The analysis of tetracyclines, quinolones, macrolides, lincosamides, pleuromutilins, and sulfonamides in chicken feathers using UHPLC-MS/MS in order to monitor antibiotic use in the poultry sector

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Abstract In The Netherlands, all antibiotic treatments should be registered at the farm and in a central database. To enforce correct antibiotic use and registration, and to enforce prudent use of antibiotics, there is a need for methods that are able to detect antibiotic treatments. Ideally, such a method is able to detect antibiotic applications during the entire lifespan of an animal, including treatments administered during the first days of the animals’ lives. Monitoring tissue, as is common practice, only provides a limited window of opportunity, as residue levels in tissue soon drop below measurable quantities. The analysis of feathers proves to be a promising tool in this respect. Furthermore, a qualitative confirmatory method was developed for the analyses of six major groups of antibiotics in ground chicken feathers, aiming for a detection limit as low as reasonably possible. The method was validated according to Commission Decision 2002/657/EC. All compounds comply with the criteria and, as a matter of fact, 58% of the compounds could also be quantified according to regulations. Additionally, we demonstrated that a less laborious method, in which whole feathers were analyzed, proved successful in the detection of applied antibiotics. Most compounds could be detected at levels of 2 μg kg⁻¹ or below with the exception of sulfachloropyridazine, tylosin, and tylvalosin. This demonstrates the effectiveness of feather analysis to detect antibiotic use to allow effective enforcement of antibiotic use and prevent the illegal, off-label, and nonregistered use of antibiotics.

Keywords Antibiotics · Validation · Feathers · Monitoring · LC-MS/MS

Introduction

The use of antibiotics is common practice to treat bacterial infections in the poultry sector. Antibiotic treatments, mostly orally administered through drinking water, should be carried out according to registration in order to prevent excessive antibiotic residues in food products meant for human consumption. Through Commission Regulation 96/23/EC [1], the European Union (EU) strictly monitors the presence of antibiotics in food of animal origin on the basis of EU/37/2010 [2], which establishes maximum residue limits (MRL) for matrices such as muscle, liver, and eggs.

Besides the issue of antibiotic residues in food products through which consumer exposure can occur, there has been a growing concern of antibiotic resistance, which is currently one of the major public health threats [3]. The use of antimicrobial agents is considered to be the most important factor in the selection of resistant bacteria, where superfluous use of antibiotics is often considered to be the main factor [4]. However, also the use of smaller amounts of antibiotics, e.g., as a preventive measure or contamination, can contribute to selection and persistence of resistant bacteria [5, 6].

Recently, The Netherlands has focused on prudent use of antibiotics to fight the increasing incidence of antibiotic resistance [5]. In order to prevent extensive or unnecessary use of antibiotics in The Netherlands, policies have been implemented to restrict the use of antibiotics. These policies require that every antibiotic treatment is recorded at the farm and in a central database. Previously, the antibiotic use could only be enforced through monitoring food products. However, the
The method and validation characteristics are presented here. Especially when antibiotics are administered early in the animal’s life, no residues are expected to be detected in food products. This indicates that regular analysis of food products at the MRL, while useful for monitoring for safety and certain good agricultural practice, is not suitable for the detection and prevention of nonregistered use of antibiotics.

There is a need for methods that are able to detect the use of antibiotics administered over the entire life span of an animal, including treatments administered during the first days of the animals’ lives. In previous research focusing on the excretion of oxytetracycline [7], multiple fluoroquinolones (enrofloxacin, its metabolite ciprofloxacin [8], and flumequin [9]) and flofenicol and its metabolite flofencicol amine [10] to feathers, it has already been shown that antibiotic residues can still be detected in feathers long after treatment. In another study, different antibiotics in feather meal, originating from different countries, were detected, including antibiotics that are registered as banned substances in the country of origin [11]. These findings support the claim that feathers are a promising matrix for monitoring antibiotic use in the poultry sector.

In the research investigating the excretion of oxytetracycline to feathers, it was suggested that after oral treatment, antibiotics enter the bloodstream and dispose into the rachis of feathers [7]. It was suggested that concurrently, the antibiotics can exit the body by excretion through the uropygial gland and are dispositioned on the feathers through grooming behavior [8].

