DETERMINATION OF PENTOBARBITAL IN TALLOW USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS) CARTS: IR01702

Tara J. Nickel[1], Christine R. Casey[1], Susan Young[1], and Wendy C. Andersen[2]

[1]U.S. Food and Drug Administration, ORA Denver Laboratory[1] and Animal Drugs Research Center[2], Denver Federal Center, Denver, CO 80225-0087

Abstract

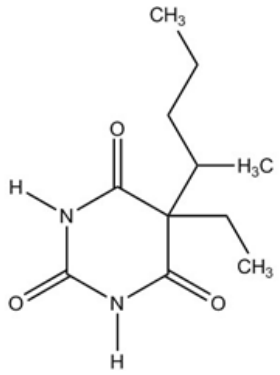
Beginning in 2017, pentobarbital contamination has triggered the recall of canned pet foods. FDA issued alerts to pet owners about potential pentobarbital contamination in canned dog food. The most recent alert, from February 2018, detailed 18 dog food products voluntarily recalled due to pentobarbital identified in these products. The source of the pentobarbital contamination has been linked to the tallow supplied to the dog food manufacturers. This LIB describes the quantitative and qualitative determination of pentobarbital in tallow, or fats of animal origin. Pentobarbital was determined by shakeout extraction with acetonitrile, dilution, followed by analysis via LC-MS/MS. Pentobarbital was separated using an Agilent Eclipse Plus-C18 liquid chromatographic column and detected 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. The method was validated for pentobarbital in tallow per FDA OFVM Level Two validation criteria. Recoveries were calculated using "standard in solvent" calibration curves with the addition of Pentobarbital-D_5internal standard. The method was validated at 12.5, 50.0, and 250 ng/g levels, and the calibration range tested was 10.0 to 1000 ng/g. The average accuracy for pentobarbital spiked beef tallow samples ranged from 98.6 to 110% over the range of 10.0 - 1000 ng/g at the 1VL (50 ng/g) target quantitative validation level. The recoveries and RSDs with and without the ISTD correction meet the requirements specified in the FDA OFVM. However, it is still recommended that the internal standard be used to correct the analytical response for fat samples of animal origin to correct for possible significant matrix effects. The method detection limit (MDL) was determined to be 2.4 ng/g and the limit of quantitation (LOQ) was 8.2 ng/g. To date, pentobarbital has been determined in two separate subs of animal fat (tallow) with concentrations 603 ng/g and 710 ng/g.

INTRODUCTION

Pentobarbital is a barbiturate drug most commonly used in animals as a sedative, anesthetic, or for euthanasia (Figure 1). Pets that eat pet food containing pentobarbital can experience drowsiness, dizziness, excitement, loss of balance, nausea, nystagmus (eyes moving back and forth in a jerky manner) and inability to stand; consuming high levels of pentobarbital can cause coma and death[1]. Tallow is promoted as an inexpensive, palatable, and stable source of energy, with a nutritional profile "sufficient for safe use as a pet food ingredient"[2]. Hence manufacturers of pet food have been using tallow in their products. When tallow is produced in meat rendering operations, pentobarbital may be introduced to the tallow product when euthanatized animal carcasses are included in the source material, as pentobarbital is not destroyed by the rendering process[3].

The US Food and Drug Administration (US FDA) has determined that any detectable level of pentobarbital in pet food is a violation of the Federal Food, Drug, and Cosmetic Act, thus, pentobarbital is considered an adulterant if it is detected in pet food[1]. Methods developed for pentobarbital residues by the FDA over 1998-2002 were based on gas and liquid chromatography (GC, LC) with mass spectrometry (MS) detection[4-6]. Surveillance studies conducted by the Center for Veterinary Medicine (CVM) during these years yielded evidence of residues in some dry dog foods with concentrations ranging from approximately 2-32 ng/g[7]. More recently, canned dog foods were found to be contaminated with significantly higher concentrations of pentobarbital[8], 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[2].

