A Determination of Veterinary Drug Residues in Milk Using Polymeric SPE and UHPLC-MS/MS Analysis

UCT Part Numbers:
ECHLD126-P – EnviroClean® HLDVB , 200 mg/6mL SPE cartridge, PE Frit
SLDA100ID21-18UM – Selectra® DA, 100 × 2.1 mm, 1.8 µm HPLC column
SLDAGDC20-18UM – Selectra® DA, 10 × 2.0 mm, 1.8 µm guard cartridge
SLGRDHLDR – Guard cartridge holder

Summary:

This application note outlines a multi-class, multi-reside method for the determination of 49 representative veterinary drugs in milk using a simple, solid-phase extraction (SPE) procedure and analysis by UHPLC-MS/MS. To achieve fast and simultaneous extraction of the various drug residues, a generic liquid extraction procedure using EDTA/acetic acid buffer is conducted prior to extraction on a polymeric SPE cartridge. UHPLC separation is carried out with a Selectra® DA column, which exhibits alternative selectivity to a C18 phase and is capable of enhanced retention for the more polar drugs. The method was evaluated for each compound at three varying concentrations (1, 10 and 100 µg/kg). For most compounds, recoveries were between 70% and 120% and reproducibility was <20%. In addition, the majority of compounds could be accurately detected at a concentration of 1 µg/kg, demonstrating that the presented method is sufficient to monitor a wide range of veterinary drugs in milk. The drugs investigated belonged to several different classes, including β-agonists, macrolides, amphenicols, sulfonamides, tetracyclines and quinolones.

Introduction:

Veterinary drugs are frequently administered to food-producing animals, including dairy cows, to treat and prevent disease and/or increase growth rates. The inappropriate or illegal use of these drugs can result in the presence of their residues in food of animal origin which could pose a potential threat to human health. Milk is an important food commodity that is consumed by a large portion of the population, including infants. To ensure food safety and prevent the unnecessary exposure of consumers to veterinary drugs, it is vital to test milk for drug residues. The United

States, European Union (EU), CODEX and other international organizations have established maximum residue limits (MRLs) for veterinary drugs in a variety of biological matrices, including milk [1-3]. The MRLs for milk are typically lower than those set for other biological matrices (muscle, liver and kidney) and span a wide concentration range (low µg/kg to >1000 µg/kg). In addition, a number of drugs are prohibited for use in food producing animals or are unauthorized for use in lactating animals and require very low detection limits (≤2 µg/kg).

Milk is a complex matrix containing dissolved fats, carbohydrates, proteins and minerals (including calcium), which can complicate the development of a fast, easy and reliable analytical method for the identification and quantification of veterinary drug residues. Development of a multi-class, multi-residue (MMR) method can be challenging not only due to the inclusion of a large number of drugs with diverse physicochemical properties, but also on account of the complex sample matrix and the instability of certain drug classes (e.g. β-lactams, tetracyclines and macrolides). A MMR method should ideally be capable of extracting a wide range of drugs, reduce major matrix interferences, obtain good analyte recovery, be reproducible and achieve adequate limits of detection (LOD's). The use of a generic sample preparation procedure, such as SPE using a polymeric sorbent, is a suitable approach for achieving these goals. Ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) is the detection system of choice for veterinary drugs as it allows rapid detection of trace-level residues in complex matrices. However, the diverse physicochemical properties of the veterinary drugs still pose challenges and analytical conditions must be optimized to obtain adequate sensitivity of all the compounds as well as good retention and peak shape of problematic compounds.

Sample Preparation Procedure:

A. Sample extraction

1. Weigh 5 g of milk into a 15 mL polypropylene centrifuge tube.
2. Add 5 mL of 0.1M EDTA-Na_2 + 2% acetic acid.
3. Vortex for 5 minutes to de-proteinize the milk.
4. Centrifuge for 5 minutes at ≥ 3500 *g*.

Note: A larger volume of extraction solvent or a second extraction of the milk sample (5mL buffer) can be carried out if deemed necessary.

B. SPE extraction

1. Condition SPE cartridge with:
   a) 1 × 3 mL methanol
   b) 1 × 3 mL ultrapure water
2. Apply the supernatant to the SPE cartridge, taking care to avoid any transfer of the lipid layer. If required, use a low vacuum to draw the sample through (≤5 mL/min).

