

Supplementary Materials

# Evaluation of antibiotic dissemination into the environment and untreated animals, by analysis of oxytetracycline in poultry droppings and litter.

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**Table S1.** Weights of the experimental birds from group A, which were recorded during the treatment period, for calculation of the individual dose of oxytetracycline.

| Treatment<br>day | Bird weight (g) |        |        |        |        |        |
|------------------|-----------------|--------|--------|--------|--------|--------|
|                  | Bird 1          | Bird 2 | Bird 3 | Bird 4 | Bird 5 | Bird 6 |
| 1                | 156             | 190    | 118    | 168    | 126    | 123    |
| 2                | 167             | 205    | 121    | 175    | 141    | 135    |
| 3                | 184             | 216    | 133    | 190    | 170    | 142    |
| 4                | 195             | 232    | 143    | 209    | 167    | 154    |
| 5                | 214             | 263    | 157    | 243    | 185    | 162    |
| 6                | 230             | 272    | 165    | 258    | 192    | 168    |
| 7                | 225             | 270    | 258    | 286    | 204    | 186    |
| 8                | 270             | 308    | 195    | 296    | 244    | 203    |
| 9                | 262             | 310    | 192    | 301    | 250    | 207    |
| 10               | 305             | 349    | 220    | 335    | 300    | 234    |

**Table S2.** Individual dose of oxytetracycline administered to birds of group A birds for 10 consecutive days.

| Treatment<br>day   | Administered doses (mg) |        |        |        |        |        | Total <sup>2</sup> |
|--------------------|-------------------------|--------|--------|--------|--------|--------|--------------------|
|                    | Bird 1                  | Bird 2 | Bird 3 | Bird 4 | Bird 5 | Bird 6 |                    |
| 1                  | 12,48                   | 15,2   | 9,44   | 13,44  | 10,08  | 9,84   | 70,48              |
| 2                  | 13,36                   | 16,4   | 9,68   | 14     | 11,28  | 10,8   | 75,52              |
| 3                  | 14,72                   | 17,28  | 10,64  | 15,2   | 13,6   | 11,36  | 82,8               |
| 4                  | 15,6                    | 18,56  | 11,44  | 16,72  | 13,36  | 12,32  | 88                 |
| 5                  | 17,12                   | 21,04  | 12,56  | 19,44  | 14,8   | 12,96  | 97,92              |
| 6                  | 18,4                    | 21,76  | 13,2   | 20,64  | 15,36  | 13,44  | 102,8              |
| 7                  | 18                      | 21,6   | 12,64  | 22,88  | 16,32  | 14,88  | 106,32             |
| 8                  | 21,6                    | 24,64  | 15,6   | 23,68  | 19,52  | 16,24  | 121,28             |
| 9                  | 20,96                   | 24,8   | 15,36  | 24,08  | 20     | 16,56  | 121,76             |
| 10                 | 24,4                    | 27,92  | 17,6   | 26,8   | 24     | 18,72  | 139,44             |
| Total <sup>1</sup> | 176,64                  | 209,2  | 128,16 | 196,88 | 158,32 | 137,12 | 1006,32            |

<sup>1</sup> Total oxytetracycline administered to each bird; <sup>2</sup> Total oxytetracycline administered per day of treatment.

**Table S3.** Substance specific mass spectrometric conditions.

| Analyte                 | Precursor ion<br>(m/z) | Product ion<br>(m/z) | DP <sup>4</sup> (V) | EP <sup>5</sup> (V) | CE <sup>6</sup> (V) | CXP <sup>7</sup> (V) |
|-------------------------|------------------------|----------------------|---------------------|---------------------|---------------------|----------------------|
| OTC <sup>1</sup>        | 461.000                | 426.0 <sup>8</sup>   | 72.0                | 10.0                | 28.0                | 25.0                 |
|                         |                        | 381.0                | 73.0                |                     | 36.0                | 22.0                 |
|                         |                        | 444.0                | 70.0                |                     | 30.0                | 15.0                 |
| 4-epi-OTC <sup>2</sup>  | 461.000                | 426.0 <sup>8</sup>   | 72.0                | 10.0                | 28.0                | 25.0                 |
|                         |                        | 381.0                | 73.0                |                     | 36.0                | 22.0                 |
|                         |                        | 444.0                | 70.0                |                     | 30.0                | 15.0                 |
| TC D6 (IS) <sup>3</sup> | 451.000                | 160.0                | 34.0                | 10.0                | 25.0                | 30.0                 |

<sup>1</sup> OTC: Oxytetracycline; <sup>2</sup> 4-epi-OTC: 4-epimer-oxytetracycline; <sup>3</sup> Tc D6 (IS): Tetracycline D6 (Internal Standard); <sup>4</sup> DP: Declustering potential; <sup>5</sup> EP: Entrance potential; <sup>6</sup> CE: Collision energy; <sup>7</sup> CXP: Collision cell exit potential; <sup>8</sup> quantitative ions.