Figure 1: Pentobarbital Structure



For continuous monitoring of pentobarbital in tallow and dog food products, methodology was required that would yield accurate quantitative and confirmatory results with simple extraction procedures that could be applied to a variety of different product types and be suitable for routine regulatory analysis. Previously published FDA methods were based on lengthy sample extraction and cleanup procedures and older instrument technologies that are no longer routinely used in our laboratory[4-6]. More recent methods have been developed using a QuEChERS extraction with GC-MS analysis[9], an ethyl acetate extraction with dispersive solid phase EMR Lipid clean-up[10] and high-resolution (HR) LC-MS analysis[11], and a simple acetonitrile extraction with analysis by LC-MS/MS[12]. This LIB describes a modification of the latter method for the quantitative and qualitative determination of pentobarbital in tallow. Validation data was initially collected from

fortified tallow samples with pentobarbital concentrations of 12.5, 50, and 250 ng/g to cover the lower concentrations observed in the 1998-2000 dog food surveys[7] and higher concentration contamination determined in recent events[8]. Additional validation data was collected for tallow samples fortified at 5 and 10 ng/g to establish the method detection limit, and support a regulatory target testing level of 10 ng/g.

METHODS AND MATERIALS

Equipment

a) LC-MS instrument. – 5500 QTRAP hybrid quadrupole linear ion trap mass spectrometer (Sciex, Foster City, CA, USA) 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, USA) 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).
b) Agilent Zorbax Eclipse Plus-C18 2.1 x 50 mm, 1.8 µm column, (959757-902, Agilent Technologies, Santa Clara, CA, USA).
c) Eppendorf pipettes – Variable (5 µL to 1000 µL) volume (Brinkman Instruments, Inc., Westbury, NY), or equivalent.
d) Centrifuge – refrigerated to 4 ºC, capable of accelerating 50 mL tubes to 6000 rpm (4032 *x g*) 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 – 15 mL and 50 mL disposable, conical, graduated polypropylene tubes with cap (Falcon® Blue Max™, P/N:50 mL tubes 352070, 15 mL tubes 352097, Becton Dickinson, Franklin Lakes, NJ).
i) Syringe filters – PALL Life Science Acrodisc 13 mm Syringe Filters 0.2 µm nylon syringe filters (P/N 4427T, Pall Life Sciences) with 1-mL disposable syringe (P/N 309602, Becton Dickinson, Franklin Lakes, NJ).
j) Microcentrifuge tubes – 1.7 mL snap cap tubes, polypropylene, non-sterile (P/N: CLS3622-500EA) Sigma Aldrich, St. Louis, IL, USA)
k) LC vials –2 mL glass amber autosampler vials LC vials with pre-slit snap caps (Thermo Scientific P/N: C4011-6W and C4011-55)
l) Appropriate mixers, blenders, food processors, *etc.* used to homogenize sample matrix if necessary

REAGENTS AND SOLUTIONS

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

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, Cerilliant, 1.000 ± 0.005 mg/mL in methanol, 1 mL/ampoule, p/n P-010
2. Pentobarbital-D_5, Cerilliant, 1.000 ± 0.005 mg/mL in methanol, 1 mL/ampoule, p/n P-013

e) Negative control – All tallow controls were acquired from local markets when available, online retailers, or previous samples tested to determine that pentobarbital was not present above the stated method detection level (MDL).

Preparation of Standards

Pentobarbital and Pentobarbital-D_5 are ordered premade at 1000 µg/mL (1 mg/mL).

a) Prepare pentobarbital working solution at 2,500 ng/mL by combining 25 µL of the 1000 µg/mL stock solution with acetonitrile for a total volume of 10.0 mL.
b) Prepare an ICV working solution of pentobarbital at 2,500 ng/mL by combining 25 µL of the 1000 µg/mL stock solution with acetonitrile for a total volume of 10.0 mL.
c) Prepare an ISTD spiking solution of pentobarbital-D_5 at 5,000 ng/mL by combining 50 µL of the 1000 µg/mL deuterated stock solution with acetonitrile for a total volume of 10.0 mL

Note: All solutions are stable for 1 year and stored at 4°C.

Tables 1 and 2 are examples of the working solution and the solvent calibrant preparation. Table 3 demonstrates the equivalent concentrations of the calibrants to the concentration in the samples for use in the processing method.

All eight calibration standards shown in Table 2 are not required for regulatory sample analysis; however, a minimum of 5 calibration standards are used with every batch of samples including the Cal-1. The Cal-1 calibration standard is 1.0 ng/mL, which is equivalent to an in-tallow concentration of 10.0 ng/mL (Table 3) and is considered the lower limit of quantitation (LLOQ).