Recently, more in-depth research on the disposition of antibiotics to feathers was done in order to explore the future possibilities of the use of this matrix for monitoring purposes [12]. This study involved segmentation of incurred feathers containing enrofloxacin, confirming that antibiotics are incorporated inside the feathers after oral treatment, as was already suggested by earlier results found based on segmentation of incurred feathers containing oxytetracycline [7]. This mechanism allows discrimination of different exposure routes and provides promising results for anedating antibiotic treatments.

In order to effectively monitor antibiotic use in the poultry sector, a broad confirmatory method is needed, covering multiple groups of antibiotics, applicable to feathers. To our knowledge, this is the first time a multi-residue method for the qualitative confirmatory analysis of tetracyclines, quinolones, macrolides, lincosamides, pleuromutilins, and sulfonamides in chicken feathers, using ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) is presented. The method was fully validated according to Commission Decision 2002/657/EC [13]. The method and validation characteristics are presented here.

Note that to cover an even wider range of relevant compounds, multiple analytical methods can be applied.

**Materials and methods**

The following antibiotics are referred to when different antibiotic classes are mentioned. Tetracyclines: chlortetracycline, oxytetracycline, tetracycline, and doxycycline. (Fluoro)quinolones: ciprofloxacin, danofloxacin, difloxacin, flumequin, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, and sarafloxacin. Macrolides: erythromycin, gamithromycin, josamycin, natamycin, neospiramycin, spiramycin, tildipirosin, tilmicosin, tulathromycin, tylosin, and tylvalosin. Lincosamides: lincomycin and pirlimycin. Pleuromutilins: tiamulin and valnemulin. Sulfonamides: dapsone, sulfacetamide, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadimidine, sulfadoxine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfamoxole, sulfaphenazole, sulfapyridine, sulfathiazole, sulfisoxazole, and sulfamonomethoxine.

**Reference standards**

The reference standards of chlortetracycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, flumequin, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, sarafloxacin, erythromycin, josamycin, lincomycin, spiramycin, tiamulin, tylosin, valnemulin, dapsone, sulfacetamide, sulfachloropyridazine, sulfadimethoxine, sulfadimidine, sulfadoxine, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfamoxole, sulfaphenazole, sulfapyridine, sulfathiazole, sulfisoxazole and sulfosulfadiazine were purchased at Sigma-Aldrich (St. Louis, MO, USA). Neospiramycin, pirlimycin, and natamycin were purchased at Toronto Research Chemicals (Toronto, ON, Canada). Doxycycline and sulfadiazine were purchased at Council of Europe (EDQM, Strasbourg, France). Gamithromycin and tulathromycin were purchased at Santa Cruz Biotechnology (Dallas, TX, USA). Tilmicosin was purchased at Dr. Ehrenstorfer GMBH (Augsburg, Germany), tylosin at ECO Animal Health (London, UK), tildipirosin at MSD Animal Health (Boxmeer, The Netherlands), and sulfamonomethoxine at TCI Europe (Zwijndrecht, Belgium).

The internal standards norfloxacin-d5, ciprofloxacin-d8, enrofloxacin-d3, sarafloxacin-d9, difloxacin-d3, oxolinic acid-d5, nalidixic acid-d5, flumequin-13C3, sulfathiazole-13C6, sulfapyridine-13C6, sulfamerazine-13C6, sulfadimidine-13C6, sulfoximethoxazole-13C6, sulfamethoxypyridazine-13C6, sulfadoxine-d3, sulfisoxazole-13C6, sulfadimethoxine-d6, and sulfadiazine-d6 were purchased at Witega (Berlin, Germany). Erythromycin-13C5-d6, spiramycin-d3, lincomycin-d3,
sulfadiazine-d$_4$, and dapsone-d$_8$ were purchased at Toronto Research Chemicals. Tetracycline-d$_6$ and gamithromycin-d$_4$ were purchased at Santa Cruz Biotechnology. Demeclocycline was purchased at Sigma-Aldrich and tildipirosin-d$_{10}$ at MSD Animal Health.

