MN Dept of Agriculture (MDA) Verification/Validation of CTC & OTC by HPLC

Michele Swarbrick
DEPARTMENT OF AGRICULTURE

Background

- MDA lab has been analyzing CTC via JAOAC Vol 80, pp 961-965, 1997 "Assay of Chlortetracycline in Animal Feeds by Liquid Chromatography with Fluorescence Detection"
  - Over the past years, this method has experienced some problems during chromatography analysis using HPLC. On an intermittent basis, precipitates form in the instrument, i.e. salting out, after injection of filtered sample preparations. The resulting excessively high pressure caused instrument malfunctions and shutdowns. The lab has been unable to determine the cause or a satisfactory remedy.

Ideas for Alternative Method

- MDA lab evaluated alternative methodology and AOAC 2008.09 "Oxytetracycline/Oxytetracycline Hydrochloride in Animal Feed, Fish, Feed, and Animal Remedies – Liquid Chromatography" had similar HPLC conditions and with modification maybe extended it to the determination of CTC.
- Two (2) improvements from JAOAC Vol 80, pp 961-965, 1997 that could address the "salting out" issue are (i) using methanol instead of acetone for the extraction solvent for consistency with the mobile phase solvent system, and (ii) diluting the extract with 50% water.
- MDA lab has decided to move ahead with a single-laboratory verification/validation to determine suitability and fit for purpose for matrices typically submitted by its customer.

Method Parameters

- MDA lab modified AOAC 2008.09 method to extend it for the determination of chlortetracycline (CTC) / chlortetracycline hydrochloride (CTC-HCl).
- Summary of method parameters that were not altered:
  - **Aqueous Mobile Phase** – 0.1 M sodium acetate, 0.055 M calcium chloride, 0.020 M disodium EDTA.
  - **Extraction Solution** – Acidic Methanol (1 part HCl : 50 part Methanol)
  - **FLD detector** - 390 nm excitation, 512 nm emission
  - **Flow rate** - 1.5 mL/min
  - **Final dilution** - working calibration standards and sample extracts contain 50% water
  - **Analytical Column** - Phenomenex Prodigy ODS-3 4.6 x 150 mm, 5 um

Modified Method Parameters

- One curve instead of a high or low curve. Extending curve range ~0.2-10 µL/mL
- Listed Standard Prep
  - 1000ug/mL OTC Stock - 25mg dissolved in 3 mL methanol and diluted to 25 mL with 0.01 M HCl
    - 100 ug/mL Intermediate standard A in acid-methanol
      - 10 ug/mL Intermediate standard B acid-methanol
- New Stock Standard Prep
  - 250 ug/mL OTC and CTC (prepped separately) - 25mg dissolved in acid-methanol to 100 mL
    - 25 ug/mL OTC and CTC (combined) Intermediate standard in acid-methanol

Modified Method Parameters

- Extraction time changed from 45 – 60 min to 1.5 – 2 hours
  - Lengthening extraction time improved recoveries of CTC
- HPLC Gradient
  - Increasing % MeOH in gradient helped with CTC elution