Table 1: Working Solution Preparation (in acetonitrile)

Standard Name	starting conc. µg/mL	volume Pent added (mL)	Final volume (mL)	Final conc. ng/mL
Pentobarbital-2500	1,000	0.025	10.0	2,500
Pentobarbital ICV	1,000	0.025	10.0	2,500
Pent-D_5 Spiking (ISTD)	1,000	0.050	10.0	5,000

Table 2: Example of Solvent Calibration Curve Preparation (in 50:50 acetonitrile: water)

Calibration Curve	Initial conc pentobarbital (ng/mL)	volume of pentobarbital std added (mL)	Volume (mL) D5-ISTD Added (5,000 ng/mL)	Final Volume (mL)	Final conc pentobarbital (ng/mL)	Final Conc. D_5 ISTD (ng/mL)
Cal-1	2,500	0.020	0.050	50.0	1.00	5.00
Cal-2		0.010	0.020	20.0	1.25	
Cal-3		0.010	0.010	10.0	2.50	
Cal-4		0.020		10.0	5.00	
Cal-5		0.040		10.0	10.0	
Cal-6		0.100		10.0	25.0	
Cal-7		0.200		10.0	50.0	
Cal-8		0.400		10.0	100	

Table 3: Conversion of Solvent Standards to Sample Concentration

Calibration Curve	In Vial Final Conc (ng/mL)	Tallow sample wt. (g)	Vol ACN extraction (mL)	Dilution	Equivalent In tallow concentration (ng/g)	Final Conc. D_5 ISTD (ng/g)
Cal-1	1.00	2.00	10	2.00	10.0	50.0
Cal-2	1.25				12.5	
Cal-3	2.50				25.0	
Cal-4	5.00				50.0	
Cal-5	10.0				100	
Cal-6	25.0				250	
Cal-7	50.0				500	
Cal-8	100.0				1000	
ICV	10.0				100	

Sample Preparation

A minimum of a 25 g sample portion is necessary for the analysis of tallow. If sample appears heterogeneous, stir the 25 g portion manually with a spatula or spoon to ensure homogeneity. A 2.0-gram aliquot is used for each analysis.

Spike Recovery Control Checks

Fortify spike/duplicate by adding 10 µL of working solution (2,500 ng/mL) and 20 µL of ISTD (5,000 ng/mL) is added samples to 2.00 $\pm$ 0.05 grams of negative control tissue to yield the 12.5 ng/g concentration level for all compounds.

Example spiking calculation:

40 µL	2500 ng	= 50.0 ng
2.00 grams of negative control	mL	g

In vial concentration:

50 ng	2 g sample amount	0.500 mL extraction	= 5.00 ng in vial
g	10 mL extraction volume	1.000 mL final volume	mL

Extraction Procedure

Record the sample weight to at least three significant figures and use calibrated pipettes/volumetric glassware.

a) Weigh 2.00± 0.05 g of each homogenized sample into a 50 mL centrifuge tube. For each unknown sample, weigh out two portions. For each batch, include an empty tube to serve as Reagent Blank (RB). Weigh out three portions of negative control material to serve as negative control (NC), matrix spike (SPK), and matrix spike duplicate (DUP).
b) For all samples in the batch, including RB, NC, SPK, and DUP, add 20 µL of 5,000 ng/mL pentobarbital-D_5.
c) Fortify spike (SPK) and duplicate (DUP) portions with 40 µL of 2,500 ng/mL pentobarbital spiking standard, resulting in a 50 ng/g in sample spike. (Fortification level may be adjusted as necessary, as long as the samples fall within the calibration curve).
d) Add 10 mL of acetonitrile to each tube.
e) Cap and shake on geno grinder @ 500 rpm for 5 minutes.

f) Sonicate for 30 minutes.
g) Vortex 30 seconds.
h) Centrifuge at 6000 *x g* and 4°C for 10 minutes.
i) Combine 500 µL of sample supernatant with 500 µL of water in a microcentrifuge tube; vortex to mix, then filter using a 0.2 µm Nylon syringe filter into a LC vial.
j) Analyze via LC-MS/MS.

Instrumentation

a) LC-MS/MS system – The 5500 Q TRAP (hybrid quadrupole linear ion trap (QqLIT) is a combination system in which the final quadrupole can operate as conventional mass filter or as linear ion trap with axial ion ejection. For the purpose of this method the instrument 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 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 4 shows the values of the parameters optimized and the MRM transitions used for the confirmation and quantification of pentobarbital residue.

b) HPLC system – Agilent 1260 HPLC system 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 ºC 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 5 for the LC gradient. The Agilent autosampler injection volume was 5 µL. 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 carryover.

Table 4: Pentobarbital MS parameters: Retention times (RT), transitions, declustering potential (DP), collision energy (CE), cell exit potential (CXP), and the resulting typical ion ratios for the product ions of each analyte from the ABI SCIEX 5500 QTRAP analysis.