C. Wash cartridge

1. 1 × 3 mL ultrapure water.
2. 1 × 3 mL 10% methanol.
3. Dry cartridge under vacuum (≥10 inHg) for 5-10 minutes to remove residual water.
4. 1 × 3 mL hexane.
5. Dry cartridge under vacuum (≥10 inHg) for 1 minute to remove residual hexane.

D. Elution

1. Elute with 3 mL acetone.
2. Evaporate the sample to dryness at 35-40°C under a gentle stream of nitrogen.
3. Reconstitute in 1 mL of methanol:water (50:50, v/v).
4. Filter extract with a 0.22 µm nylon (or other suitable membrane) syringe filter into an autosampler vial.

LC-MS/MS Conditions:

HPLC Conditions	
Instrumentation	Thermo Scientific™ Dionex™ Ultimate™ 3000
HPLC column	UCT Selectra® DA, 100 × 2.1 mm, 1.8 µm (p/n: SLDA100ID21-18UM)
Guard column	UCT Selectra® DA, 10 × 2.0 mm, 1.8 µm (p/n: SLDAGDC20-18UM)
Guard column	p/n: SLGRDHLDR
Column temp.	60°C
Flow rate	400 µL/min
Injection volume	5 µL
Autosampler temp.	10°C
Wash solvent	Methanol
Divert valve	Divert to waste at 0-1.5 and 12-16.5 min to reduce ion source

Time (min)	Mobile phase A Water + 0.1% formic acid	Mobile phase B Methanol + 0.1% formic acid
0.0	95%	5%
0.5	70%	30%
4.0	70%	30%
5.0	40%	60%
8.0	40%	60%
8.5	0%	100%
12.0	0%	100%
12.1	95%	5%
16.5	95%	5%

MS Conditions	
Instrumentation	Thermo Scientific™ TSQ
Ionization mode	ESI^+ & ESI^-
Spray voltage	4000 V
Vaporizer	450°C
Capillary	350°C
Sheath gas	55 arbitrary units
Auxiliary gas	45 arbitrary units
Ion sweep gas	0 arbitrary units
Declustering	0 V
Q1 and Q3 peak	0.7 Da
Collision gas	Argon
Collision gas	1.7 mTorr
Acquisition	EZ method (scheduled SRM)
Cycle time	0.5 sec
Software	Xcalibur™ version 2.2

SRM Transitions							
Analyte	t_R (min)	Precursor ion	Product ion 1	CE 1 (V)	Product ion 2	CE 2 (V)	S-lens (V)
Sulfanilamide	2.14	156.00	92.11	12	108.10	10	45
Albuterol	2.55	240.12	121.07	28	148.07	18	51
Albuterol-D_3 (IS)	2.55	243.13	124.15	28	151.15	17	50
Lincomycin	3.13	407.16	126.11	29	359.23	17	93
Ampicillin	3.82	350.08	106.07	17	192.03	14	70
Trimethoprim	3.90	291.10	123.06	24	230.11	22	88
Trimethoprim-$^{13}C_3$ (IS)	3.90	294.09	233.19	22	264.16	25	70
Thiamphenicol(ESI^-)	4.10	353.92	121.08	54	184.99	21	70
Sulfadiazine	4.15	251.02	92.10	25	156.02	15	66
Sulfathiazole	4.30	255.98	92.08	27	156.01	13	66
Norfloxacin	4.35	320.09	233.08	24	276.11	16	82
Ormetoprim	4.53	275.11	123.08	26	259.12	26	90
Thiabendazole	4.57	202.01	131.07	32	175.05	25	75
Thiabendazole-D_6 (IS)	4.57	208.02	137.14	33	181.10	25	70
Oxytetracycline	4.60	461.08	337.09	28	426.14	18	82
Cefalexin	4.85	348.04	157.98	6	174.00	14	55
Ofloxacin	5.06	362.10	261.09	26	318.15	17	92
Ciprofloxacin	5.25	332.09	231.05	34	288.15	17	82
Ciprofloxacin-^{15}N-$^{13}C_3$ (IS)	5.25	336.09	235.10	36	291.21	16	80
Tetracycline	5.30	445.08	154.04	15	410.18	17	76
Sulfamethoxazole	5.38	254.09	92.07	26	148.08	17	55
Sulfamethoxazole-$^{13}C_6$ (IS)	5.38	260.03	98.15	26	162.07	15	61
Sulfamerazine	5.41	265.03	92.09	28	155.96	16	72
Lomefloxacin	6.04	352.09	265.08	22	308.16	16	78
Sulfamethizole	6.12	270.99	92.08	26	156.01	13	62
Chloramphenicol (ESI^-)	6.43	320.93	121.04	35	152.01	19	70
Cefotaxime	6.50	455.99	124.97	43	166.96	19	74

