection limit (MDL) and limit of quantitation (LOQ) were determined using the formulas below. The MDL for each matrix was calculated using the replicates of 5 ng/g spikes and the LOQ was calculated using the replicates of 10 ng/g spikes for the initial matrices tested (tallow, dry and wet dog food, and horsemeat). For the remaining matrices, the MDL and LOQ were both determined using the standard deviation data for the 10 ng/g spikes.

MDL = Method Detection Limit = $\sigma t_{(df=N-1,1-\propto=0.99,\ one\ sided)}$

Where: σ = standard deviation
t = Student's t value for df = N-1 at the 99% confidence level, one sided
N= number of replicate spikes

LOQ = Limit of Quantitation = $\sigma(10)$

Where: σ = standard deviation

MDL and LOQ are provided in Table 7. The MDL and LOQ are highest for tallow at 3.6 and 11.6 ng/g, respectively. For the initial validation in tallow presented in LIB 4648, nine replicates of tallow were spiked at 12.5 ng/g, and the MDL and LOQ calculated from that data was determined to be

2.4 ng/g and 8.2 ng/g, respectively. The MDL was below 3 ng/g for all matrices other than tallow. The LOQ was less than 10 ng/g for all other matrices, which demonstrated method sensitivity appropriate to analyze low part per billion levels of pentobarbital contamination in several different and complex matrices.

Qualitative Identification/Confirmation of Identity

For qualitative identification, CVM GFI # 118 criteria was used[7]. The identity of pentobarbital was considered confirmed if the following criteria were met:

- LC-MS presents a chromatographic peak with RT within $\pm$ 5% of the chromatographic peak relative to the standard.
- The chromatographic peak should exceed a signal-to-noise (s/n) threshold of 3:1. The MultiQuant software is used to calculate signal to noise.
- Two ion ratios are $\leq$ I20%I or one ion ratio is $\leq$ I10%I of the average ion ratios from the calibration standards analyzed in the same sequence.
- Negative controls and reagent blanks do not contain a positive identification for the analyte at or above the LOQ (i.e. no lab contamination or carryover).

For this validation study all the pentobarbital fortified samples met the conditions to be positively identified, with product ion transition ratios within ± 20% of the average ion ratios of the calibrants. None of the negative controls or reagent blank samples met the criteria for identification of pentobarbital.

As previously discussed, some meat and bone meal (MBM) sources were observed to have an interference peak and noisy baseline for the m/z 225→85 product ion transition. This resulted in inconsistent peak integration by the MultiQuant software for the m/z 225→85 transition at low concentration levels (10 ng/g) in the meat and bone meal (MBM) matrices. This inconsistency resulted in product ion ratios that exceeded the ion ratio criteria for pentobarbital identity confirmation. To improve the ability to confirm the identity of pentobarbital in meat and bone meal (MBM) matrices, an additional product ion transition was acquired for m/z 225→42 that was not affected by a matrix interference. However, the peak intensity for the m/z 225→42 transition was greater than the peak intensity for the m/z 225→182 quantitative transition, thus the product ion ratio of the m/z 225→42 qualifier transition to the m/z 225→182 quantitative transition was greater than 100% (typically ~275%). This additional transition provides additional criteria to support the identification of pentobarbital in meat and bone meal (MBM) or other matrices which demonstrate similar matrix interferences. Figures 8 and 9 show the additional transition added for meat and bone meal (MBM).

Quantitative Analysis

This method provides accurate quantitative results for pentobarbital within a variety of matrices within the concentration range of 10-250 ng/g as shown in Table 7. Tables 8 and 9 document typical composition profiles of retail pet foods, tallow, meat and bone meal (MBM), and various meats used as pet food ingredients. The difference in protein, fat and moisture in retail dry and canned pet food did not seem to have a significant effect on method performance. Muscle and offal meats have similar compositions as compared to finished canned pet foods and tended to perform similarly. Meat and bone meal (MBM) has more protein and very little moisture as compared to the pet foods or meats, yet performed similarly for quantitative analysis.. Unsurprisingly, samples of tallow, containing only fats, and no protein or moisture present more performance variability between replicate analyses, being difficult matrices to extract. Regardless of the matrix composition variability, this method performs suitably for all matrices for the validation levels tested. Using the isotopically labeled internal standard to correct analyte response for quantitative analysis facilitated mitigation of differences in extraction efficiencies between matrices.