**Table S4.** Validation parameters and acceptance criteria following guidelines 2002/657/EC and VICH GL49 for validation of analytical methodology.

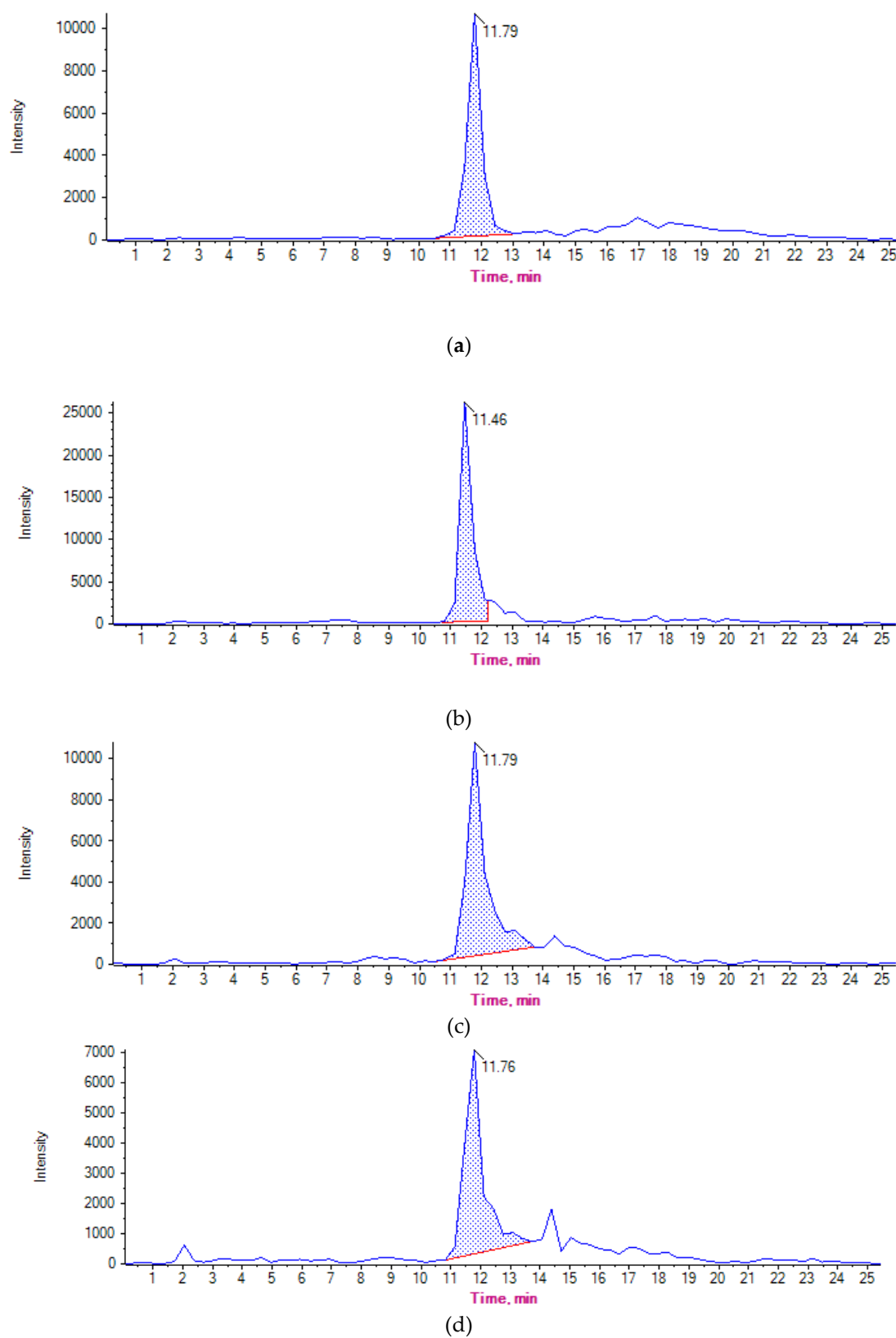
| Parameter                                  | Description of analysis performed                                                                                             | Criteria                                                                                                    |
|--------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|
| <b>Retention time</b>                      | 6 injections of certified standard                                                                                            | RSD <sup>1</sup> < 2.5%                                                                                     |
| <b>LOD<sup>2</sup> and LOQ<sup>3</sup></b> | 1 calibration curve at concentrations of 0.005, 0.010, 0.020, 0.050 y 0.100 µg/mL (5, 10, 20, 50 y 100 µg kg <sup>-1</sup> ). | LOQ criteria: Signal-to-noise ratio 3:1 RSD ≤ 10%<br><br>LOD criteria: Signal-to-noise ratio 10:1 RSD ≤ 10% |
| <b>Linearity</b>                           | 3 x 5 levels (0.25, 0.5, 1 and 2 times the limit of 50 µg kg <sup>-1</sup> , including zero)                                  | R <sup>2</sup> ≥ 0.99<br><br>CV ≤ 25%                                                                       |
| <b>Recovery</b>                            | 6 per level (one day) x 3 levels (0.5, 1, and 1.5 times the limit of 50 µg kg <sup>-1</sup> )                                 | 90%–110%                                                                                                    |
| <b>Specificity</b>                         | 20 blank samples                                                                                                              | No interferences                                                                                            |
| <b>Repeatability (Precision)</b>           | 6 x 3 levels (0.5, 1, and 1.5 times the limit of 50 µg kg <sup>-1</sup> ), under the same conditions (one level per day)      | RSD < reproducibility                                                                                       |
| <b>Reproducibility (Precision)</b>         | 6 x 3 levels (0.5, 1, and 1.5 times the limit of 50 µg kg <sup>-1</sup> ), under different conditions (one level per day)     | RSD Between 16% and 23%                                                                                     |
| <b>LOD and LOQ in matrix</b>               | 7 repetitions at the level of the IQL <sup>4</sup>                                                                            | LOQ criteria: Signal-to-noise ratio 3:1 RSD ≤ 10%<br><br>LOD criteria: Signal-to-noise ratio 10:1 RSD ≤ 10% |