Listed Mobile phase gradient

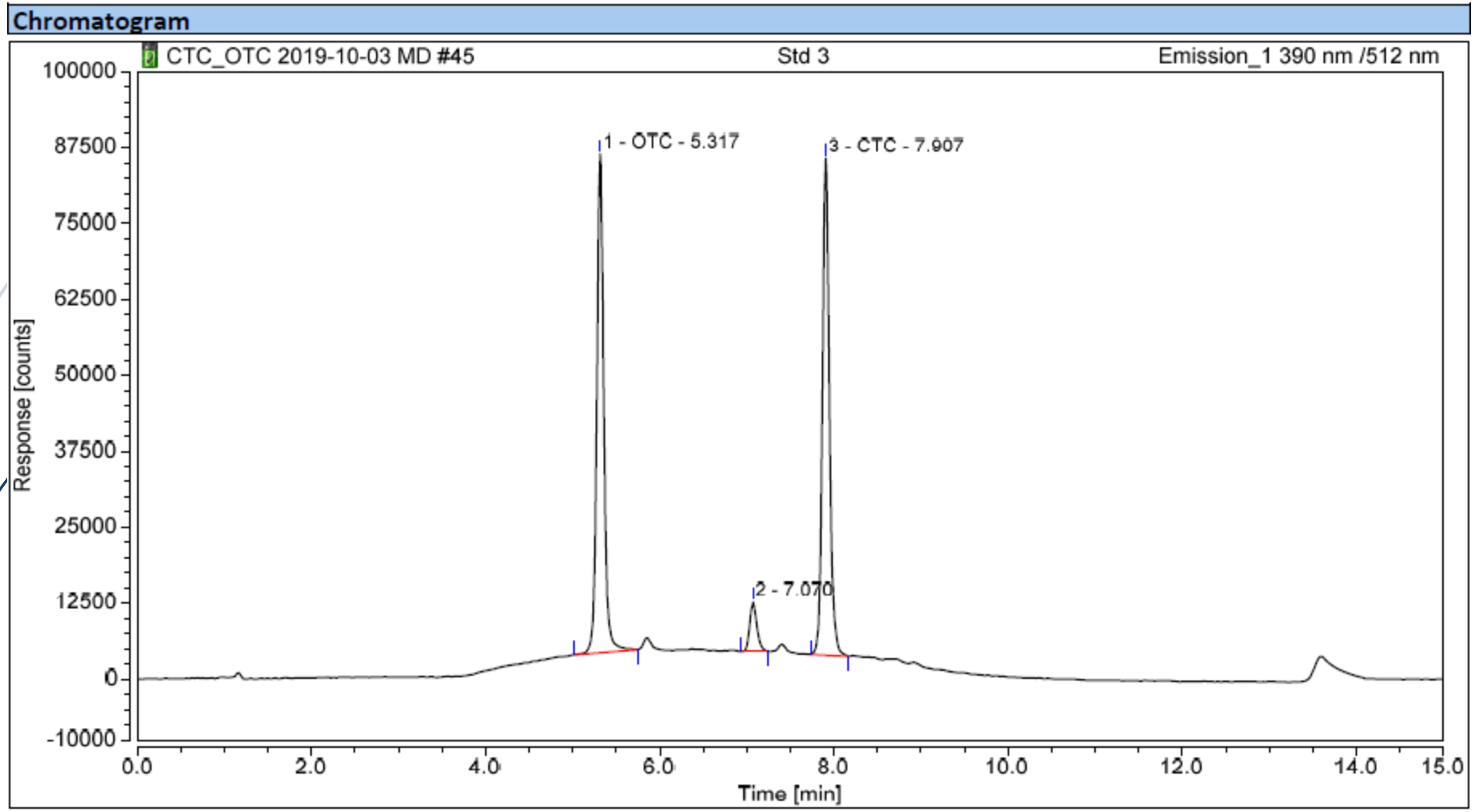
Time	% MeOH	% Aqueous
0	15	85
1	15	85
9	35	65
16	35	65
16.6	15	85
25	15	85

Modified Mobile phase gradient

Time	% MeOH	% Aqueous
0	85	15
2	85	15
7	30	70
12	30	70
12.1	85	15
16	85	15

Modified Method Parameters

- Injection volume changed form 20 uL to 5 uL
  - Reducing injection volume helped with carry over issues.
  - Also added that the injection loop wash before and after injection.
- Injecting mid-level standard beginning, middle (if more than 5 samples extracted) and end of run, instead of running whole calibration curve at end of run and averaging the two curves.