Reagents

Methanol ULC/MS grade (MeOH) and acetonitrile ULC/MS grade (ACN) were purchased at Actu-All Chemicals (Oss, The Netherlands). Formic acid (FA), citric acid monohydrate, sodium hydroxide (NaOH), and disodium hydrogen phosphate dihydrate were purchased at VWR International (Darmstadt, Germany). Ammonium hydroxide (25%) was purchased at Merck Millipore (Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased at Sigma-Aldrich. Milli-Q water, referred to as water from here on, was prepared using a Milli-Q system with a resistivity of at least 18.2 MΩ cm$^{-1}$ (Merck Millipore). McIlvain-ethylenediaminetetraacetic acid (EDTA) buffer was prepared by dissolving 74.4 g disodium EDTA (VWR International) in 500 mL 0.1 M citric acid and 280 mL 0.2 M phosphate buffer. The pH was adjusted to 4.0 by adding 0.1 M citric acid or 0.2 M phosphate buffer. The total volume was adjusted to 2 L.

Stock solutions of the reference standards and internal standards were made at 1000 mg L$^{-1}$ for tetracyclines, macrolides, lincosamides, pleuromutilins, and sulfonamides and at 100 mg L$^{-1}$ for quinolones. Tetracyclines and sulfonamides were dissolved in MeOH, quinolones in 2% 2M ammonia hydroxide in MeOH, lincosamides, tylosin, tiamulin, and valnemul in water, tildipirosin and natamycin in MeOH, and the remainder of the macrolides and pleuromutilins in ACN.

A mixed solution of reference standards was made at 0.5/0.1 mg L$^{-1}$ (tetracyclines, quinolones, macrolides, lincosamides, pleuromutilins/sulfonamides) in MeOH and a mixed solution of internal standards was made at 0.5 mg L$^{-1}$ for all compounds in MeOH.

Analysis procedure

The main purpose of analyzing feathers is to determine whether and which antibiotic residues are present. A distinction can be made between freely extractable and non-freely extractable residues if required. Freely extractable residues are those that can be extracted from whole feathers, as opposed to not freely extractable residues that can only be extracted after grinding, which indicates they are incorporated inside the feather [12]. The validated method is designed to allow three options. The first option is to extract whole feathers yielding only the freely extractable residues. This approach is simple and fast and is useful to efficiently and effectively detect what antibiotics are present. The second option is to grind the whole feathers before extraction, yielding the total amount of antibiotic residues. This approach is especially relevant if the total antibiotic concentration is to be determined. Last, the non-freely extractable residues can be determined by grinding the feathers.
feathers after a washing procedure of the whole feathers has been applied. This procedure is most laborious but is mandatory if treatments should be antedated using a segmentation procedure [7, 12]. A schematic overview of the individual steps for the different applications is displayed in Fig. 1.

Sample extraction

Detection of freely extractable residues

Weigh 1 g of feathers (approximately three large wing feathers) into a 50 mL polypropylene (PP) centrifuge tube (Greiner Bio-One, Alphen aan de Rijn, The Netherlands). Add internal standard solution and 2 mL of 0.125% TFA in MeOH to all samples. Shake thoroughly by hand and add 16 mL of McIlvain-EDTA buffer. Shake for 60 min using a rotary tumbler (Heidolph REAX-2, Schwabach, Germany) and centrifuge for 5 min at 3500 g. From here, extracts are submitted to sample clean-up.

Detection of total residues

Transfer 1 g of feather sample, cut to fit using secateurs, into a zirconium grinding bucket. Add a zirconium grinding ball (20 mm diameter) and grind two times for 4 min using a mixer mill (Type MM301, Retsch Haan, Germany). Weigh 100 mg of ground feather into a 12 mL PP centrifuge tube. Add internal standard solution to each sample and wait for 5 min. Add 2 mL 0.125% TFA in MeOH and place the centrifuge tubes in a water bath at 45 °C during 60 min. Subsequently, add 2 mL of McIlvain-EDTA buffer, shake for 5 min using a rotary tumbler and centrifuge at 3500 g for 10 min. Decant the supernatant into a 50 mL PP centrifuge tube and dilute with 14 mL McIlvain-EDTA buffer. From here, extracts are submitted to sample clean-up.