Table 8: Retail pet food sources comparison of composition[8]

	Crude Protein (min) (g/100 g)	Crude Fat (min) (g/100 g)	Moisture (max) (g/100 g)
Canned Dog Food Source 1	1	5	78
Canned Dog Food Source 2	1	3	80
Canned Dog Food Source 3	8	6	78
Canned Cat Food Source 1	11	3	79
Dry Dog Food Source 2	21	13	10
Dry Dog Food Source 3	21	10	12
Dry Dog Food Source 4	21	8	18
Dry Cat Food Source 1	30	11	12

Table 9: Typical composition of other matrices of animal origin[9,10]

	Protein (g/100 g)	Fat (g/100 g)	Water (g/100 g)
Beef Fat/Tallow	0	100	0
Meat and bone meal (MBM)	48-52	8-12	4-7
Muscle Meat and Offal Meat	19-25	2-12	70-75

Pentobarbital may be found in animal feed or feed ingredients with concentrations significantly higher than the concentration range validated in this study[3]. To obtain accurate quantitative determination in these cases, a common analytical approach would be to dilute the final sample extract to the midpoint of the calibration curve using the 50:50 acetonitrile:water diluent. However, in this method, the quantitative determination is corrected based on the response of the deuterated internal standard. For samples with pentobarbital concentrations that are much higher than the range of the calibration curve, the dilution that would be required for the extract may decrease the internal standard concentration to the point where it is unreliable for concentration correction. To evaluate the performance of the method without internal standard correction, validation data for some matrices was evaluated without the correction. Precision and accuracy, detection limits and linearity were evaluated, and the results are provided in Appendix A. For some matrices, similar method performance is achieved for with and without internal standard correction. The appropriateness of this uncorrected dilution approach for high pentobarbital concentrations must be evaluated on a case by case basis when applied to regulatory samples.

CONCLUSION

This LIB describes a rapid, sensitive, and selective method suitable for the identification and quantification of pentobarbital in numerous matrices of animal origin including fats, meat and bone meal (MBM), meats, as well as in finished pet foods. The average accuracy for pentobarbital spiked into all matrices at 50 ng/g was 97% with 3% RSD. Accuracy and precision using internal standard correction met the chemical methods of analysis requirements specified by FDA for the matrices investigated, with a few exceptions of high recoveries in one spike level each for dry dog food, elk, and beef liver. Across all 17 matrices, the average method detection limit was 1.6 ng/g and the average limit of quantitation was 5.3 ng/g.

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Figures 1-9 provide the MultiQuant result summary for calibrants, controls and fortified samples. Each summary includes the analyte peak precursor name, retention time, calculated concentration, analyte response, calculated ion ratio, and ratio confirmation. Each summary also includes three chromatograms for the graphical representation of the precursor to product ion transition for the three different product ion transitions (four transitions for Figures 8 and 9) acquired for pentobarbital.

Figure 1: Solvent calibration standard at 50 ng/g in sample equivalent (In vial concentration: 5.0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.24	4.20	52.28	86106.0		
Pentobarbital 2 (225->85.0)	4.24	4.20		12809	14.9% (14.3%)	✓
Pentobarbital 3 (225->138.0)	4.24	4.20		5270	6.1% (6.9%)	✓

Pentobarbital 1 Pentobarbital 2 Pentobarbital 3

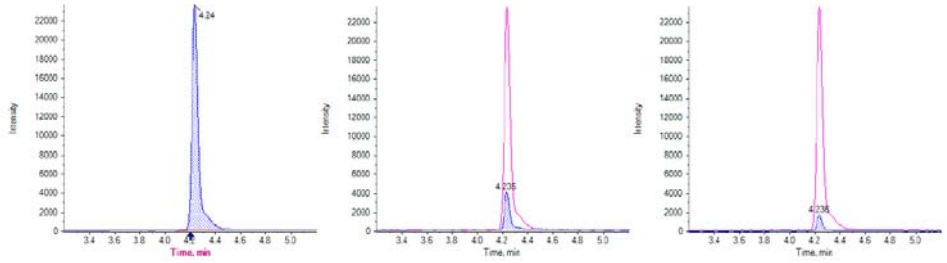


Figure 2: Dry dog food source # 3 negative control
(In vial concentration: 0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	N/A	4.20	N/A	N/A		
Pentobarbital 2 (225->85.0)	3.95	4.20		4152	0.0% (14.3%)	No
Pentobarbital 3 (225->138.0)	4.46	4.20		523	0.0% (6.9%)	No