Chromatogram
CTC_OTC 2019-10-03 MD #45
Std 3
Emission_1 390 nm /512 nm
1 - OTC - 5.317
2 - 7.070
3 - CTC - 7.907
Response [counts]
Time [min]
100000
87500
75000
62500
50000
37500
25000
12500
0
-10000
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
15.0

Performance Characteristics Evaluated

- Linear Calibration Range
- Determination of Minimum Detection Limit (MDL)
- Accuracy (Reproducibility)
- Precision (Repeatability)
- Specificity

Linear Calibration Range

- Determined the linear calibration range using a 6-point curve with concentrations at approximately 0.2, 0.5, 1, 2, 5, and 10 µL/mL.

Linear Calibration Range – CTC

Standard	Recovery (%)			
	Analysis 1	Analysis 2	Analysis 3	Analysis 4
11.8	99.8	100	99.9	100
4.73	102	101	102	101
2.37	100	100	99.9	99.5
1.42	98.5	98.7	98.4	98.9
0.47	97.9	101	99.7	103
0.24	97.7	91.0	88.7	99.2
r^2	0.9999	1.0000	0.9999	1.0000
Offset	282.6	230.6	217.5	156.4

Linear Calibration Range – OTC

Standard	Recovery (%)		
	Analysis 1	Analysis 2	Analysis 3
11.7	96.7	100	100
4.67	97.4	99.7	100
2.33	96.4	99.0	99.3
1.40	96.2	99.0	98.8
0.47	97.0	104	103
0.23	94.1	111	104
r^2	1.0000	1.0000	1.0000
Offset	18.06	13.64	19.62

Minimum Detection Limit (MDL)

- Verified that MDA lab could obtain a MDL level ≤ 10 mg/kg CTC (equivalent to 0.5 µg/mL in solution) concentration present in feed material / mineral premix

Matrix: Soybean Meal
Fortification: 0.24 ug/mL in solution

Replicate	CTC µg/mL
1	0.2522
2	0.3068
3	0.2727
4	0.2682
5	0.2574
6	0.2634
7	0.2616
Standard Deviation:	0.0167
Student t-value:	3.1430
MDL (µg/mL):	0.0524

Matrix: Soybean Meal
Fortification: 0.24 ug/mL in solution

Replicate	OTC µg/mL
1	0.239
2	0.237
3	0.236
4	0.241
5	0.240
6	0.238
7	0.230
8	0.231
9	0.228
Standard Deviation:	0.0044
Student t-value:	2.8960
MDL (µg/mL):	0.0129

Accuracy (Reproducibility)

- Based on samples analyzed for CTC by MDA lab, the range of concentrations is greater than 1 order of magnitude
  - Repeatability evaluated at 3 concentrations (low, mid, and high).
  - Due to the unavailability of certified reference material(s), MDA lab prepared and analyzed fortifications that have concentrations equivalent to low-level standard (approximately 2 µg/mL), mid-level standard (approximately 4 µg/mL), and high-level standard (approximately 10 µg/mL) on three (3) separate occasions.
- **Acceptance criteria** –
  - Mid & high concentration: Recovery between 85-110%
  - Low concentration: Recovery between 60-140%

Accuracy (Reproducibility)

CTC – Fortified Blank Matrix – concentration in solution

CTC	Low Level Spike		Mid Level Spike		High Level Spike	
Replicate	Result (ug/mL)	Recovery (%)	Result (ug/mL)	Recovery (%)	Result (ug/mL)	Recovery (%)
1	0.20	86.0	3.88	95.2	9.78	96.0
2	0.21	90.9	3.93	96.4	9.96	97.7
3	0.22	92.3	3.98	97.6	9.97	97.8
1	0.21	87.6	3.93	96.3	9.93	97.4
2	0.23	96.7	3.95	97.0	9.99	98.0
3	0.21	89.0	3.99	97.7	9.98	97.9
1	0.24	103.2	3.99	97.9	9.97	97.8
2	0.25	104.8	4.07	99.9	10.02	98.3
3	0.22	91.9	4.08	100.0	10.06	98.7
Bias:	-3.06	---	0.70	---	6.68	---
Average:	0.22	93.6	3.98	97.6	9.96	97.7
Standard Deviation:	0.015	---	0.062	---	0.072	---
% RSD:	6.691	---	1.555	---	0.722	---