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

Table 5: LC gradient for pentobarbital

@Step	Total 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

RESULTS AND DISCUSSION

Method Development

Development of this pentobarbital method was based on modifications to the FDA Forensic Chemistry Center's T064 method[4] (FCC) and methods developed by the California Animal Health and Food Safety Laboratory (CAHFS) at the University of California at Davis (UCD)[10,11]. The T064 method was developed for dog food and is based on a shakeout extraction with acetonitrile, followed by dilution then analysis via ultrahigh performance liquid chromatography (UPLC)-MS/MS. The UCD extraction protocol was developed for tallow with an extraction with ethyl acetate, followed by dispersive solid phase extraction (dSPE) with two-step EMR lipid removal, solvent exchange, and analysis by a UPLC residue screening procedure with high resolution (HR)MS. Three separate variables were evaluated from the different methods before the Denver Laboratory (DENL) developed a final procedure and performed method validation for this LIB.

The first variable investigated was the extraction procedure. Animal fat samples containing pentobarbital were shipped to the DENL by UCD, and these samples were used to compare extraction procedures. DENL analyzed the samples using both the UCD ethyl acetate extraction plus dSPE EMR lipid clean-up procedure and the FCC T064 acetonitrile extraction procedure. For comparison, a positive tallow sample originally found to contain 240 ng/g pentobarbital[10], yielded 185 ng/g pentobarbital by the ethyl acetate/dSPE procedure and 228 ng/g pentobarbital by the acetonitrile extraction. These results indicated that the two extraction procedures yielded comparable results for tallow, and the simpler acetonitrile extraction would be sufficient be routine analysis.

The second variable was the analytical column: The FCC T064 method uses an UPLC Peptide BEH C18 column, however the DENL LC-MS/MS system used for this analysis was a conventional high performance liquid chromatography (HPLC) system. Performance is sacrificed when a Waters UPLC column is used on a conventional HPLC system[13]. The FCC T064 method was initially tested with the UPLC column but the back pressure would approach the upper limit of the conventional HPLC pressure limits. The UCD method protocol used an Agilent C18 HPLC column with similar dimensions as the Waters UPLC column, with the exception of the particle and pore sizes. The Waters peptide column had particle size 1.7 µm and pore size 300 Å versus 1.8 µm particle size for the Agilent with 95 Å pore size. With the Agilent column, the back pressure was within the range of the conventional HPLC, therefore the Agilent C18 provided superior results for the DENL method.

The last component was the difference in the mass spectrometers. The UCD screening method is based on analysis with a Q-Exactive Orbitrap HRMS, but this technology was not available for regulatory sample analysis in DENL. The FCC T064 method was validated with a SCIEX QTRAP 5500 mass spectrometer with MultiQuant processing software, which is instrumentation routinely used for regulatory analysis in DENL.

The final method used in DENL for pentobarbital in tallow analysis combined the simplicity of the FCC acetonitrile T064 extraction, the LC column type recommended by UCD for residue screening, suitable chromatography parameters for HPLC separation, and pentobarbital analysis using a SCIEX QTRAP 5500 MS system. Three pentobarbital-containing tallow samples were tested to compare results between the DENL acetonitrile extraction HPLC-MS/MS method and the UCD ethyl acetate dSPE HRMS method. The results in Table 6 demonstrate the difference in results between the quick acetonitrile DENL/FCC extraction and the more time and reagent consuming UCD extraction.

Table 6: Incurred animal fat samples - method comparison between UC Davis and DENL

Sample #	UCD Conc. (ng/g)[10]	DENL Conc. (ng/g)	% Difference
002 (n=2)	110	92.5	15.9
004 (n=1)	56.0	38.1	31.9
005 (n=1)	16.0	12.6	21.3

Method Validation

The objective of this study was to validate a sensitive and rapid method for the determination of pentobarbital in tallow. The method was validated according to the FDA OFVM Level Two Chemical Method Validation guidelines[14]. The validation levels for tallow were 12.5 ng/g, 50 ng/g, 250 ng/g. Three replicates were tested at each validation level using fortified samples in three different negative control tallow sources. The samples were validated over a three-day period. Calibration curves were established from the eight solvent calibrant standards (1.00, 1.25, 2.50, 5.00, 10.0, 25.0, 50.0, and 100 ng/mL in vial corresponding to 10.0, 12.5, 25, 50,100, 250, 500, and 1000 ng/g in sample) with the concentration on the x-axis and internal standard corrected peak response on the y-axis. All calibration curves were generated with the SCIEX MultiQuant software, and a linear curve with 1/x weighting, not forced through zero, was used for all recovery calculations. Correlation coefficients (r^2) were typically greater than 0.998, and the average r^2 was 0.9991 over the three days of validation data collection.

