Compendium of Analytical Laboratory Methods for Food and Feed Safety: Chemical Analytical Manual (CAM)

METHOD NUMBER: C-005.01

POSTING DATE: 2/1/2019

POSTING EXPIRATION DATE: **1/31/2021**

PROGRAM AREA: Chemical Contaminants and Toxins

METHOD TITLE: Determination of Pentobaribital in Tallow Using Liquid Chromatography Tandem Mass Spectrometry

VALIDATION STATUS: Level 2, Single laboratory validation

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METHOD SUMMARY/SCOPE:
Identification and quantitative determination of pentobarbital in tallow and other grease products of animal origin using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), using the negative electrospray ionization (ESI) mode. Pentobarbital is extracted from a homogenized tallow sample aliquot using acetonitrile. After centrifugation, supernatant is diluted 1:1 with water and analyzed via LC-MS/MS using a solvent standard curve. Deuterated pentobarbital (pentobarbital-D5) is used as an internal standard (I.S.) to correct for sample matrix suppression and/or loss of analyte. Identification of pentobarbital in a sample is based on both correlation of pentobarbital chromatographic retention time (RT) with that of a standard, and ion ratio match.

Analytes(s): Pentobarbital

Matrices: tallow and other grease products of animal origin

REVISION HISTORY:

1. Purpose & Scope

The objective of this work instruction is to provide direction on the routine quantitative determination of pentobarbital in tallow using LC-MS/MS based on methodology from the Forensic Chemistry Center (5.1). Complete method performance details and validation data is summarized in DEN-LB-V.18-4 (5.7).

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2. Procedure

2.1 Summary of Method

Pentobarbital is extracted from a homogenized tallow sample aliquot using acetonitrile. After centrifugation, supernatant is diluted 1:1 with water and analyzed via LC-MS/MS using a solvent standard curve. Deuterated pentobarbital (pentobarbital-D5) is used as an internal standard (I.S.) to correct for sample matrix suppression and/or loss of analyte. Identification of pentobarbital in a sample is based on both correlation of pentobarbital chromatographic retention time (RT) with that of a standard, and ion ratio match.

2.2 Sample Preparation

2.2.1 For tallow, at minimum a 25 g sample portion is necessary. If sample appears heterogeneous, stir the 25 g manually with a spatula or spoon to ensure homogeneity. A 2 g aliquot is used for each analysis.

2.3 Preparation of Standards

2.3.1 Pentobarbital and pentobarbital-D5 are ordered premade (Cerriliant) with concentration 1000 µg/mL (1 mg/mL) in methanol.

2.3.2 Prepare a pentobarbital standard at 2,500 ng/mL with 25 µL of the 1000 µg/mL stock diluted to 10.0 mL with acetonitrile.

2.3.3 Prepare a pentobarbital ICV standard at 2,500 ng/mL with 25 µL of the 1000 µg/mL stock, from a different source or different ampoule, diluted to10.0 mL with acetonitrile.

2.3.4 Prepare a pentobarbital-D5 internal standard spiking solution at 5,000 ng/mL with 50 µL of the 1000 µg/mL pentobarbital-D5 stock diluted to 10.0 mL with acetonitrile.

2.3.5 All prepared solutions have an expiration date of one year when stored at 4°C.

2.3.6 Table 2.3.8 and 2.3.9 are examples of the preparations of working solutions and the solvent calibrants. Table 2.3.10 demonstrates the equivalent concentrations of the calibrants to the concentration in the samples for use in the processing method.

2.3.7 All eight calibration standards shown in Table 2.3.10 are not required for regulatory sample analysis; however, a minimum of 5 calibration standards are used with every batch of samples with a 10.0 ng/g standard (in sample amount), which is equivalent to an in vial concentration of 1.0 ng/mL as the lower limit of quantitation (LLOQ).