Accuracy (Reproducibility)

OTC – Fortified Blank Matrix – concentration in solution

OTC	Low Level Spike		Mid Level Spike		High Level Spike	
Replicate	Result (ug/mL)	Recovery (%)	Result (ug/mL)	Recovery (%)	Result (ug/mL)	Recovery (%)
1	0.24	98.5	3.75	90.9	9.37	91.0
2	0.24	97.7	3.75	91.1	9.41	91.4
3	0.24	97.3	3.73	90.4	9.43	91.6
1	0.24	103.1	3.35	95.6	10.18	94.8
2	0.24	102.6	3.31	94.6	10.23	95.3
3	0.24	102.0	3.37	96.1	10.24	95.3
1	0.23	98.5	3.22	92.1	9.75	90.8
2	0.23	99.0	3.19	91.2	9.82	91.5
3	0.23	97.7	3.20	91.4	9.91	92.2
Bias:	-3.05	---	0.15	---	6.54	---
Average:	0.24	99.6	3.43	92.6	9.82	92.7
Standard Deviation:	0.004	---	0.228	---	0.334	---
% RSD:	1.889	---	6.649	---	3.406	---

Precision (Repeatability)

- Selected previously analyzed samples at low (no claim), mid (approximately 100 to 180 mg/kg) and high (> 1500 mg/kg) concentrations. Prepared and analyzed 7 replicates of each in a single batch.
- **Acceptance Criteria** –
  - Mid & high concentration: %RSD ≤ 5%
  - Low concentration: %RSD ≤ 10%

Precision (Repeatability)

Repeatability Data for CTC

CTC	Low Level - In-house Sample [1] Claim 0 g/ton		Mid Level - In-house Sample Claim 100 g/ton		High Level - In-house Sample Claim 1600 g/ton	
Replicate	Result (g/ton)	Pass	Result (g/ton)	Pass	Result (g/ton)	Pass
1	< 5.0	---	78.57	---	1609	---
2	< 5.0	---	82.94	---	1615	---
3	< 5.0	---	82.85	---	1622	---
4	< 5.0	---	81.23	---	1614	---
5	< 5.0	---	82.69	---	1569	---
6	< 5.0	---	78.38	---	1602	---
7	< 5.0	---	81.64	---	1582	---
Mean:	N/A	---	81.19	---	1602	---
Standard Deviation:	N/A	---	1.82	---	17.96	---
RSD_r:	N/A	Pass	2.24	Pass	1.12	Pass

[1] All results < 5 g/ton (Method Reporting Limit)

Precision (Repeatability)

Repeatability Data for OTC

OTC	Low Level - In-house Sample [1] Claim 0 g/ton		Mid Level - In-house Sample Claim 500 g/ton	
Replicate	**Result (g/ton)**	**Pass**	**Result (g/ton)**	**Pass**
1	< 5.0	---	409.00	---
2	< 5.0	---	412.00	---
3	< 5.0	---	411.00	---
4	< 5.0	---	411.00	---
5	< 5.0	---	410.00	---
6	< 5.0	---	404.00	---
7	N.A.[2]	---	413.00	---
Mean:	N/A	---	410.00	---
Standard Deviation:	N/A	---	2.73	---
RSD_r:	N/A	Pass	0.66	Pass

[1] All results < 5 g/ton (Method Reporting Limit)