Pentobarbital 1 Pentobarbital 2 Pentobarbital 3

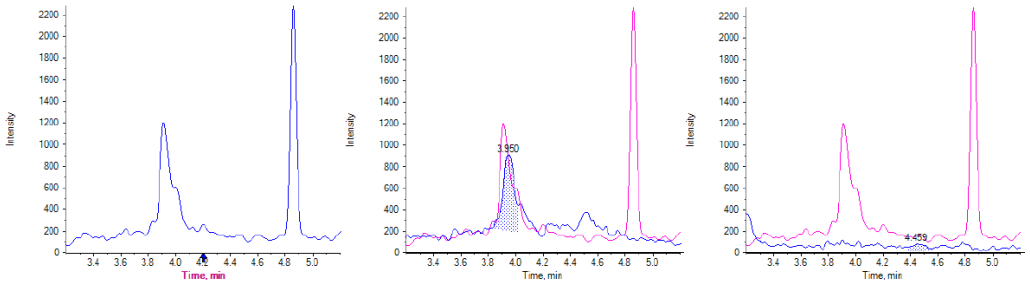


Figure 3: Dry dog food source # 3 - fortified at 50 ng/g
(In vial concentration: 5.0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.22	4.20	51.67	66910.0		
Pentobarbital 2 (225->85.0)	4.22	4.20		9140	13.7% (14.3%)	✓
Pentobarbital 3 (225->138.0)	4.22	4.20		4941	7.4% (6.9%)	✓

Pentobarbital 1 Pentobarbital 2 Pentobarbital 3

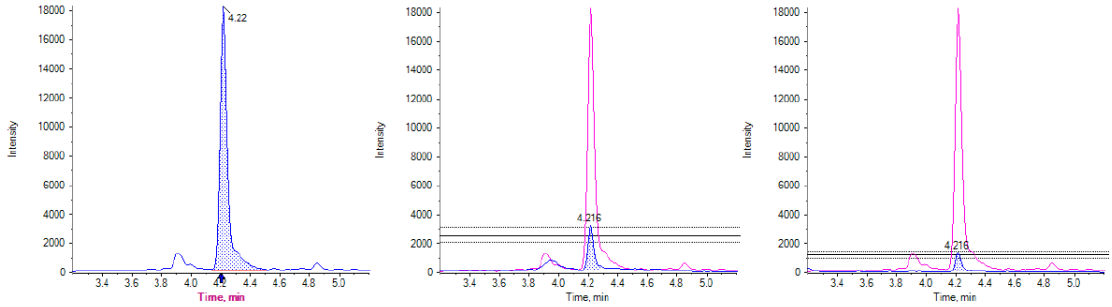


Figure 4: Wet dog food source # 3 negative control
(In vial concentration: 0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	3.92	4.20	<0	5051.0		
Pentobarbital 2 (225->85.0)	4.54	4.20		1339	26.5% (15.6%)	No
Pentobarbital 3 (225->138.0)	4.57	4.20		630	12.5% (7.0%)	No