2.3.8 Working Solution Preparation (in acetonitrile)

Standard Name	starting conc (µg/mL)	Standard added (mL)	Final vol (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-D5 Spiking (ISTD)	1,000	0.050	10.0	5,000

2.3.9 Solvent Calibration Standard Preparation (in 50:50 acetonitrile:water)

Calibration Curve	Initial conc pentobarbital (ng/mL)	volume of pentobarbital std added (mL)	Volume D5-ISTD Added (5000 ng/mL)	Final Volume (mL)	Final Conc pentobarbital (ng/mL)	Final Conc d5-Pent (ng/mL)
Cal-1	2,500	0.020	0.050	50.0	1.00	5.00
Cal-2	2,500	0.010	0.020	20.0	1.25	5.00
Cal-3	2,500	0.010	0.010	10.0	2.50	5.00
Cal-4	2,500	0.020	0.010	10.0	5.00	5.00
Cal-5	2,500	0.040	0.010	10.0	10.00	5.00
Cal-6	2,500	0.100	0.010	10.0	25.00	5.00
Cal-7	2,500	0.200	0.010	10.0	50.00	5.00
Cal-8	2,500	0.400	0.010	10.0	100.00	5.00
ICV	2,500	0.040	0.010	10.0	10.00	5.00

2.3.10 Conversion of Solvent Standards to Sample Concentration

Cal Curve/Unique ID	In Vial Final Conc (ng/mL)	Tallow sample wt. (g)	Vol ACN extraction (mL)	Dilution	Equivalent pentobarbital In tallow concentration (ng/g)	Equivalent d5-pent (ISTD) In tallow concentration (ng/g)
Cal-1	1.00	2.00	10	2.00	10.0	50.0
Cal-2	1.25	2.00	10	2.00	12.5	50.0
Cal-3	2.50	2.00	10	2.00	25.0	50.0
Cal-4	5.00	2.00	10	2.00	50.0	50.0
Cal-5	10.0	2.00	10	2.00	100	50.0
Cal-6	25.0	2.00	10	2.00	250	50.0
Cal-7	50.0	2.00	10	2.00	500	50.0
Cal-8	100.0	2.00	10	2.00	1000	50.0
ICV	10.0	2.00	10	2.00	100	50.0

2.3.11 Example calculation of in vial versus in sample concentration for tallow:

=10.0 ng/mL x10 mL/2.00 grams x 2 = 100 ng/g

=conc. Std (ng/mL) x sample Vol mLs/sample weight grams x dilution factor

2.3.12 Spiking Solution Preparation (demonstrates typical fortification levels for pentobarbital in tallow)

initial conc of pentobarbital (ng/mL)	Volume of standard used (mL)	sample weight (g)	final conc of pentobarbital in tallow (ng/g)
2500	0.010	2.00	12.5
2500	0.040	2.00	50.0
2500	0.200	2.00	250

2.4 Reagents

2.4.1 Water, Fisher, LC-MS grade

2.4.2 Acetonitrile, Fisher, LC-MS Grade

2.4.3 Pentobarbital, Cerilliant, 1.000 ± 0.005 mg/mL in methanol, 1 mL/ampoule, p/n P-010

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

2.4.5 Diluent for Standards: 50/50 water/acetonitrile (v/v)

2.5 Equipment (equivalent equipment may be substituted)

2.5.1 Vortexer/ Mixer, Troemner, (500-2500 rpm)

2.5.2 SPEX Geno/Grinder 2000

2.5.3 Sonicator (Branson 2510 or 8510)

2.5.4 Appropriate mixers, blenders, food processors, etc. used to homogenize sample matrix if necessary

2.5.5 Centrifuge capable of 6000 rpm with refrigeration (4 °C) for 50 mL tubes

2.5.6 Plastic centrifuge tubes with caps, 15mL and 50mL

2.5.7 Microcentrifuge tubes, at least 1 mL capacity

2.5.8 Nylon syringe filters, PALL Life Science Acrodisc 13 mm Syringe Filters 0.2 μm

2.5.9 Luer slip 1 mL syringes

2.5.10 2 mL glass amber autosampler vials and pre-slit snap caps (#66030-608)

2.5.11 Calibrated pipettes and volumetric glassware

2.6 Instrumentation (equivalent instrumentation may be substituted)

2.6.1 Agilent 1200 HPLC system, Combi Pal autosampler, with an AB SCIEX 5500 QTRAP MS instrument operated in the negative mode with an ESI source using Multiple Reaction Monitoring (MRM). AB SCIEX 1.6.2 software was used for instrument control and MultiQuant 3.0 was used for data processing.