Detection of non-freely extractable residues

The analysis of non-freely extractable residues is similar to the procedure for analysis of total residues, but is preceded by a washing procedure. Weigh 1 g feathers (approximately three large whole feathers) into a 50 mL PP centrifuge tubes. Add 20 mL 0.125% TFA in MeOH and wash the feathers by shaking for 5 min using a rotary tumbler. Decant the washing solvent and repeat this procedure two more times and combine the wash solvent fractions. Dry the washed feathers overnight at room temperature and continue the procedure for analysis of total residues.

Sample clean-up

Condition a Strata-X reversed-phase polymeric SPE cartridge (Phenomenex, Torrance, CA, USA) with 5 mL MeOH and subsequently 5 mL water. Transfer the complete extract onto the cartridge and slowly pass it through (if needed by applying vacuum) to allow interaction between the SPE material and the antibiotic residues. Rinse the cartridges with 5 mL of water and dry by applying vacuum for 1 min. Elute the residues with 5 mL MeOH into a 14 mL glass tube. Evaporate the solvent (40 °C, N₂) using a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) and reconstitute the residues in 200 µL MeOH by using a vortex mixer (IKA, Staufen, Germany). Dilute with 300 µL water and transfer the extract into a glass vial suitable for LC-MS/MS analysis.

UHPLC-MS/MS

The UHPLC system consists of an Acquity model (Waters, Milford, MA, USA) with an Acquity HSS-T3 C₁₈ analytical column of 2.1 × 100 mm, 1.7 µm (Waters), placed in a column oven at 30 °C. The mobile phase consists of 2 mM ammonium formate and 0.16% FA in water (Solvent A) and 2 mM ammonium formate and 0.16% FA in MeOH (Solvent B). The gradient: 0–1.0 min, 0% mobile phase B, 1.0–2.5 min, linear increase to 25% B, 2.5–5.4 linear increase to 70% B, and 5.4–5.5 min linear increase to 100% with a final hold of 1.0 min. The gradient is returned to its initial conditions within 0.1 min and the column is allowed to equilibrate for 0.9 min before the next injection is initiated, resulting in a total run of 7.5 min. The flow rate is 0.4 mL min⁻¹ and the injection volume is 5 µL. Detection is carried out by MS/MS using a Xevo TQS (Waters) in positive electrospray ionization (ESI) mode. The operating parameters are: capillary voltage, 3.0 kV; source temperature, 130 °C; desolvation temperature, 450 °C; cone gas flow, 150 L h⁻¹; and desolvation gas, 650 L h⁻¹. The antibiotics were fragmented using collision induced dissociation (argon). SRM transitions were selected based on the abundance of the signal and, if multiple options were available, the selectivity of the transition [14] (Table 1). Data were acquired and processed using MassLynx 4.1 software (Waters).