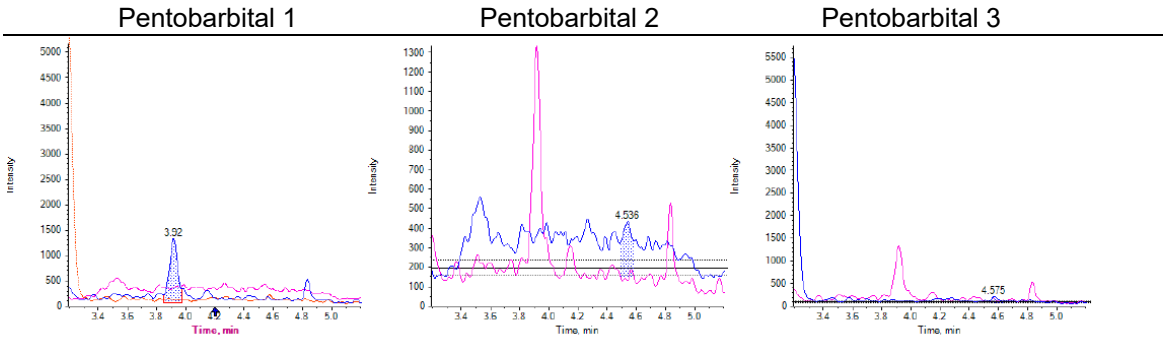


Figure 5: Wet dog food source # 3 - fortified at 50 ng/g
(In vial concentration: 5.0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.21	4.20	<0	125496.0		
Pentobarbital 2 (225->85.0)	4.20	4.20		21782	17.4% (15.6%)	✓
Pentobarbital 3 (225->138.0)	4.21	4.20		9255	7.4% (7.0%)	✓

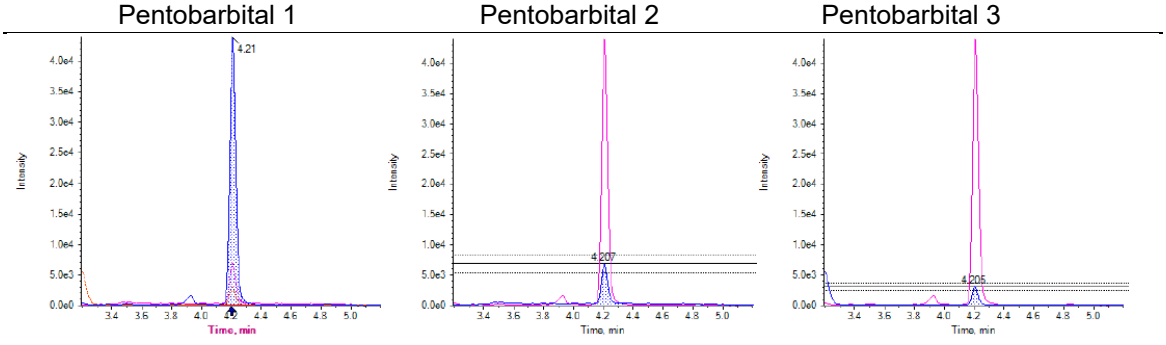


Figure 6: Horsemeat negative control
(In vial concentration: 0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.21	4.69	N/A	N/A		
Pentobarbital 2 (225->85.0)	4.61	4.69		770	0.0% (14.9%)	No
Pentobarbital 3 (225->138.0)	4.55	4.69		489	0.0% (6.9%)	No

Pentobarbital 1 Pentobarbital 2 Pentobarbital 3

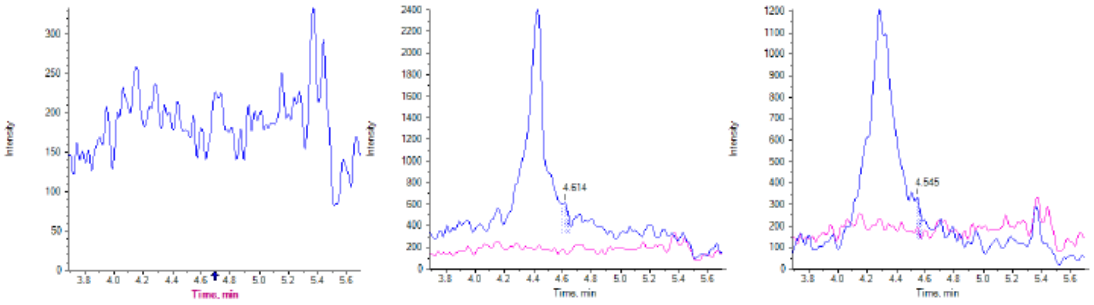


Figure 7: Horsemeat- fortified at 50 ng/g
(In vial concentration: 5.0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.69	4.69	50.29	70189.0		
Pentobarbital 2 (225->85.0)	4.69	4.69		11187	15.9% (14.9%)	✓
Pentobarbital 3 (225->138.0)	4.69	4.69		4374	6.2% (6.9%)	✓