2.6.2 AB SCIEX QTRAP 5500 settings:

2.6.2.1.1 Curtain gas: 30 psi

2.6.2.1.2 GS1: 50 psi

2.6.2.1.3 GS2: 60 psi

2.6.2.1.4 Collision gas: medium

2.6.2.1.5 Ion spray voltage (IS): -3500V

2.6.2.1.6 Source temperature: 400˚C

2.6.2.1.7 Entrance Potential (EP): -10V

2.6.2.2 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	Typical 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-D5	-100	-19 -18 -21	-13 -9 -10	100 15 7
pentobarbital-D5	4.20	230	→	187		-100	-17	-10	N/A

2.6.2.3 Combi Pal autosampler settings

2.6.2.3.1 sample injection volume: 5 µL

2.6.2.3.2 autosampler tray temperature: 15˚C

2.6.2.3.3 Combi Pal Injector wash solution 1: 95% water/5% acetonitrile

2.6.2.3.4 Combi Pal Injector wash solution 2: 95% acetonitrile/5% water

2.6.2.4 Agilent 1200 HPLC settings

2.6.2.4.1 A divert valve directed column effluent to waste before (0-0.5 minutes), and during the system re-equilibration time (4.5-8.50)

2.6.2.4.2 Column temperature was 40°C

2.6.2.4.3 LC flow rate was 0.350 mL/min.

2.6.2.4.4 The mobile phase was water (A) and acetonitrile (B), and the LC gradient is described in Table 2.6.1.5 below

2.6.2.4.5 HPLC column – Agilent Zorbax Eclipse Plus C18, 2.1 x 50 mm, 1.8 micron size column (part # 959757-902)

2.6.2.4.6 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

2.7 Extraction

2.7.1 Weigh 2.00± 0.05 g of each homogenized sample. 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).

2.7.2 For all samples in the batch, including RB, NC, SPK, and DUP, add 20 µL of 5000 ng/mL pentobarbital-D5.

2.7.3 Fortify spike (SPK) and duplicate (DUP) portions with 40 µL of 2500 ng/mL pentobarbital spiking standard, resulting in a 50 ng/g in sample spike. (Fortification level may be adjusted as necessary, as long as they samples fall within the calibration curve).

2.7.4 Add 10 mL of acetonitrile to each tube.

2.7.5 Cap and shake on geno grinder @ 500 rpm for 5 minutes.

2.7.6 Sonicate for 30 minutes.

2.7.7 Vortex 30 seconds.

2.7.8 Centrifuge at 6000 x g and 4°C for 10 minutes.

2.7.9 Combine 500µL of sample supernatant with 500µL of water in a microcentrifuge tube; vortex to mix, then filter using a 0.2 µm Nylon syringe filter into a LC vial.

2.7.10 Analyze via LC-MS/MS.

2.8 Data Analysis and Quality Acceptance

2.8.1 Calibration curves are established from a multi-point solvent calibrant standard curve, ranging from 1-100 ng/mL in vial, equivalent 10-1000 ng/g in sample, with the concentration on the x-axis and internal standard corrected peak response on the y-axis. Suggested eight point curve for routine tallow analysis is 1.00, 1.25, 2.5, 5.0, 10.0, 25.0, 50.0, 100 ng/mL (equivalent to 10.0, 12.5, 25, 50, 100, 250, 500, 1000 ng/g in sample).

2.8.2 Quantitative results will be reported for samples with responses that fall within the standard curve range and meet identity confirmation criteria.

2.8.3 If 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. The original sample extract may be diluted with 50:50 acetonitrile:water.

2.8.4 The calculated method limit of detection (MDL) and calculated method limit of quantitation (LOQ), measurement of uncertainty, and typical linearity (r^2) are indicated in Table 2.8.4 below. Data was analyzed with and without the pentobarbital-D5 internal standard correction (ISTD).

