# Application Note #00543

## Detection of Oxytetracycline and Chlorotetracycline in Animal Feed and Tissue Extracts using the Varian 320-MS Triple Quadrupole Mass Spectrometer

Bethany V. Pond and Annabel Mitchell, Varian, Inc.

### Introduction

Tetracyclines are broad spectrum antibiotics that have been widely used as therapeutics in food producing animals because of their effectiveness and low cost[1]. These compounds have also been widely added to animal feed as a means for promoting growth. Current EU tolerance levels for oxytetracycline (OTC) and chlorotetracycline (CTC) are in the hundreds of ppb depending on the type of matrix. Most of these reported matrices include milk, eggs, muscle, liver, and kidney.[2]

The analysis of Tetracyclines has proven difficult because the molecular structure of these compounds lend themselves to easily chelate to metal ions and to interact with silanol groups. During LC analysis, the addition of a chelating agent can solve the tailing of peaks, however, these conditions are not suitable for ESI/MS analysis[2].

In this application note, a LC/MS/MS methodology using the 320-MS Triple Quadrupole Mass Spectrometer is presented for the analysis of OTC and CTC in animal feed. In addition, a low-level matrix spike of CTC in chicken breast tissue is presented.

### Instrumentation

Varian 212-LC Binary Gradient LC/MS Chromatography Pump (2)

Varian 320-MS Triple Quadrupole Mass Spectrometer equipped with an ESI source

Varian Prostar™ 430 Autosampler

### Materials and Reagents

All solvents (reagent or HPLC Grade) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Chlorotetracycline (CTC) HCl (Part # C4881-5G) and Oxytetracycline (OTC) HCl (Part # 05875-10G) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Both the OTC and CTC samples in animal feed extract were provided by a Dept of Agriculture Laboratory.

### Sample Preparation

A standard solution (1 mg/mL) of OTC and CTC was prepared in Methanol. Further dilutions of the standard solution were carried out in a 1:1 mixture of Water:Methanol.

**Sample Preparation:**

Solutions ranging in concentration from 0.2 – 20 ng/µL from the standard solution were prepared for calibration curve purposes in animal feed samples. For the tissue sample, a calibration curve was prepared at a much lower range (0.02 – 2.0 ng/µL).

### Instrument Conditions

**LC Conditions:**

Column: Polaris C18-A 5 µ 100 X 2 mm (Varian Part# A2000100X020)

Guard Column: Metaguard 2.0 mm Polaris 5 µ C18-A (Varian Part # A20000MG2)

Column Temp: 45 °C

Solvent A: Water

Solvent B: 0.1 % Formic Acid in 1:1 (v/v) Acetonitrile:Methanol

Flow rate: 0.2 µL per min

### Injection Volume: 5 µL

**LC Program:**

| Time (min) | % B |
|---|---|
| 0 | 15 |
| 5:00 | 15 |
| 10:00 | 40 |
| 11:00 | 90 |
| 12:00 | 90 |
| 14:00 | 15 |
| 18:00 | 15 |

### Mass Spectrometry Conditions:

Ionization Mode: ESI positive

Nebulizing Gas Pressure: 55 psi

Drying Gas Pressure: 36 psi at 400 °C

Dwell Time: 0.2 s for each transition

## MS/MS Conditions:

| | OTC | CTC |
|---|---|---|
| Precursor Ion (m/z) | 461 | 479 |
| Capillary (V) | 45 | 60 |
| Product Ions (m/z) | 443<br>426 | 444<br>154 |
| Collision Energy (V) | 10.5<br>16.0 | 18.5<br>23.5 |

## Discussion

Figure 1 shows SRM chromatograms of CTC and OTC at a concentration of 5 ng/µL.