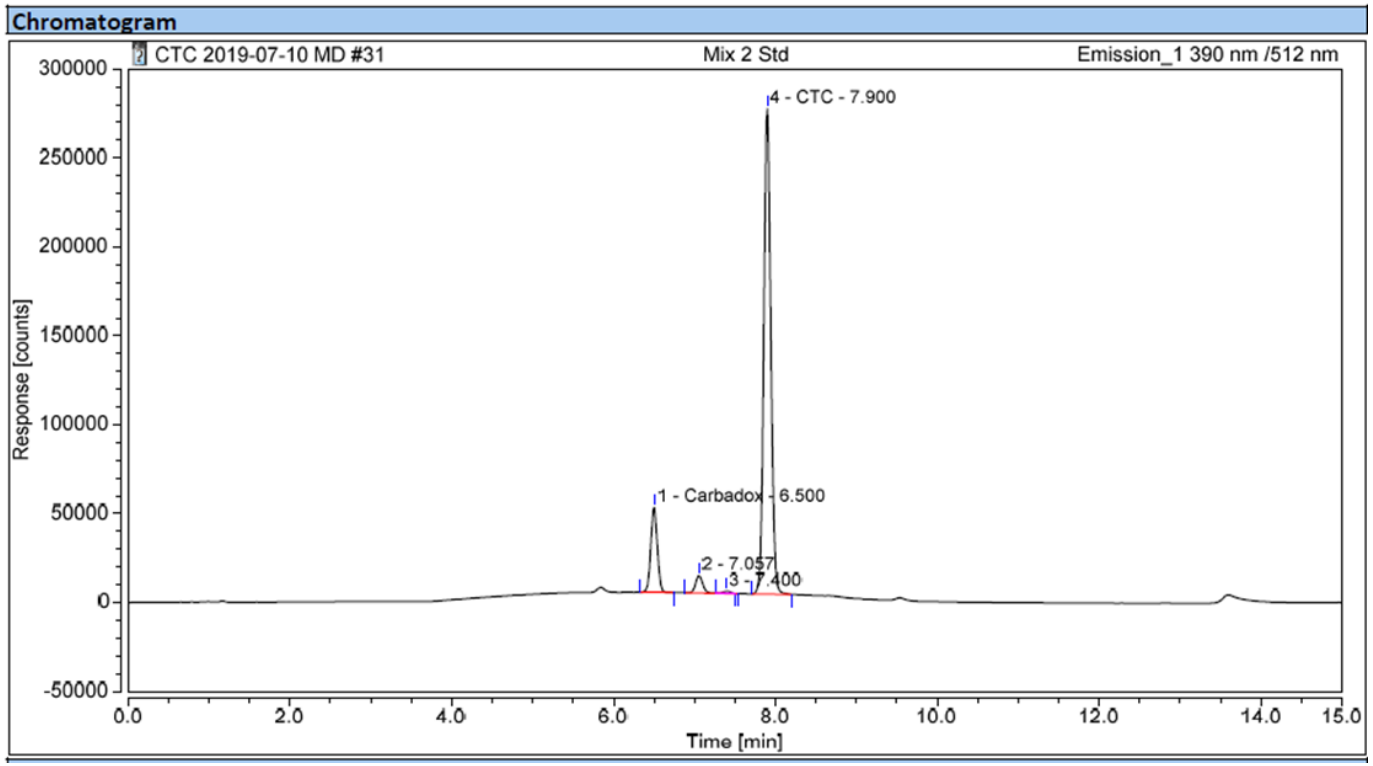
[2] There was not enough sample for 7th replicate

Specificity

- Evaluated chromatography for interference from:
  - Approved combinations of medically important drugs which include Decoquinate, Laidlomycin, and Lasalocid.
  - Oxytetracycline (OTC) and Epi-CTC (a degradation product of CTC in feeds).
  - Matrix –Evaluate chromatography of MDA samples for matrix interferences.
- Injected a number of drugs individually and as mixes to see what showed up in chromatography.
  - Mix 1 Contained Decoquinate, Lasalocid, Laidomyosin, OTC and Tylosin
  - Mix 2 contained Amprolium, Monensin, Carbadox, Sulfamethazine

Specificity

- The method is free of interferences from matrix and other drugs. Only drug that showed a peak at the 390 nm/512 nm was Carbadox and peak well separated from CTC (and OTC – retention time of 5.3 minutes)