The validation data was also processed without the internal standard correction. Internal standard correction is used for routine regulatory sample analysis. However, if a tallow sample demonstrates a response above the highest calibration point, the sample can be further diluted with the diluent to approximately the midpoint of the solvent curve. The data can then be processed without the internal standard correction. Care must be taken to ensure the concentration range of the solvent curve reflects the dilution of the sample. For further information on the validation data without the use of the deuterated pentobarbital internal standard refer to Appendix A.

The accuracy and precision results from the validation are summarized in Table 7 for fortified beef tallow. Representative ion chromatograms for pentobarbital in tallow are shown in Figures 2-4. Method accuracy (trueness) was determined by calculating the percent recovery of pentobarbital based on a solvent curve with the use of the deuterated pentobarbital internal standard. The results demonstrated the method accuracy was satisfactory for pentobarbital, according to FDA OFVM guidelines for chemical method validation[14]. Average recoveries for pentobarbital in tallow ranged from 98.6% to 110% for all three validation levels as shown in Table 7. The FDA OFVM guidelines 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[14].

Table 7: Tallow accuracy and precision at each fortification level, n=9 for each level

Analytes	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	12.5 ng/g		50 ng/g		250 ng/g	
Pentobarbital with ISTD	98.6%	6.68%	105.1%	3.75%	110%	2.57%

Method Detection Level

The method detection limit (MDL) was calculated from the quantitative product ion transition and reported in Table 8. The method detection limit was evaluated by analyzing nine replicates fortified at the 12.5 ng/g concentration, where MDL = t x s ("t" is the Student t value at the 99% confidence level, and "s" is the standard deviation of the tested concentration. The limit of quantitation (LOQ) was calculated taking the standard deviation of the 12.5 ng/g spike level and multiplying by a factor of 10. The measurement of uncertainty (U) was calculated by U=RSD (12.5 ppb spikes) x K. Table 8 includes: method detection level (MDL), limit of quantification (LOQ), measurement of uncertainty, and the average correlation coefficient (r^2).

To test the accuracy of this method to quantify pentobarbital in tallow samples with concentrations below 12.5 ng/g, additional validation data was collected. In this low concentration validation set, one source of tallow was fortified with pentobarbital at levels of 5 ng/g and 10 ng/g (n=7 per level). The 5 ng/g spikes averaged 97.7% recovery and 18.1% RSD, and the 10 ng/g spikes yielded 97.8 % average recovery with 9.8 % RSD. The average recovery for these low-level spikes are consistent with the 12.5-250 ng/g validation data. The 18.1 % RSD (and related 29.0 % measurement uncertainty) for the 5 ng/g spikes represents higher variability than what was observed for the 12.5-250 ng/g validation set, but this data is still within the expected performance guidelines[14]. The MDL and LOQ calculated from the 5 ng/g fortification level are 2.8 and 8.8 ng/g, respectively, consistent with the values reported in Table 8, indicating that the method is suitable for quantitative analysis at a regulatory testing level of 10 ng/g.

Table 8: Tallow LOD (MDL) and LOQ Results (n=9)

Analyte	Calculated MDL (ng/g)	Calculated LOQ (ng/g)	Measurement of Uncertainty (%)	r^2 (n=3) for tallow
Pentobarbital with ISTD	2.4	8.2	15.4	0.9991

Quantitative Analysis

For routine regulatory sample analysis, quantitative results are reported for samples with responses that fall within the standard curve range and meet identity confirmation of identity criteria. If a larger dilution is required due to pentobarbital level in a sample above the highest calibration standard, the internal standard may be omitted from use of calculation of concentration of analyte in the sample, refer to Appendix A. The original sample extract may be diluted with 50:50 acetonitrile:water.

At the date of publication, this validated pentobarbital analysis method was used by the DENL to determine pentobarbital in two separate subsamples of tallow with concentrations of 603 ng/g and 710 ng/g. An ion chromatogram for the 603 ng/g finding is shown in Figure 5.