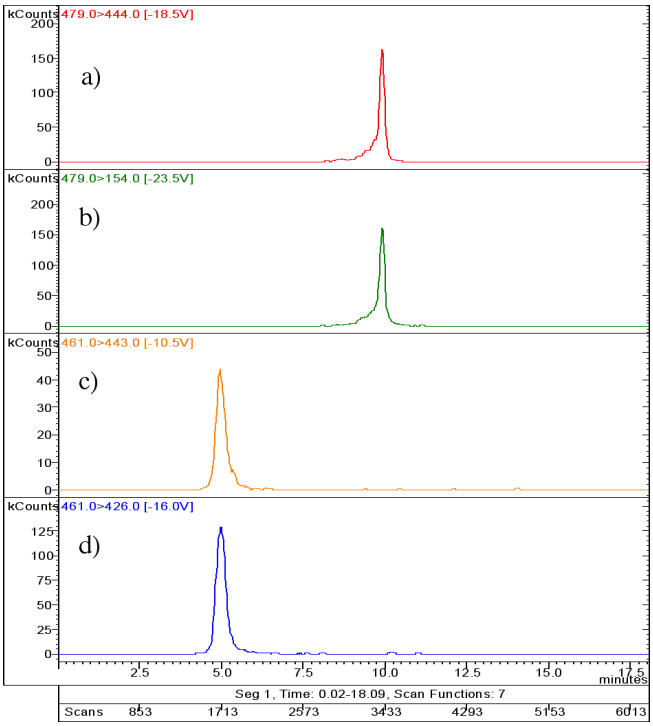


*Figure 1 SRM chromatograms of: a) CTC: 479 -> 444 b) CTC: 479 -> 154, c) OTC: 461 -> 443, and d) OTC: 461 -> 426 at a concentration of 5 ng/µL.*

For CTC, recent studies have shown that the chromatographic peak has excessive fronting as seen in Figure 1 a) and 1 b). This phenomenon is due to the rapid epimerization and formation of CTC keto tautomers. This process can adversely affect the quantification of the original antibiotic[3].

The sample solvent and the LC conditions used help minimize the appearance of the keto tautomer which is prevalent in CTC analyses.[3]

A standard calibration curve was run over the concentration range of 0.2 – 20 ng/µL. The calibration curve for oxytetracycline is shown in Figure 2.

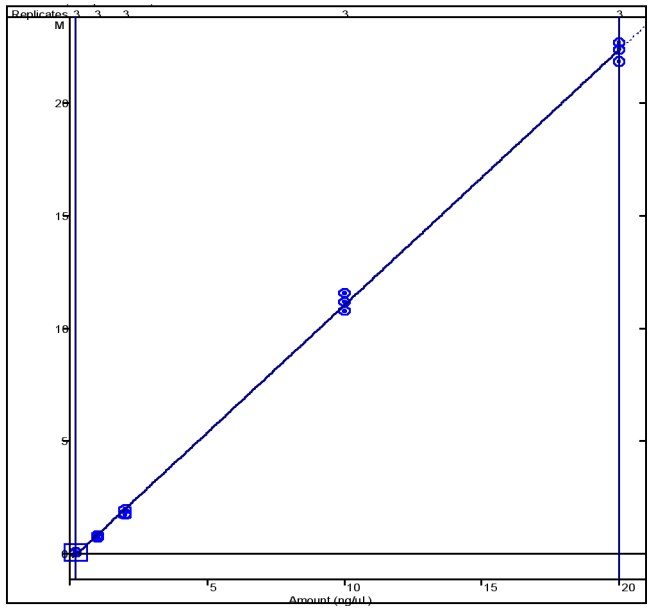


*Figure 2 Calibration curve of oxytetracycline (461 -> 443) over the concentration range of 0.2 – 20.0 ng/µL with three replicates.*

Calibration curves were run using two transitions for both antibiotics. For each of these curves, an $R^2 > 0.997$ was obtained.

The animal feed sample injected was at a concentration of 400 mg/kg. The samples were extracted in 65% Acetone:35% water and 0.2 M HCl. The final concentration of the extracted sample was ~ 45 µg/µL. Figure 3 shows the SRM chromatograms of OTC in animal feed.

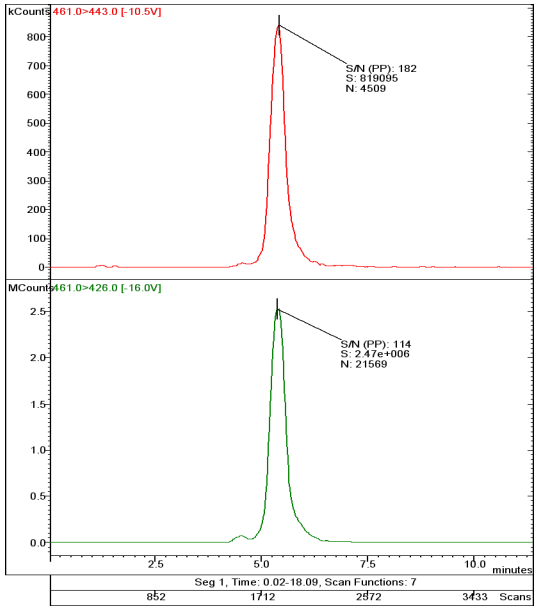


*Figure 3 SRM chromatograms of oxytetracycline (upper: 461 -> 443 and lower: 461 -> 426) in animal feed extract*

The SRM chromatogram of animal feed extract containing CTC showed a large signal of the keto tautomer. Accurate area reproducibility measurements could not be obtained on the 479 -> 444 transition, due to a high evidence of tautomerization, but the 479 -> 154 transition provided good area reproducibility, and could be used for quantitative analysis.