<sup>1</sup> Relative standard deviation; <sup>2</sup> Limit of detection; <sup>3</sup> Limit of quantification; <sup>4</sup> Instrumental quantification limit.

**Table S5.** Validation of analytical methodology: Precision and recovery for droppings and litter.

| Analyte                | Matrix    | Work Concentration<br>( $\mu\text{g kg}^{-1}$ ) | RSD <sup>3</sup> of<br>Repeatability<br>(%) | RSD of<br>Reproducibility<br>(%) | Average<br>Recovery<br>(%) |
|------------------------|-----------|-------------------------------------------------|---------------------------------------------|----------------------------------|----------------------------|
| OTC <sup>1</sup>       | Droppings | 25                                              | 3.33                                        | 4.74                             | 104.3                      |
|                        |           | 50                                              | 3.15                                        | 5.17                             | 95.7                       |
|                        |           | 75                                              | 1.09                                        | 1.63                             | 101.4                      |
|                        | Litter    | 25                                              | 3.64                                        | 7.12                             | 91.9                       |
|                        |           | 50                                              | 3.54                                        | 6.06                             | 108.1                      |
|                        |           | 75                                              | 1.20                                        | 2.24                             | 97.3                       |
| 4-epi-OTC <sup>2</sup> | Droppings | 25                                              | 3.32                                        | 21.30                            | 98.4                       |
|                        |           | 50                                              | 3.22                                        | 20.62                            | 101.6                      |
|                        |           | 75                                              | 1.10                                        | 7.02                             | 99.5                       |
|                        | Litter    | 25                                              | 3.93                                        | 5.29                             | 96.0                       |
|                        |           | 50                                              | 3.82                                        | 4.89                             | 104.0                      |
|                        |           | 75                                              | 1.30                                        | 1.72                             | 98.7                       |

<sup>1</sup> OTC: Oxytetracycline; <sup>2</sup> 4-epi-OTC: 4-epimer-oxitetracycline; <sup>3</sup> Relative standard deviation.



**Figure S1.** Representative OTC chromatograms of the first sampling (1-day post the end of treatment) from samples of (a) droppings from group B; (b) droppings from group C; (c) litter from group B; (d) litter from group C. The chromatographic signal of OTC reaches at least 3 times the signal noise of the baseline.