2.8.5 MDL, LOQ, Uncertainty, Linearity

	Calculated MDL (ng/g)	Calculated LOQ (ng/g)	Lower Limit of Quantitation (lowest calibrator (ng/g)	Measurement of Uncertainty (%)	r^2 (n=3) for tallow (n=1 for dry/wet food)
Pentobarbital with ISTD	2.4	8.2	10.0	15.4	0.9991
Pentobarbital no ISTD	3.0	10.2	10.0	20.8	0.9988

2.8.6 All calibration curves were generated with the AB SCIEX MultiQuant software. A linear fit with 1/x weighting (not forced through zero) was used for all recovery calculations. If a smaller dynamic range is used, a linear curve with no weighting may be used. All calibration curves should have $r^2 \geq 0.995$.

2.8.7 Precision and accuracy general guidance (may vary with sample matrix, especially for different tallow/animal fat matrices):

2.8.7.1 Validation data demonstrated satisfactory quantitative analysis for pentobarbital determined in tallow at spiking levels 12.5, 50, and 250 ng/g, with method accuracy generally ranging from 86-115%, and $RSD_r \leq 7\%$.

2.8.7.2 The FDA OFVM specifies that analyte recovery should be within the range 60%-115% corresponding to concentration from 10-100 mg/kg, $RSD_r \leq 22\%$.

2.8.7.3 The FCC T064 SOP specifies a recovery range from 80%-120%, which gives more leeway on the higher end of the recovery range.

2.8.7.4 The aforementioned ranges are for guidance on data acceptability. Ranges for precision and accuracy should be determined in house after a sufficient number of data points are obtained.

2.8.7.5 RB and NC should have responses below the lowest calibration standard.

2.8.7.6 Any QC failures must be investigated prior to reporting results.

2.8.8 Positive confirmation of identity for positive samples, and spiked samples

2.8.8.1 Signal to noise must be >3:1 (The AB SCIEX MultiQuant software is used to calculate signal to noise, if required.)

2.8.8.2 Retention time must match the comparison standard(s) within 5%.

2.8.8.3 Ion ratios must match the comparison standard(s) by an absolute value of 20%. (MultiQuant software uses the average ion ratio of all standards in the calibration curve).

2.8.9 Sample Result Reporting

2.8.9.1 Numerical sample results greater than the lower limit of quantitation (LLOQ) of 10 ng/g shall be reported.

2.8.9.2 Samples with calculated amounts less than the lower limit of quantitation (LLOQ) of 10 ng/g, but greater than the MDL shall be reported as "pentobarbital detected at <10 ng/g, but greater than MDL", along with the value for the MDL.

2.8.9.3 Samples with calculated amounts < MDL shall be reported as "pentobarbital not detected at or above MDL".

3. Glossary/Definitions

A. RB: Reagent Blank. Used to verify reagents are uncontaminated by interfering components, the reagent blank is an extract that contains no sample matrix. Carried thorough the extraction as if it were a sample, one must be extracted with each batch and display no interference peaks at the reference times of interest at or above lowest calibration.

B. NC: Negative Control. Used to verify the lack of matrix effects, the control is an aliquot of matrix material known to contain no analytes of interest. One must be extracted with each batch, and must display no interference peaks at reference times of interest at or above lowest calibration.

C. SPK/DUP: Matrix spike/matrix spike duplicate. Used to demonstrate effective and reproducible extraction, the matrix spike and duplicate are two aliquots of negative control matrix material, each fortified at a level near the midpoint of the curve. A pair of matrix spikes must be extracted and analyzed with each batch.

D. ICV: Independent Calibration Verification. Used to assure the accuracy of the calibration curve, the ICV is a solvent standard prepared from a secondary standard source.

E. CCV: Continuing Calibration Verification. Used to check the calibration during a run, the CCV is a re-injection of a midpoint solvent standard curve. A CCV is injected after every ten extracts and at the end of the analytical sequence.

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4. References & Supporting Documents

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

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

C. Pentobarbital in Beef Tallow March 2018, Personnel Correspondence with UC Davis, "Pentobarbital in Beef Tallow March 2018 (004) UC Davis extraction march 2018" location: H:\ALL_LAB\1_Chemistry\1. Chemistry Unit A\Pentobarbital in dog food 2018\Tallow\References

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

E. ORA Laboratory Manual, Volume II-Methods, Method Verification and Validation ORA-LAB.5.4.5 https://www.fda.gov/downloads/ScienceResearch/FieldScience/LaboratoryManual/UCM092147.pdf

F.DENL QMS # 18-3 and QmiS RPRT-000060 (Determination of pentobarbital in Tallow, wet pet food, and dry pet food using liquid chromatography tandem mass spectrometry).