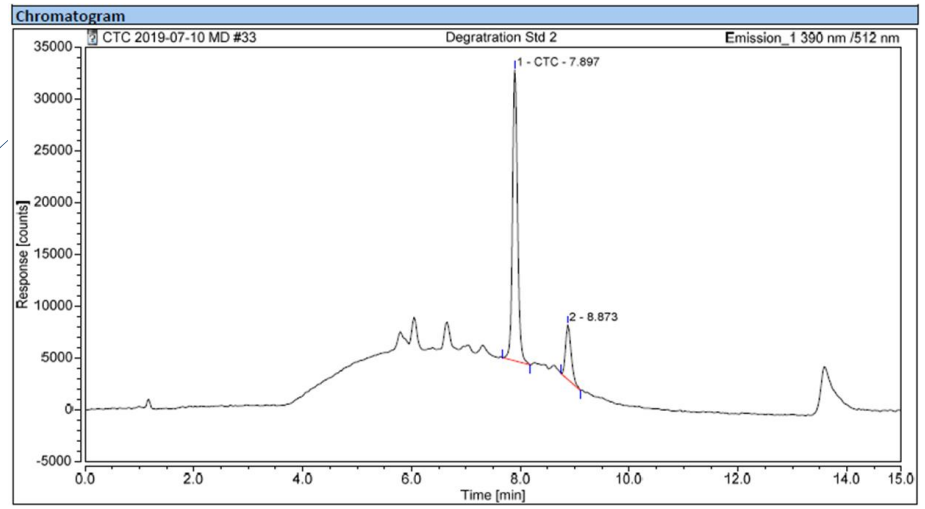


CTC Degradation Standards

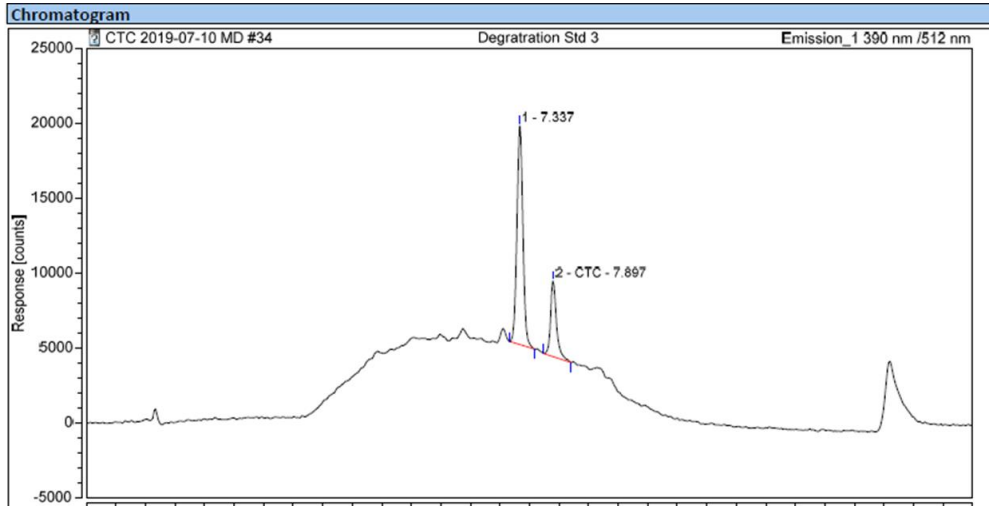
- Obtained a CTC degradation set from Bioaustralis to confirm that degrades do not interfere with OTC and CTC
  - Anhydro-CTC HCl eluted at 8.87 minutes – minor peak,
  - Epianhydro-CTC HCl eluted at 7.337 minutes – minor peak
  - Epi-CTC HCl eluted at 5.93 minutes

CTC Degradation Standards

Anhydro-CTC HCl (8.87 min)