Method validation

Although the method was designed as a qualitative confirmatory method, a full validation was performed according to quantitative confirmatory criteria as described in Commission Decision 2002/657/EC, which implements Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. The full
| Compound               | Precursor ion | Product ion | Cone (V) | Collision energy (eV) |
|------------------------|---------------|-------------|----------|-----------------------|
| Chlortetracycline      | 478.9         | 153.9       | 2        | 26                    |
|                        |               | 444.0       |          |                       |
| Tetracycline           | 444.9         | 410.0       | 2        | 18                    |
|                        |               | 153.9       |          |                       |
| Tetracycline d₅        | 450.9         | 416.0       | 2        | 18                    |
| Oxytetracycline        | 460.9         | 426.0       |          | 16                    |
|                        |               | 200.9       |          |                       |
| Demeclocycline         | 465.3         | 430.2       | 2        | 20                    |
| Doxycycline            | 444.9         | 428.0       | 2        | 20                    |
|                        |               | 320.9       |          |                       |
| Marbofloxacin          | 363.0         | 319.9       | 20       | 14                    |
| Norfloxacin            | 320.0         | 231.0       | 20       | 19                    |
| Norfloxacin d₅         | 325.1         | 231.0       | 20       | 37                    |
| Ciprofloxacin          | 332.0         | 231.0       | 20       | 33                    |
|                        |               | 288.0       |          | 17                    |
| Ciprofloxacin d₅       | 340.1         | 235.0       | 20       | 34                    |
| Danofloxacin           | 358.0         | 82.0        | 20       | 55                    |
|                        |               | 255.1       |          | 37                    |
| Danofloxacin d₅        | 361.0         | 85.0        | 20       | 55                    |
| Enrofloxacin           | 360.1         | 316.0       | 20       | 18                    |
|                        |               | 286.0       |          | 31                    |
| Enrofloxacin d₅        | 365.1         | 321.0       | 20       | 18                    |
| Sarafloxacin           | 386.1         | 342.0       | 20       | 18                    |
|                        |               | 299.0       |          | 25                    |
| Sarafloxacin d₆        | 394.0         | 350.0       | 20       | 18                    |
| Difloxacin             | 400.0         | 356.0       | 20       | 18                    |
|                        |               | 299.0       |          | 26                    |
| Difloxacin d₅          | 403.0         | 359.0       | 20       | 19                    |
| Nalidixic acid         | 233.1         | 215.2       | 25       | 15                    |
|                        |               | 187.1       |          | 25                    |
| Nalidixic acid d₅      | 238.1         | 220.2       | 25       | 15                    |
| Oxolinic acid          | 262.1         | 160.2       | 25       | 35                    |
|                        |               | 244.2       |          | 20                    |
| Oxolinic acid d₅       | 267.1         | 249.2       | 25       | 20                    |
| Flumequine             | 262.1         | 244.2       | 25       | 20                    |
|                        |               | 202.1       |          | 30                    |
| Flumequine ¹³C₅        | 265.1         | 247.2       | 25       | 20                    |
| Tildipirosin           | 368.0         | 98.3        | 25       | 20                    |
|                        |               | 174.2       |          | 20                    |
| Tildipirosin d₁₀       | 372.9         | 108.0       | 25       | 15                    |
| Tulathromycin          | 404.0         | 72.1        | 25       | 38                    |
|                        |               | 158.3       |          | 20                    |
| Lincomycin             | 407.1         | 126.1       | 25       | 22                    |
|                        |               | 359.0       |          | 17                    |
| Lincomycin d₃          | 410.1         | 129.1       | 25       | 23                    |
| Spiramycin I           | 422.3         | 101.3       | 25       | 20                    |
|                        |               | 174.1       |          | 18                    |
| Compound          | Precursor ion | Product ion<sup>a</sup> | Cone (V) | Collision energy (eV) |
|-------------------|---------------|--------------------------|----------|-----------------------|
| Spiramycin I d₃  | 423.7         | 174.0                    | 25       | 18                    |
| Neospiramycin I   | 699.4         | 174.1                    | 10       | 25                    |
| Pirlimycin        | 411.0         | 112.0                    | 25       | 21                    |
|                   |               | 363.1                    |          | 15                    |
| Tilmicosin        | 435.3         | 98.9                     | 25       | 17                    |
|                   |               | 143.2                    |          | 15                    |
| Gamithromycin     | 777.5         | 116.4                    | 25       | 31                    |
| Gamithromycin d₄ | 781.5         | 158.0                    | 25       | 31                    |
| Tiamulin          | 494.1         | 192.1                    | 25       | 18                    |
|                   |               | 119.0                    |          | 37                    |
| Erythromycin      | 734.2         | 158.2                    | 25       | 25                    |
|                   |               | 576.4                    |          | 15                    |
| Erythromycin C₁₃ d₃ | 738.2      | 162.0                    | 25       | 25                    |
| Tylosin           | 916.2         | 174.0                    | 25       | 31                    |
|                   |               | 772.2                    |          | 29                    |
| Valnemulin        | 565.1         | 263.1                    | 25       | 17                    |
|                   |               | 164.1                    |          | 29                    |
| Josamycin         | 828.1         | 109.0                    | 25       | 55                    |
|                   |               | 174.0                    |          | 27                    |
| Tyvalosin         | 1042.2        | 109.3                    | 25       | 30                    |
|                   |               | 174.0                    |          | 34                    |
| Natamycin         | 666.3         | 485.3                    | 25       | 5                     |
|                   |               | 503.3                    |          | 25                    |
| Sulfadiazine      | 251.0         | 156.0                    | 25       | 13                    |
|                   |               | 91.9                     |          | 23                    |
| Sulfadiazine d₄  | 255.0         | 160.0                    | 25       | 15                    |
| Sulfacetamide     | 215.1         | 92.0                     | 25       | 18                    |
|                   |               | 155.9                    |          | 15                    |
| Sulfapyridine     | 250.2         | 91.9                     | 25       | 30                    |
|                   |               | 155.9                    |          | 15                    |
| Sulfapyridine<sup>13</sup>C₆ | 256.2 | 97.9                     | 25       | 30                    |
| Sulfathiazole     | 256.0         | 155.9                    | 25       | 14                    |
|                   |               | 91.9                     |          | 23                    |
| Sulfathiazole<sup>13</sup>C₆ | 262.0 | 161.9                    | 25       | 14                    |
| Sulfamerazine     | 265.0         | 91.9                     | 25       | 30                    |
|                   |               | 155.9                    |          | 15                    |
| Sulfamerazine<sup>13</sup>C₆ | 271.0 | 97.9                     | 25       | 25                    |
| Dapsone           | 249.0         | 155.9                    | 25       | 13                    |
|                   |               | 107.9                    |          | 19                    |
| Dapsone d₈        | 257.0         | 160.0                    | 25       | 14                    |
| Sulfamoxole       | 268.2         | 156.1                    | 25       | 13                    |
|                   |               | 92.2                     |          | 25                    |
| Sulfamethizole    | 271.0         | 155.9                    | 25       | 13                    |
|                   |               | 92.0                     |          | 25                    |
| Sulfamethizole<sup>13</sup>C₆ | 277.0 | 162.0                    | 25       | 14                    |
| Sulfadimidine     | 279.0         | 186.0                    | 25       | 15                    |
|                   |               | 124.0                    |          | 21                    |
| Sulfadimidine<sup>13</sup>C₆ | 285.0 | 185.9                    | 25       | 17                    |
validation was carried out using the worst-case scenario: determination of total residue as described above as this will result in the most challenging sample extracts (excluding washing and including grinding). The following parameters related to a qualitative confirmatory method were determined: selectivity, stability, confirmation of the identity, decision limit (CCα), and detection capability (CCβ). Limit of detection (LOD) and limit of confirmation (LOC) were additionally determined for the analysis of freely extractable residues. In order to critically assess the applicability of the method, quantitative confirmatory parameters were assessed additionally. These include trueness, repeatability (RSDr), repeatability including matrix variation (RSDr*), within-laboratory reproducibility (RSDRL), and linearity.