Enrofloxacin	6.51	360.12	245.12	24	316.19	17	86
Demeclocycline	6.55	465.03	430.12	19	448.13	14	92
Sulfachloropyridazine	6.76	284.98	92.10	27	156.01	14	70
Sulfamethazine	6.80	279.05	124.09	25	186.03	16	68
Sulfamethazine-$^{13}C_6$ (IS)	6.80	285.06	124.17	24	186.07	16	76
Azithromycin	6.85	749.11	116.00	38	591.45	24	128
Sarafloxacin	6.88	386.07	299.06	27	342.17	17	90
Clindamycin	6.96	425.10	126.10	29	377.19	17	95
Chlortetracycline	7.05	479.04	153.99	27	444.13	19	95
Cefazolin	7.15	454.97	111.92	31	155.97	14	68
Doxycycline	7.24	445.08	321.08	28	428.18	16	79
Diphenhydramine	7.34	256.12	115.07	65	165.05	62	45
Carbadox	7.40	263.04	129.06	30	231.06	12	69
Sulfadimethoxine	7.60	311.03	108.05	29	156.04	19	87
Erythromycin	7.91	734.37	157.99	28	576.41	16	107
Erythromycin-$^{13}C_2$ (IS)	7.91	736.36	160.07	28	578.41	13	108
Cephalothin	8.10	418.95	204.01	16	359.05	10	55
Penicillin G	8.15	367.09	114.04	31	160.02	14	71
Anhydroeythromycin	8.23	716.36	158.01	27	558.36	15	112
Clarithromycin	8.53	748.38	157.99	27	590.41	15	120
Ceftiofur	8.73	523.95	124.92	54	240.96	14	100
Penicillin V	8.83	383.09	114.04	32	160.03	15	70
Tylosin	8.97	916.42	173.92	34	772.47	23	173
Roxithromycin	9.30	837.43	157.95	31	679.47	16	120
Oxolinic acid	9.39	262.03	160.05	36	216.04	29	66
Oxacillin	9.52	434.08	144.01	31	160.00	15	75
Cloxacillin	10.10	468.04	160.00	16	177.96	31	85
Flumequine	10.40	262.04	126.05	48	202.03	32	70
Virginiamycin	10.80	526.19	337.08	19	355.08	16	83

CE = collision energy

Results and Discussion:

Summary of the recovery, reproducibility and method performance data generated (n=12 each).

	1 µg/kg		10 µg/kg		100 µg/kg			
Analyte	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**LCL (µg/kg)**	**Linearity (R^2)**
Sulfanilamide	87.1[a]	1.6	82.6	12.2	75.2	8.2	0.5	0.9993
Albuterol	100.1	6.5	106.6	4.4	105.9	1.7	0.5	0.9996
Lincomycin	109.3	5.8	87.4	9.1	94.0	4.6	0.5	0.9974
Ampicillin	109.8	10.4	92.3	7.8	96.3	3.6	0.5	0.9983
Trimethoprim	94.8	5.6	105.6	3.6	103.1	1.8	0.5	0.9992
Thiamphenicol	81.3	23.3	103.3	7.7	80.2	17.2	0.5	0.9962
Sulfadiazine	88.7	10.1	89.7	7.8	86.7	6.1	0.5	0.9985
Sulfathiazole	90.0	8.9	92.4	12.8	98.4	4.4	0.5	0.9990
Norfloxacin	96.4	4.7	98.0	5.5	98.1	3.3	0.5	0.9992
Ormetoprim	95.3	6.1	101.6	10.1	98.0	4.2	0.5	0.9974
Thiabendazole	96.5	5.7	102.3	4.5	101.6	1.5	0.5	0.9997
Oxytetracycline	98.8	11.6	90.9	13.8	96.2	4.2	0.5	0.9984
Cefalexin	69.3[a]	7.8	78.4	25.9	82.1	21.6	1.0	0.9981
Ofloxacin	101.2[a]	4.9	83.7	9.1	100.1	7.3	0.5	0.9988
Ciprofloxacin	95.0	3.5	99.4	4.2	100.9	1.8	0.5	0.9988
Tetracycline	108.8	10.0	99.0	5.2	99.4	7.7	0.5	0.9993
Sulfamethoxazole	89.0	9.2	101.6	3.6	101.7	2.5	0.5	0.9994
Sulfamerazine	87.7	10.7	88.9	10.8	100.1	12.4	0.5	0.9975
Lomefloxacin	107.1	4.4	111.0	4.6	108.5	1.9	0.5	0.9989
Sulfamethizole	91.0	13.5	95.2	12.2	98.8	5.0	0.5	0.9990
Enrofloxacin	96.4	4.2	84.4	5.6	99.5	4.8	0.5	0.9985
Chloramphenicol	98.0	12.2	110.3	7.4	101.5	4.1	0.5	0.9959
Cefotaxime	96.6	11.3	90.7	6.2	92.1	3.2	0.5	0.9979
Demeclocycline	100.3	11.1	95.2	5.8	95.9	7.3	0.5	0.9991
Sulfachloropyridazine	85.6	9.0	82.7	15.1	88.7	14.3	0.5	0.9994
Sulfamethazine	97.7	9.1	99.4	4.3	99.7	2.4	0.5	0.9996