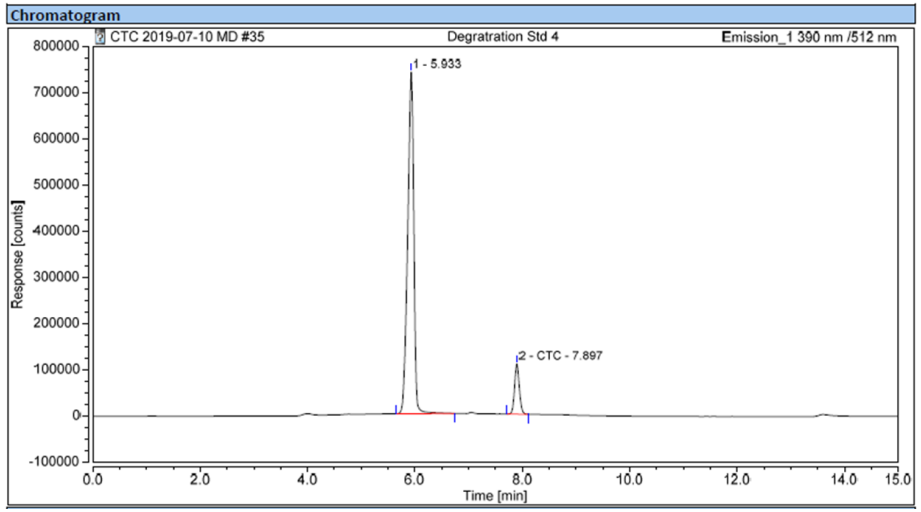


Epianhydro-CTC HCl (7.34 min)

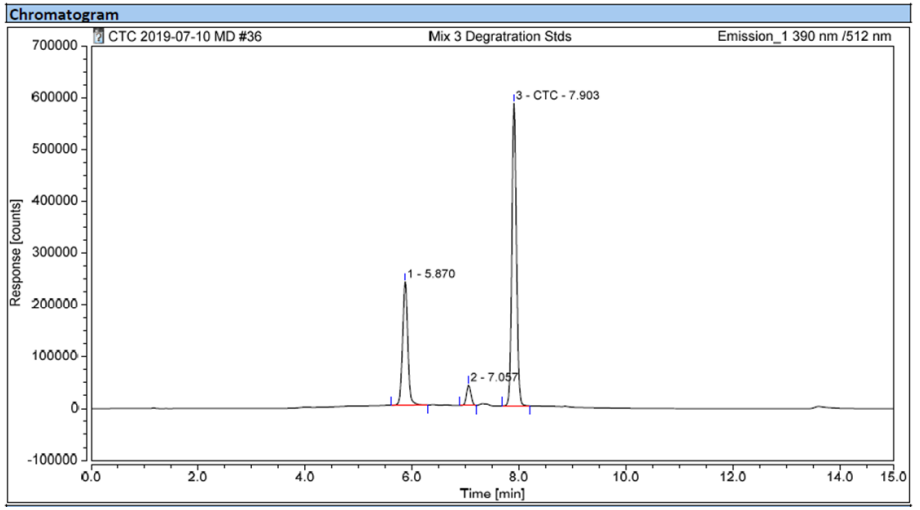


CTC Degradation Standards

Epi-CTC HCl (5.93 min)