The validation was carried out using blank chicken feather samples from different origins (n = 21). Ultimately, a validation is carried out using incurred certified reference materials. Because these are not available for this specific application, the second best option was used: spiking. Since there is no MRL or other target level for antibiotics in feathers, target levels were chosen based on MRLs in other animal matrices and the performance of the method during method development. The target level for tetracyclines, quinolones, macrolides, lincosamides, and pleuromutilins was set at 100 µg kg⁻¹ and for sulfonamides at 20 µg kg⁻¹. The validation was carried out at three different levels (0.5, 1.0, and 1.5 * target level), on three different occasions by two different technicians.

Quantitative results were obtained through correction with the corresponding isotopically labeled internal standards, if available. In case no isotopically labeled internal standard was available, corrections were made using another internal standard. Chlortetracycline, oxytetracycline, and doxycycline were corrected utilizing demeclocycline; marbofloxacin using norfloxacin-d₅; josamycin, natamycin, tiamulin, and valnemulin using erythromycin-¹³C-d₃; tylosin, neosporamycin I, pirlimycin, tilmicosin, and tylosin using gamithromycin-d₄; tulathromycin using lincomycin-d₃; sulfamonomethoxine using sulfisoxazole-¹³C₆, and sulfamoxole, sulfacetamide, and sulfaphenazole using sulfadimethoxine-d₆.

In the stage of method development, it was already determined that when analyzing the total residues, chlortetracycline and natamycin could not be detected at relevant concentration levels. For this reason, they were excluded from the method validation.