G. DENL QMS #18-4 and QmiS RPRT-000055 (determination of pentobarbital in tallow, wet pet food, and dry pet food using liquid chromatography tandem mass spectrometry).

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

I. CVM # GFI 118. Guidance for Industry Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052658.pdf

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5. Document History

Version #	Status* (D, I, R, C)	Date	Author Name and Title	Approving Official Name and Title
00	I	11/30/2018	Tara Nickel, Chemist Christine Casey, Chemist	R. Stadtmuller, Quality System Manager
01	R	12/4/18	Tara Nickel, Chemist	R. Stadtmuller, Quality System Manager
* - D: Draft, I: Initial, R: Revision, C: Cancel				

6. Change History

Version	Change
00	*Original*
01	Change 10 uL to 20 uL on 2.7.2. Add reporting statement on 2.8.9.3 for amounts below 10ng/g and >MDL.

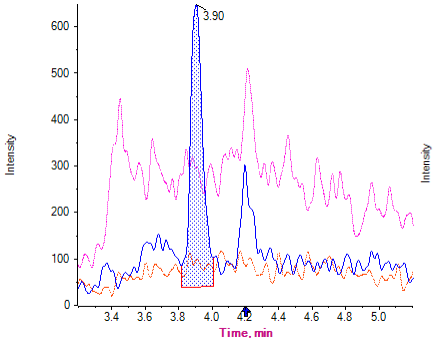
Appendix A: Representative chromatograms (A1: Tallow control, A2: mid-level solvent calibrant, A3: spiked tallow sample)

Result Summary for Figure A1: Tallow Negative Control, Source T1

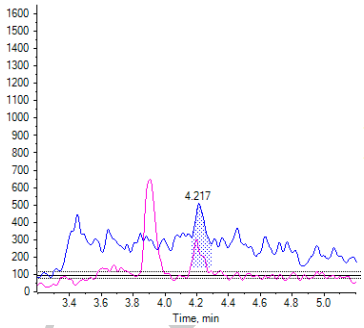
Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Pentobarbital 1 (**225->182.0**)	3.90	4.20	< 0	3190.0		
Pentobarbital 2 (**225->85.0**)	4.22	4.20		1920	60.1% (15.8%)	
Pentobarbital 3 (**225->138.0**)	**4.06**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

Chromatograms – Bars on peaks are expected ion ratio ± 20% of comparison standard(s)