Pentobarbital 1 Pentobarbital 2 Pentobarbital 3

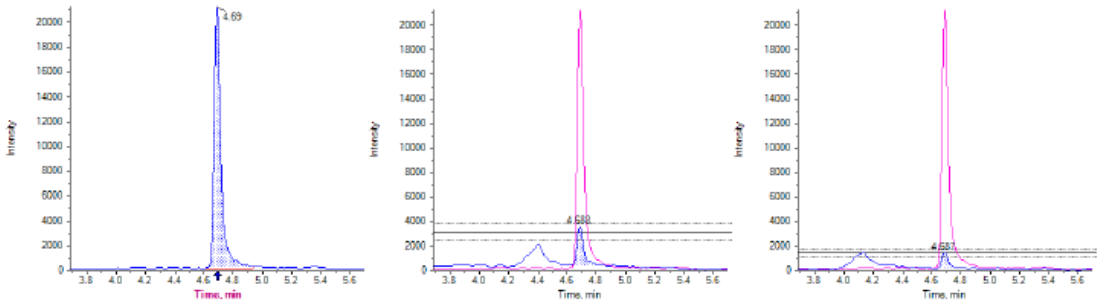
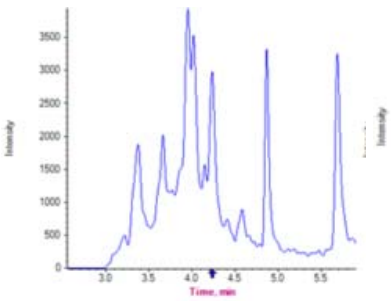


Figure 8: Result Summary for Meat and bone meal (MBM) Negative Control (source 2)

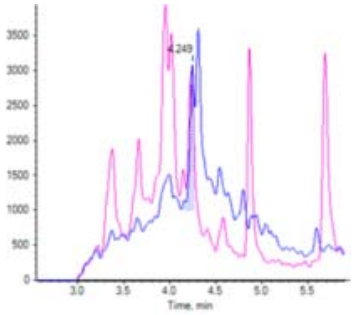
Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	N/A	4.23	N/A	N/A		
Pentobarbital 2 (225->85.0)	4.25	4.23		7.07e3	0.0% (15.9%)	
Pentobarbital 3 (225->138.0)	N/A	4.23		N/A	0.0% (6.1%)	
Pentobarbital 4 (225->42.0)	4.23	4.23		5.24e3	0.0% (281.7%)	

Chromatograms – Bars on peaks are expected ion ratio ± 20% of comparison standards

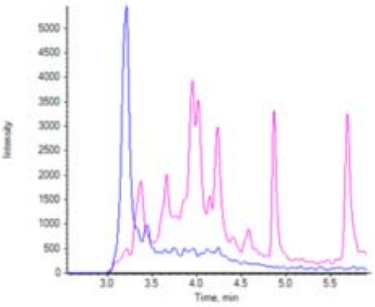
Pentobarbital 1



Pentobarbital 2



Pentobarbital 3



Pentobarbital 4

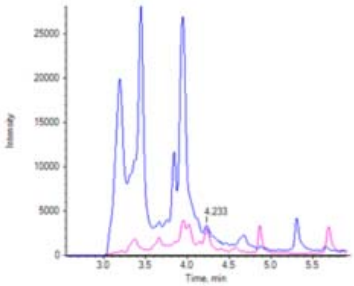
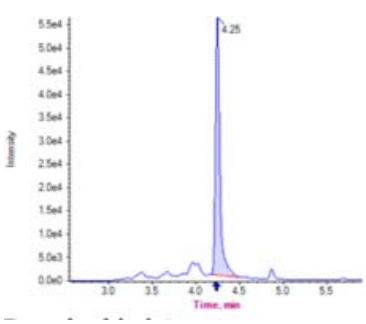


Figure 9: Result Summary for Meat and bone meal (MBM) Fortified at 50 ng/g (source 2)