Azithromycin	71.0	7.7	83.4	8.0	82.8	6.2	0.5	0.9981
Sarafloxacin	79.0	16.2	102.4	11.5	98.5	6.1	0.5	0.9994
Clindamycin	82.4	19.2	81.0	12.3	86.2	19.0	0.5	0.9978
Chlortetracycline	108.1	4.7	89.7	8.6	92.2	5.4	0.5	0.9999
Cefazolin	96.1	1.9	97.3	7.2	101.7	3.8	1.0	0.9980
Doxycycline	102.9	4.3	87.2	6.1	92.7	5.3	0.5	0.9988
Diphenhydramine	92.3	8.2	96.6	6.0	97.8	8.0	0.5	0.9999
Carbadox	82.3	11.4	95.3	12.2	95.9	6.5	0.5	0.9962
Sulfadimethoxine	74.4	14.0	74.6	7.4	81.4	5.5	0.5	0.9991
Erythromycin	97.1	8.0	102.6	3.8	100.1	1.4	0.5	0.9970
Cephalothin	105.4	5.4	96.5	11.1	99.8	6.0	0.5	0.9997
Penicillin G	103.2	5.4	98.0	7.1	99.9	2.5	0.5	0.9995
Anhydroeythromycin	112.7	9.8	107.8	6.7	100.2	4.4	0.5	0.9971
Clarithromycin	104.5	7.1	99.3	7.3	100.8	4.9	0.5	0.9986
Ceftiofur	57.1	7.7	67.8	20.3	66.2	25.9	0.5	0.9982
Penicillin V	101.7	10.1	88.9	6.2	96.7	5.9	0.5	0.9990
Tylosin	82.5	7.4	71.4	5.2	79.4	6.1	0.5	0.9995
Roxithromycin	97.2	9.1	92.4	10.5	95.6	4.2	0.5	0.9986
Oxolinic acid	101.0	5.4	97.5	7.9	99.5	3.9	0.5	0.9986
Oxacillin	92.8	10.5	83.4	5.8	88.6	6.6	0.5	0.9990
Cloxacillin	87.6	11.5	79.0	6.7	84.2	3.5	0.5	0.9982
Flumequine	80.8	13.9	93.0	6.8	91.8	10.5	0.5	0.9992
Virginiamycin	89.3	16.1	91.4	9.3	92.7	10.0	0.5	0.9979

[a]n=6.

Chromatographic separation

The unique chemistry of the Selectra® DA column, which contains a polyaromatic stationary phase, provides orthogonal selectivity to a traditional C18 column and offers a high degree of retention and selectivity for aromatic compounds. The stationary phase is capable of retaining analytes through hydrophobic (dispersive) interactions as well as through pi-pi (π- π) interactions which exhibit a substantial increase in retention for dipolar, unsaturated or conjugated analytes. The Selectra® DA column is ideally suited for the analysis of veterinary drug residues, as most compounds (and metabolites) possess aromatic functionality.

In the final UHPLC-MS/MS method, methanol was chosen as the organic mobile phase solvent, as it was found to give better overall peak shape than acetonitrile, particularly for the tetracycline and fluoroquinolone antibiotics. A hold was included in the gradient to improve chromatographic separation and all compounds were successfully eluted in <12 min. The enhanced retention of the Selectra® DA column ensured that the most polar compound included in the method, sulfanilamide, didn't elute until >2 minutes (30% methanol). Although it was possible to start the gradient at a higher percentage of organic solvent (20%) and reduce the overall run time, this required the use of a smaller injection volume (2 µL) which negatively affected the method sensitivity. Ultimately, the best sensitivity was obtained by starting the gradient at 5% methanol and using a 5 µL injection volume.