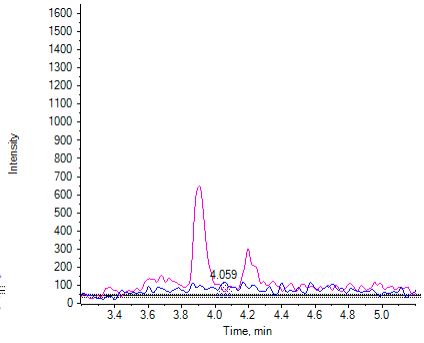
Pentobarbital 1



Pentobarbital 2



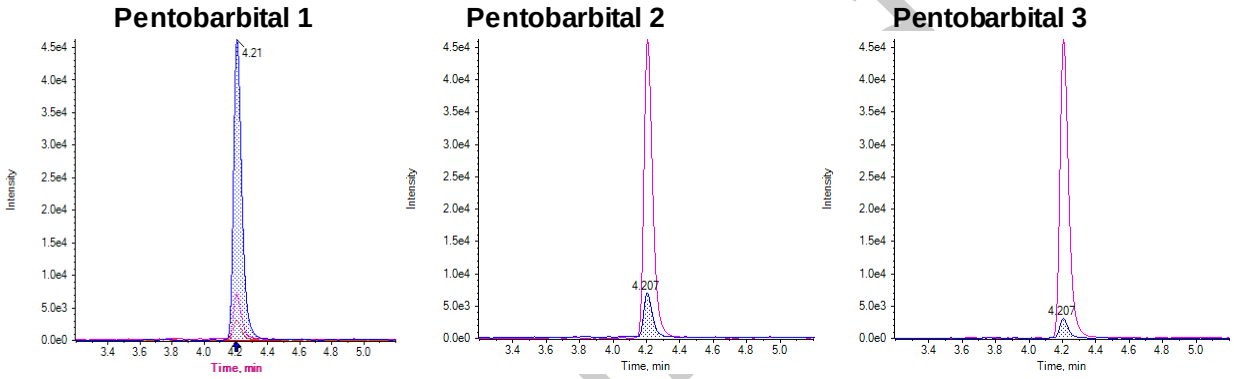
Pentobarbital 3



Result Summary for Figure A2: Solvent Cal 3- for Tallow 50 ng/g in sample equivalent (In vial concentration: 5.0 ng/mL pentobarbital, 5.0 ng/mL d5-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**)	4.21	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%)**	✓

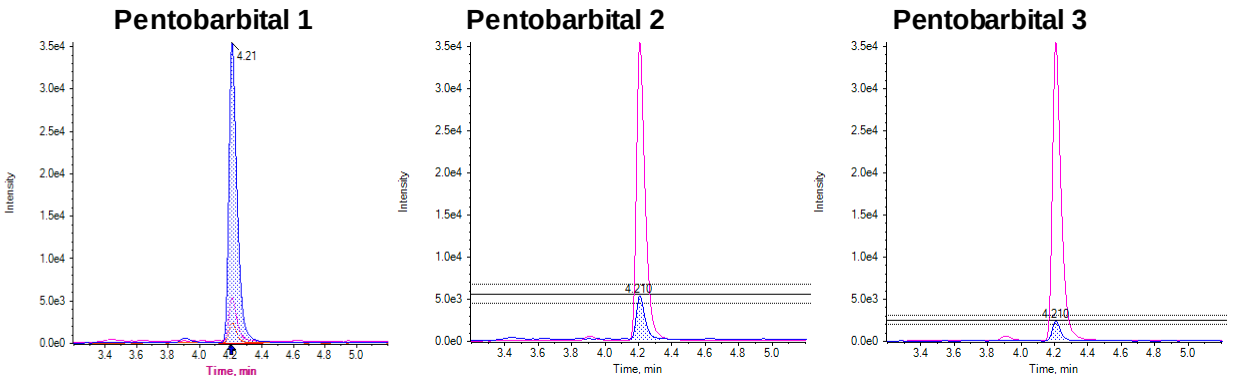
Chromatograms – Bars on peaks are expected ion ratio ± 20% of comparison standard(s)



Result Summary for Figure A3: Tallow - Fortified at 50 ng/g

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%)**	✓

Chromatograms – Bars on peaks are expected ion ratio ± 20% of comparison standard(s)



Appendix D: AB SCIEX QTRAP 5500 data acquisition method

Comment: Inst #: 1701725
HPLC column – Agilent Zorbax Eclipse Plus C18, 2.1 x 50 mm, 1.8 micron size column
M.P. A: Water
M.P. B: Acetonitrile

Synchronization Mode: LC Sync
Auto-Equilibration: Off
Acquisition Duration: 8min31sec
Number Of Scans: 2318
Periods In File: 1
Acquisition Module: Acquisition Method
Software version Analyst 1.6.2

MS Method Properties:
Period 1:

Scans in Period: 2318
Relative Start Time: 1000.00 msec
Experiments in Period: 1

Period 1 Experiment 1:

Scan Type: MRM (MRM)
Scheduled MRM: No
Polarity: Negative
Scan Mode: N/A
Ion Source: Turbo Spray
Resolution Q1: Unit
Resolution Q3: Unit
Intensity Thres.: 0.00 cps
Settling Time: 0.0000 msec
MR Pause: 5.0070 msec
MCA: No
Step Size: 0.00 Da

@Q1 Mass (Da) Q3 Mass (Da) Dwell(msec) Param Start Stop ID
225.000 182.000 50.00 CE -19.00 -19.00 Pentobarbital 1
CXP -13.00 -13.00