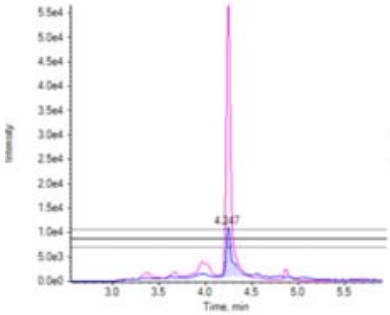
Analyte Peak Name (m/z)	Analyte RT (min)	Expected RT (min)	Calculated Concentration (ng/g)	Analyte Response (peak area)	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.25	4.23	45.76	1.93e5		
Pentobarbital 2 (225->85.0)	4.25	4.23		4.87e4	25.3% (15.9%)	✓
Pentobarbital 3 (225->138.0)	4.24	4.23		1.54e4	8.0% (6.1%)	✓
Pentobarbital 4 (225->42.0)	4.24	4.23		5.16e5	267.4% (281.7%)	✓

Chromatograms – Bars on peaks are expected ion ratio ± 20% of comparison standards

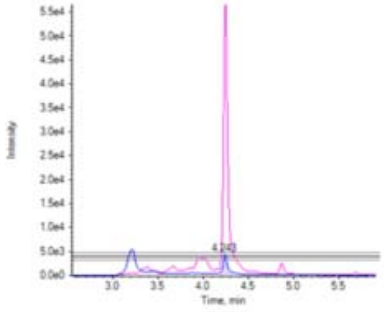
Pentobarbital 1



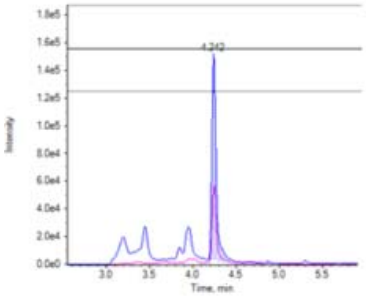
Pentobarbital 2



Pentobarbital 3



Pentobarbital 4



APPENDIX A

Quantitation of pentobarbital without an isotopically labeled internal standard

Pentobarbital contamination may be found in animal feed or feed ingredients with concentrations greater than the highest calibration point of 1000 ng/g. Due to the wide range of potential pentobarbital contamination, the same analytical validation data described in the LIB was also processed without internal standard correction. In general, if a sample has a response above the highest calibrant on a calibration curve, it is not always possible to dilute the sample to cause the analyte response to fall within the range of the curve since the dilution may affect the internal standard in a non-reproducible way (e.g. ISTD response could be diluted below the detection level). If the concentration of the pentobarbital can be determined without internal standard correction due to the absence of significant matrix effects, then extract dilution into the range of the calibration curve is a possibility. To validate the possibility of extract dilution for the regulatory analysis of high-concentration samples, quantitative results were calculated for fortified samples with and without internal standard correction. The accuracy and precision results from the validation without the internal standard (ISTD) are summarized in Tables A1 and A2 below for fortified tallow.

Table A1: Data without deuterated internal standard correction - accuracy and precision at each fortification level for dry dog food, wet dog food, and horsemeat.

Matrix	Accuracy (as % Recovery) ± % RSD			MDL (ng/g) n=7	LOQ (ng/g) (n=7)	Average Linearity (r^2)
	10 ng/g	50 ng/g	250 ng/g			
Dry Dog Food	77 ± 10	85 ± 7	82 ± 4	1.25	7.71	0.9979
Wet Dog Food	67 ± 14	75 ± 18	71± 20	1.68	9.07	0.9987
Horsemeat	75 ± 4	139 ± 9	173 ± 2	0.84	3.05	0.9991

The FDA guidelines for chemical method validation specify that analyte recovery be within the range 80%-110% with an RSD of ≤22% for analytes with concentrations ranging from 100 to 1000 µg/kg (i.e., ng/g, ppb) and 60%-115% corresponding to concentration from 10-100 µg/kg[6]. The recoveries and RSDs without the ISTD correction meet the requirements specified in the FDA for wet and dry dog food, but not for the 50 ng/g and 250 ng/g levels of fortified horsemeat. For this source of horsemeat, it would not be appropriate to omit the use of the internal standard correction. Appropriateness of internal standard omission should be evaluated on a case by case basis.