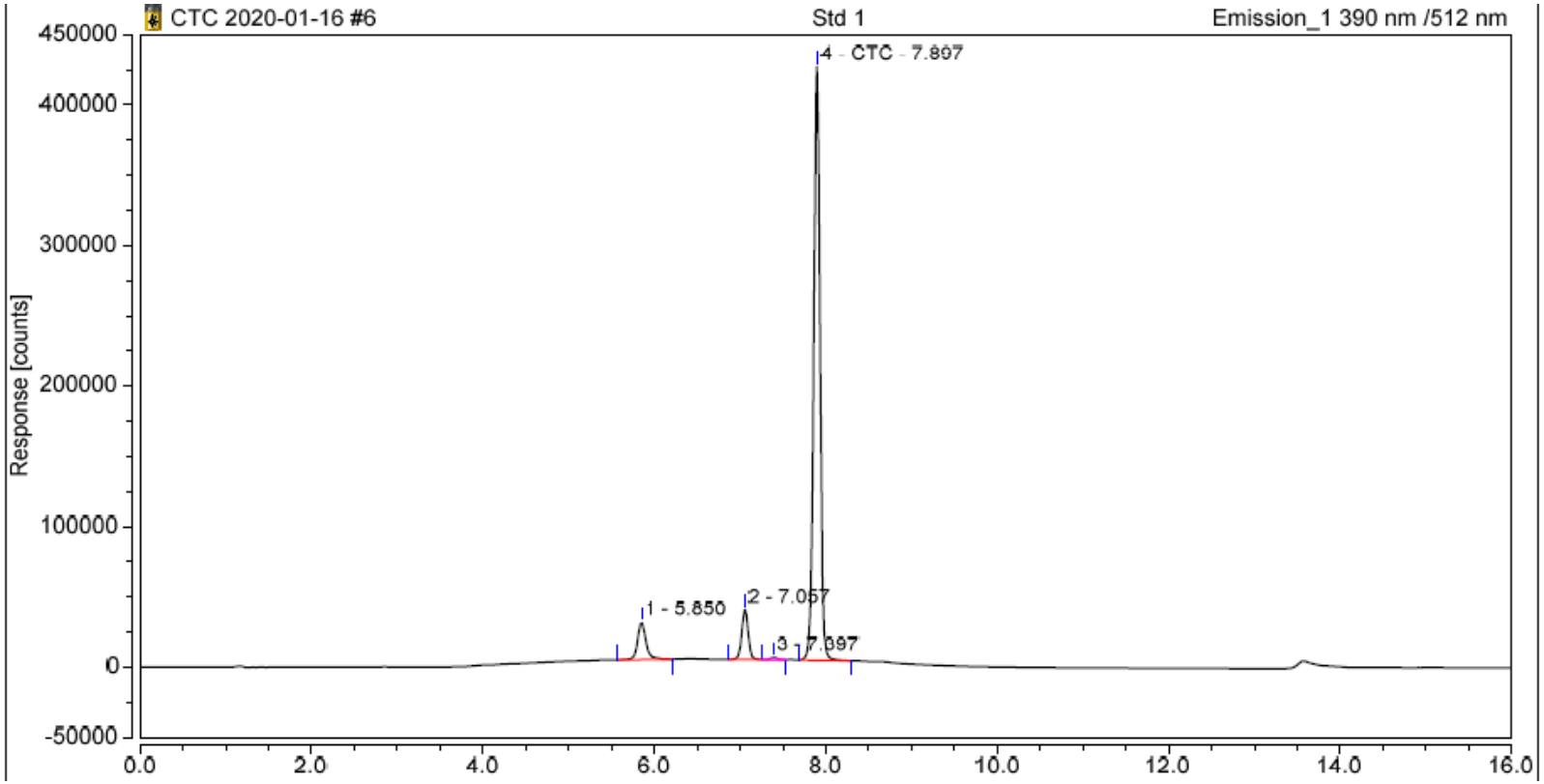


Mix of CTC and Degrades



CTC Degradation

- CTC standard that was injected two months after preparation. None of the CTC degradations detected.



Summary

- Method modifications AOAC 2008.09 "Oxytetracycline/Oxytetracycline Hydrochloride in Animal Feed, Fish, Feed, and Animal Remedies – Liquid Chromatography" to include Chlortetracycline appears fit for purposes for the Minnesota Dept. of Agriculture laboratory and will be implemented.

- **The following staff collaborated on the plan:**
- Treeske Ehresmann, Unit Supervisor
- Brian Miller, Quality Assurance Officer
- Michele Swarbrick, Research Scientist 2