@Q1 Mass (Da) Q3 Mass (Da) Dwell(msec) Param Start Stop ID
225.000 85.000 50.00 CE -18.00 -18.00 Pentobarbital 2
CXP -9.00 -9.00

@Q1 Mass (Da) Q3 Mass (Da) Dwell(msec) Param Start Stop ID
225.000 138.000 50.00 CE -21.00 -21.00 Pentobarbital 3
CXP -10.00 -10.00

@Q1 Mass (Da) Q3 Mass (Da) Dwell(msec) Param Start Stop ID
230.000 187.000 50.00 CE -17.00 -17.00 Pentobarbital-D5
CXP -10.00 -10.00

Parameter Table(Period 1 Experiment 1):
CAD: Medium
GS1: 50.00
GS2: 60.00
CUR: 30.00
TEM: 400.00
IS: -3500.00
DP -100.00
EP -10.00

Valco Valve Diverter

Total Time (min) Position
1 0.0 B
2 0.5 A
3 8.0 B
Agilent LC Pump Method Properties
Pump Model: Agilent 1260 Binary Pump
Minimum Pressure (psi): 0.0
Maximum Pressure (psi): 8702.0
Dead Volume (µl): 40.0
Maximum Flow Ramp (ml/min²): 100.0
Maximum Pressure Ramp (psi/sec): 290.0

Max Flow Ramp Up (ml/min²): 100.0
Max Flow Ramp Dn (ml/min²): 100.0

Step Table:

@Step	*Total Time(min)*	*Flow Rate(µl/min)*	*A (%)*	*B (%)*
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*

Left Compressibility: 50.0
Right Compressibility: 115.0
Left Dead Volume (µl): 40.0
Right Dead Volume (µl): 40.0
Left Stroke Volume (µl): -1.0
Right Stroke Volume (µl): -1.0
Left Solvent: A1
Right Solvent: B1

Agilent LC Pump Method Properties
Pump Model: Agilent 1260 Binary Pump
Minimum Pressure (psi): 0.0
Maximum Pressure (psi): 8702.0
Dead Volume (µl): 40.0
Maximum Flow Ramp (ml/min²): 100.0
Maximum Pressure Ramp (psi/sec): 290.0
Max Flow Ramp Up (ml/min²): 100.0
Max Flow Ramp Dn (ml/min²): 100.0

Step Table:

@Step	*Total Time(min)*	*Flow Rate(µl/min)*	*A (%)*	*B (%)*
0	*0.00*	*0*	*50.0*	*50.0*
1	*8.50*	*0*	*50.0*	*50.0*

Left Compressibility: 50.0
Right Compressibility: 115.0
Left Dead Volume (µl): 40.0
Right Dead Volume (µl): 40.0
Left Stroke Volume (µl): -1.0
Right Stroke Volume (µl): -1.0
Left Solvent: A2
Right Solvent: B2

Agilent Column Oven Properties
Left Temperature (°C): 40.00
Right Temperature (°C): 40.00

Temperature Tolerance +/- (°C): 1.00
Start Acquisition Tolerance +/- (°C): 1.00
Time Table (Not Used)
Column Switching Valve Installed 10Port2Pos
Position for first sample in the batch: Left
Use same position for all samples in the batch

CTC PAL Autosampler Method Properties
Loop Volume1 (µl): 20
Loop Volume2 (µl): 20
Injection Volume (µl): 5.000
Barcode Reading: Disabled

Method Description:

Syringe: 100ulDLW

Cycle date: 9/9/2010 3:26:06 PM

Cycle name: Analyst LC-Inj DLW Fast_Rev05

Airgap Volume (µl) 3
Front Volume (µl) 5
Rear Volume (µl) 5
Filling Speed (µl/s) 5
Pullup Delay (ms) 3
Inject to LC Vlv1
Injection Speed (µl/s) 5
Pre Inject Delay (ms) 500
Post Inject Delay (ms) 500
Needle Gap Valve Clean (mm) 3
Valve Clean Time Solvent 2 (s) 3
Valve Clean Time Solvent 1 (s) 4
Post Clean Time Solvent 1 (s) 3