The area reproducibility over 18 consecutive injections in animal feed extract is shown in Figure 4 for CTC. The % RSD was less than 6.0 % for the SRM transition of 479 -> 154. For OTC in animal feed extract, the % RSD was less than 9.0 %.

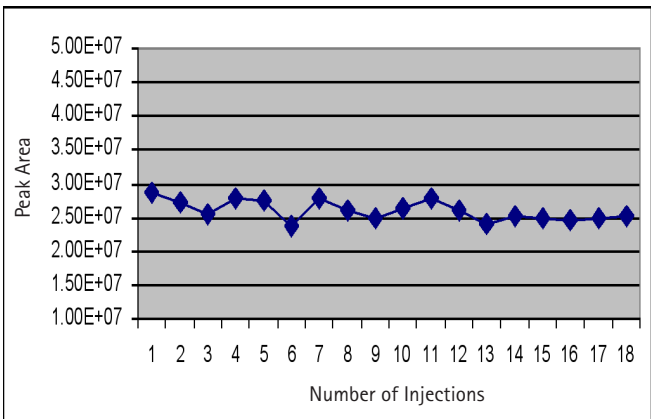


*Figure 4 Plot of area versus number injections for CTC (479 -> 154) in animal feed extract.*

Apart from the tautomerization, no observable matrix effects were seen in the animal feed sample.

The 320-MS system was also challenged with a chicken breast sample that was spiked at a much lower concentration relative to the feed samples. A minimum reporting level of 200 ug/Kg is the residue limit for these antibiotics in chicken tissue. Figure 5 shows the spiked sample in a compound report generated by the Varian Workstation software.

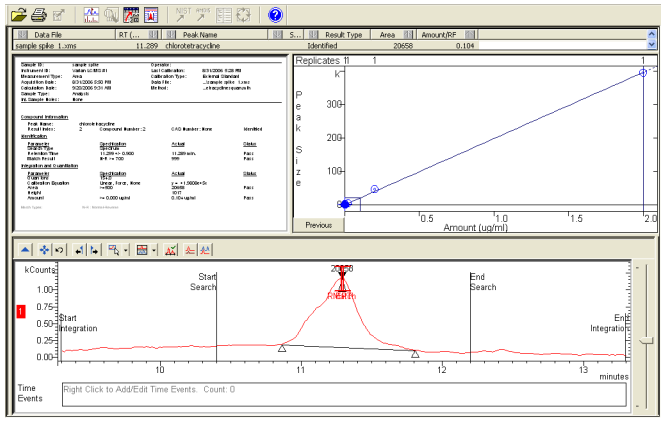


*Figure 5 Workstation report with SRM chromatogram of CTC in chicken breast extract at a concentration of 100 ug/Kg. Actual calculated amount was 104 ug/Kg. A 50 ul injection was used with a calibration curve ranging from 0. 02 to 20 ng/ul.*

## Conclusion

The analysis of CTC and OTC in animal feed extract and chicken breast tissue was carried out on the 320-MS Triple Quadrupole Mass Spectrometer. The analysis of CTC and OTC has been shown with accurate quantification and demonstrated area reproducibility. By examining the various MS/MS transitions, especially with CTC, it is possible to quantify on the SRM transitions with accurate results. The presence of the animal feed matrix did not affect the reproducibility of the results. The 320-MS can also reliably detect these antibiotics at much lower levels as required in tissue samples.

1 Anderson, C. R., Rupp, H. S., and Wu, W. H., J of Chrom. A., 2005: 1075; 23-32.

2 Gentili, A., Perret, D., and Marchese, S., Trends in Anal. Chem., 2005: 24 (7); 704 – 733.

3 Stolker, A. A. M., Brinkman, U. A. Th, J of Chrom. A., 2005: 1067; 15-53.

*The author would like to thank Crystal Holt for securing the animal feed sample extracts from the Department of Agriculture Laboratory.*