Qualitative Identification/Confirmation of Identity

For qualitative identification, CVM 118[15] guidance was used. Presence of pentobarbital was determined to be 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, if required.
- Two ion ratios are ≤ I20%I or one ion ratio is ≤ 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 the tallow validation, 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.

CONCLUSION

This LIB describes a sensitive and selective method validation for the detection, quantification, and confirmation of pentobarbital in tallow and fats of animal origin. The final method was a simple acetonitrile shakeout with dilution then analysis by negative mode HPLC-MS/MS. The method was modified from methods developed at the FDA Forensic Chemistry Center and the University of California at Davis California Animal Health and Food Safety Laboratory. The recoveries and RSDs with and without the ISTD correction meet the requirements specified in the FDA OFVM. However, it is still recommended that the internal standard be used to correct the analytical response for fat samples of animal origin to correct for possible significant matrix effects.

ACKNOWLEDGEMENTS

The authors acknowledge Robyn Noonan and Michael Filligenzi from the University of California at Davis California Animal Health and Food Safety Laboratory and Angie Mohrhaus, James Turner, and Doug Heitkemper from the FDA Forensic Chemistry Center for sharing methodology, samples, and analytical results.

REFERENCES

1. FDA Alerts Pet Owners About Potential Pentobarbital Contamination in Canned Dog Food Manufactured by The J.M. Smucker Company, Including Certain Gravy Train, Kibbles 'N Bits, Ol' Roy, and Skippy Products, February 2018.
https://www.fda.gov/animalveterinary/newsevents/ucm597135.htm

2. Pentobarbital continues to 'dog' pet food industry; beef tallow blamed, November 29, 2018
https://www.foodsafetynews.com/2018/11/pentobarbital-continues-to-dog-pet-food-industry-tainted-beef-tallow-prompts-recall/

3. O'Connor, J. J., Stowe, C. M., & Robinson, R. R. Fate of sodium pentobarbital in rendered products. *Am. J. Vet. Res*., 1985, *46*(8), 1721-1724.

4. Adam, L. A.; Reeves, V. B. Procedure for detecting and confirming pentobarbital residues in dog food by gas chromatography/mass spectrometry. *J. AOAC Int.* 1998, *81*, 359−367.

5. Heller, D. N.; Lewis, K. M.; Cui, W. Method for determination of pentobarbital in dry dog food by gas chromatography/mass spectrometry. *J. Agric. Food Chem*. 2001, *49*, 4597−4602.

6. Heller, D. N. Liquid chromatography/mass spectrometry for timely response in regulatory analyses: identification of pentobarbital in dog food. *Anal. Chem*. 2000, *72*, 2711−2716.

7. Food and Drug Administration/Center for Veterinary Medicine Report on the Risk from Pentobarbital in Dog Food , *March 1, 2002.* https://www.fda.gov/AboutFDA/CentersOffices/OfficeofFoods/CVM/CVMFOIAElectronicReadingRoom/ucm129131.htm

8. Entis, P. Year-long investigation of euthanasia drug in pet food over. *Food Safety News*, April 30, 2018 https://www.foodsafetynews.com/2018/11/pentobarbital-continues-to-dog-pet-food-industry-tainted-beef-tallow-prompts-recall/

9. Buchweitz, J. P., Johnson, M., Jones, J. L., & Lehner, A. F. Development of a Quantitative Gas Chromatography–Tandem Mass Spectrometry Method for the Determination of Pentobarbital in Dog Food. *J. Agric. Food Chem.*, 2018, *66*(42), 11166-11169.

10. Pentobarbital in Beef Tallow, Personal Communication with California Animal Health and Food Safety Laboratory (CAHFS), University of California at Davis, 2018.

11. LC-HRMS Screen CALIFORNIA ANIMAL HEALTH & FOOD SAFETY LABORATORY, DTOX-02-877 Rev. 6. November 3, 2017.

12. Turner, J.A., Mohrhaus, A.S. Determination of Pentobarbital in Pet Food Using Liquid Chromatography Tandem Mass Spectrometry, FCC SOP T064 Version 1, 3/1/2018.

13.Waters BEH Column information. http://www.waters.com/webassets/cms/support/docs/WA40001.pdf

14. U.S. Food and Drug Administration, Office of Foods and Veterinary Medicine, Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 2nd Edition 2015. https://www.fda.gov/downloads/scienceresearch/fieldscience/ucm273418.pdf

15. U.S. Food and Drug Administration (2003) Guideline for Industry: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues, *Fed. Regist*. 2003, *68*, 25617–25618. https://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm052658.pdf

Figures 2-5: The result summary states the analyte peak precursor name, retention time, calculated concentration, analyte response, calculated ion ratio, and ratio confirmation. The three chromatograms below are the graphical representation of the transition of the precursor to product ions for the three different transitions monitored for pentobarbital.