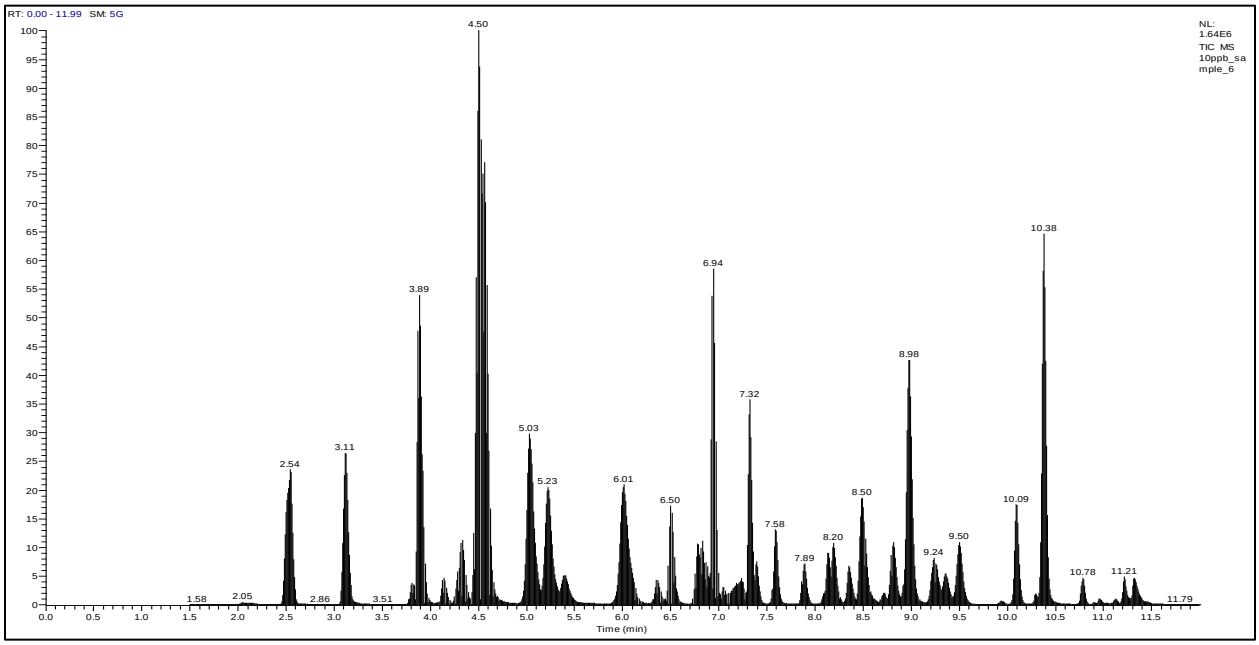

Figure 1. TIC chromatogram of an extracted milk sample (10 µg/kg) containing the 49 veterinary drugs and 7 internal standards.

Sample preparation procedure:

Prior to instrumental analysis, a sample pre-treatment step is required to concentrate the analytes of interest and eliminate non-desirable matrix components. This is particularly important for the analysis of veterinary drugs in milk because of their low regulatory limits and the larger sample size required to obtain necessary method sensitivity. One of the biggest difficulties in milk analysis is the high fat, protein, and calcium content that can often interfere with instrumental analysis. The instability of certain drug classes, namely β-lactams, tetracyclines and macrolides, causes additional complications by limiting the conditions that can be used for sample extraction and cleanup. Therefore, the sample preparation procedure was optimized to remove as much co-extracted matrix components as possible while minimizing any loss of the veterinary drug residues.

A simple deproteinization procedure using an EDTA/acetic acid buffer (sample pH should be 4-4.5) followed by centrifugation to separate the proteins and lipids was carried out prior to SPE extraction and cleanup. The inclusion of EDTA in the extraction buffer prevents the complexation of drugs with metal ions (e.g. calcium), particularly the tetracyclines and fluoroquinolones. After application of the sample supernatant to the SPE cartridge, the sorbent was washed with 10% methanol to remove polar matrix components and hexane to remove lipophilic compounds. Acetone was used as the SPE elution solvent as it was found to be more effective than methanol, particularly for hydrophobic compounds that contain multiple aromatic functional groups and are strongly retained on the DVB sorbent. Furthermore, acetone is a volatile organic solvent that is readily removed by evaporation under mild conditions (35-40°C). Filtration of the sample extract prior to LC-MS/MS analysis and the use of isotopically labeled internal standards and matrix-matched calibration curves are recommended in order to obtain the best possible results.