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (**225->182.0**)	3.90	4.20	< 0	3190.0		
Pentobarbital 2 (**225->85.0**)	4.22	4.20		1920	60.1% (15.8%)	
Pentobarbital 3 (**225->138.0**)	**4.06**	**4.20**		395	**12.4% (7.2%)**	✓

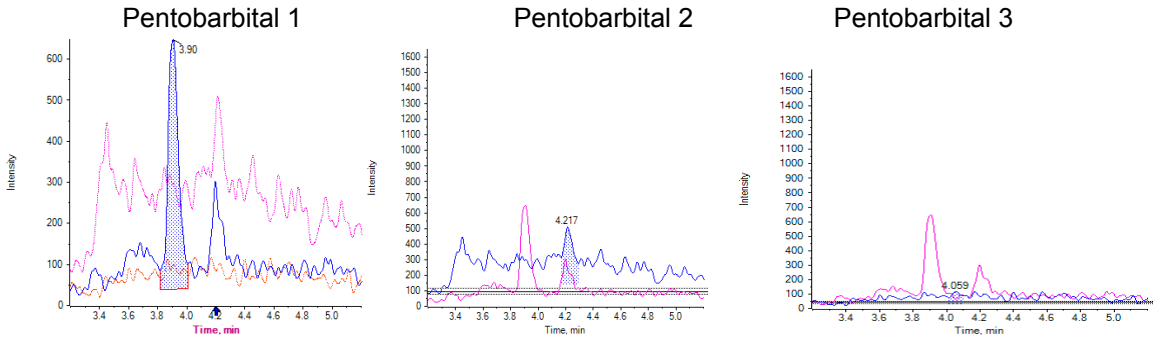


Figure 2: Tallow Negative Control, Source T1.

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.21	4.20	52.50	170000.0		
Pentobarbital 2 (225->85.0)	4.21	4.20		26600	15.6% (15.8%)	✓
Pentobarbital 3 (225->138.0)	4.21	4.20		11500	6.8% (7.2%)	✓

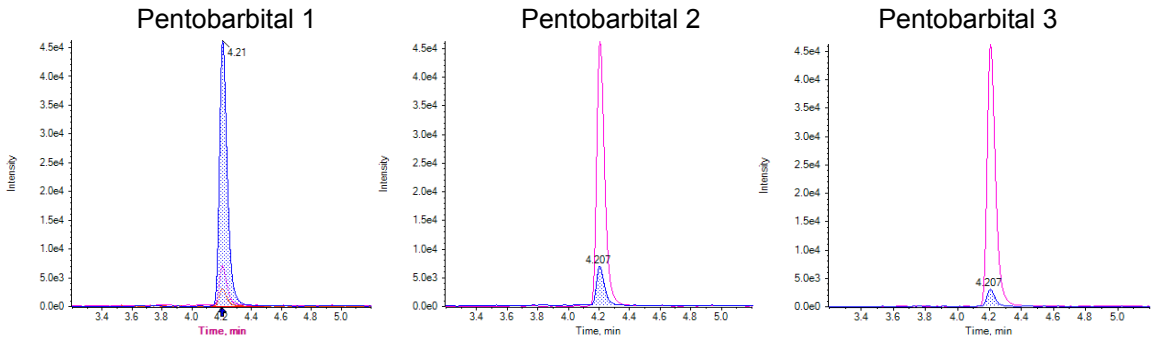


Figure 3: Solvent Cal 3- for Tallow at 50 ng/g in sample equivalent (In vial concentration: 5.0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Result Summary

Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.21	4.20	51.65	129000.0		
Pentobarbital 2 (225->85.0)	4.21	4.20		20500	15.8% (15.8%)	✓
Pentobarbital 3 (225->138.0)	4.21	4.20		8930	6.9% (7.2%)	✓