For most compounds, the recovery was between 70% and 120% and the reproducibility <20%. Only a a small number of compounds gave results outside of the acceptable limits, which was due to analyte instability (cefalexin and ceftiofur) or inadequate sensitivity at the lowest concentration level (sulfanilamide and thiamphenicol). In addition, all compounds could be accurately detected at a concentration of 10 µg/kg and the vast majority of compounds at 1 µg/kg, demonstrating that the presented method is suitable for monitoring a wide range of veterinary drug residues in milk.

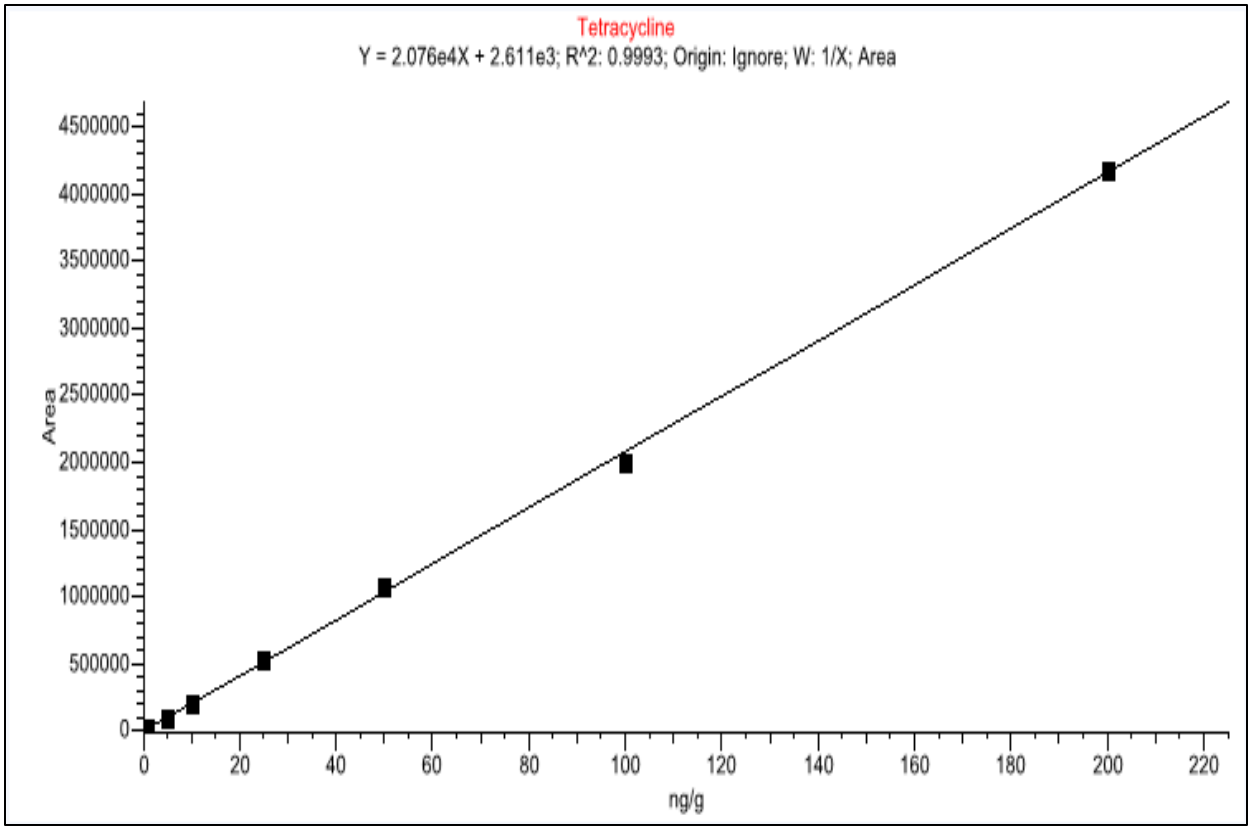

Figure 2. Example of an eight-point matrix-matched calibration curve (0.5-200 µg/kg, equivalent to 2.5-1000 ng/mL in final extract).

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