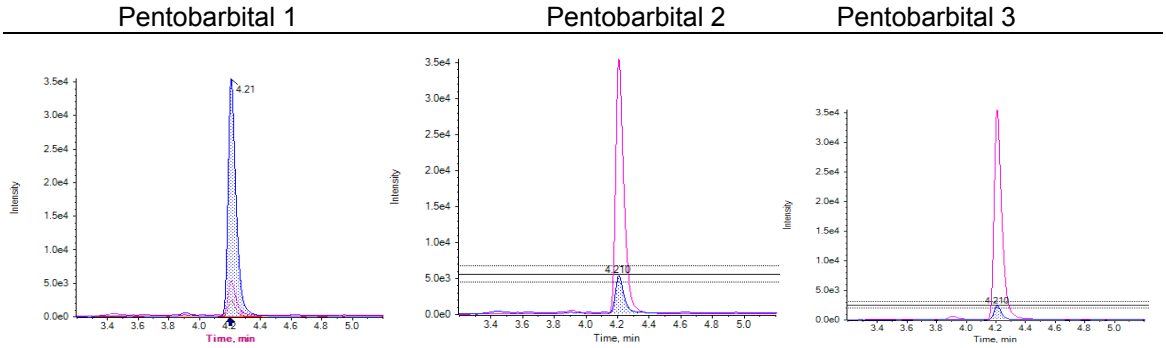


Figure 4: Tallow - Fortified at 50 ng/g (In vial concentration: 5.0 ng/mL pentobarbital, 5.0 ng/mL D_5-pent)

Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (225->182.0)	4.20	4.20	602.54	511159.0		
Pentobarbital 2 (225->85.0)	4.20	4.20		76851	15.0% (15.9%)	✓
Pentobarbital 3 (225->138.0)	4.20	4.20		40445	7.9% (5.3%)	✓

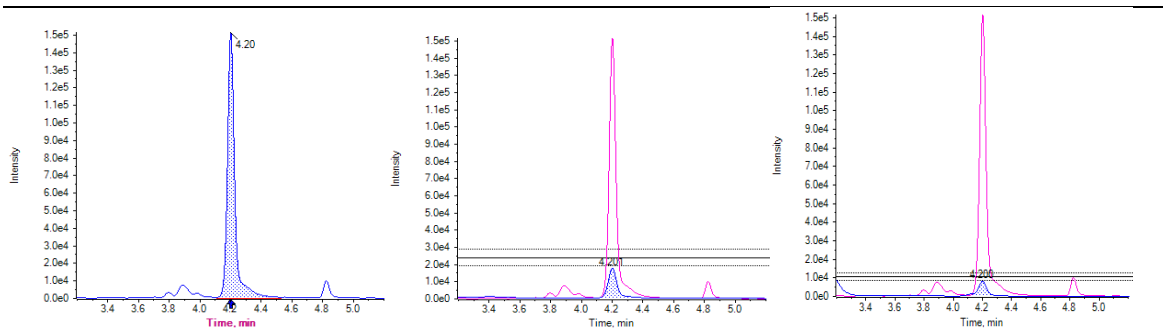


Figure 5: Regulatory animal fat sample with 603 ng/g of pentobarbital determined

APPENDIX A

Quantitation of pentobarbital in tallow without an isotopically labeled internal standard

Pentobarbital contamination may be found in animal feed or feed ingredients with concentration above 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 analyte 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 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: Comparison of use of deuterated internal standard correction pentobarbital in tallow accuracy and precision at each fortification level, n=9 for each level

Analytes	(Recovery, %)	%RSD	(Recovery, %)	%RSD	(Recovery, %)	%RSD
	12.5 ng/g		50 ng/g		250 ng/g	
Tallow						
Pentobarbital with ISTD	98.6%	6.68%	105.1%	3.75%	110%	2.57%
Pentobarbital no ISTD	90.8%	9.03%	85.6%	3.99%	88.7%	4.57%

Table A2: Comparison of use of deuterated internal standard correction for pentobarbital in tallow LOD (MDL) and LOQ Results (n=9)

Analyte	Calculated MDL (ng/g)	Calculated LOQ (ng/g)	Measurement of Uncertainty (%)	r^2 (n=3) for tallow
Pentobarbital with ISTD	2.4	8.2	15.4	0.9991
Pentobarbital no ISTD	3.0	10.2	20.8	0.9988

The FDA OFVM 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[14]. The recoveries and RSDs with and without the ISTD correction meet the requirements specified in the FDA OFVM. Thus, if a sample demonstrates a pentobarbital response above the highest calibration point, the sample could be further diluted with the diluent to approximately the midpoint of the solvent curve, and the concentration calculated without internal standard correction. This enables the DENL to reduce the need for re-extraction of a high-concentration positive sample and increase the throughput of the analysis. However, it is still recommended that the internal standard be used to correct the analytical response for fat samples of animal origin to correct for possible significant matrix effects.