### Table 1 (continued)

| Compound                      | Precursor ion | Product ion | Cone (V) | Collision energy (eV) |
|-------------------------------|---------------|-------------|----------|-----------------------|
| Sulfamethoxypyridazine        | 281.0         | 156.0       | 25       | 15                    |
| Sulfamethoxypyridazine d₃     | 284.0         | 156.0       | 25       | 14                    |
| Sulfamethoxazole              | 254.0         | 156.0       | 25       | 13                    |
| Sulfamethoxazole d₄           | 258.0         | 160.0       | 25       | 12                    |
| Sulfisoxazole                 | 268.0         | 156.0       | 25       | 13                    |
| Sulfisoxazole¹³C₆             | 274.0         | 162.0       | 25       | 30                    |
| Sulfamonomethoxine            | 281.2         | 156.1       | 25       | 20                    |
| Sulfachloropyridazine         | 284.9         | 155.9       | 25       | 26                    |
| Sulfachloropyridazine¹³C₆     | 290.9         | 161.9       | 25       | 14                    |
| Sulfadoxine                   | 311.1         | 155.9       | 25       | 15                    |
| Sulfadoxine d₃                | 314.0         | 155.9       | 25       | 17                    |
| Sulfaquinoxaline              | 301.0         | 155.9       | 25       | 15                    |
| Sulfaquinoxaline¹³C₆          | 307.0         | 161.9       | 25       | 15                    |
| Sulfadimethoxine              | 311.1         | 156.1       | 25       | 15                    |
| Sulfadimethoxine d₆           | 317.0         | 162.1       | 25       | 19                    |
| Sulfaphenazole                | 315.0         | 92.0        | 25       | 21                    |
Selectivity

In order to determine the selectivity, 21 blank samples were analyzed without addition of any reference standards except the internal standards. Selectivity was assessed by checking the signal of the blank materials for interferences at the retention times corresponding to the antibiotics.

Stability

Stability data for the antibiotics in solution were reported previously [15]. Stability in matrix was determined by storing one of the validation series at −80 °C for 28 d. After 28 d, the samples were re-analyzed and results were re-evaluated based on the validation criteria.

Decision limit (CCα) and detection capability (CCβ)

For the qualitative analysis of each compound, CCβ was set at the spiking concentration at which in at least 95% of the samples the identity of the compound was confirmed. Per definition, CCα is below this value. For compounds that can be analyzed quantitatively, CCα and CCβ were estimated using the linearity approach as stated in CD 2002/657/EC [13] according to the procedures established in ISO 11843 [16]. The resulting outcomes were visually verified based on the chromatograms of the samples spiked at the lowest validation level.

Limit of detection (LOD) and limit of confirmation (LOC)

For the analysis of freely extractable residues, the LOD and LOC were determined by analyzing samples spiked at 0.02, 0.05, and 0.1 g target level on four different occasions. For this assessment, three different LC-MS/MS systems that are regularly used for analysis of feathers were applied, including a Waters Xevo TQS systems and two AB Sciex QTrap 6500 (Framingham, MA, USA) systems. The LOD is established as the concentration level at which the response of the most intense ion was still detectable (S/N ≥ 3). The LOC was set at the concentration level at which the least abundant diagnostic ion was still detectable (S/N > 3). For both parameters, a worst case approach was chosen, so the LOD and LOC are well achievable on all occasions.

Confirmation of identity

In 2002/657/EC, criteria were established for the allowed deviation of both diagnostic ions (ion ratio) resulting from an unknown sample. These criteria are summarized in Table 2. Note that these criteria are currently under debate [17]. Furthermore, the relative retention time of an antibiotic should not deviate more than 2.5% from the reference relative retention time. In order to assess the possibility to confirm the identity of a detected compound using the presented method, the average ion ratio and the average relative retention time of the matrix matched calibration samples were used as the reference value. To comply with the criteria established for a confirmatory analysis, over 95% of the validation samples should comply with these confirmatory criteria.

Table 2 Criteria for the confirmation of the identity according to CD 2002/657/EC [13]

| Ion ratio reference (R) | Allowed deviation ion ratio of unknown sample |
|------------------------|---------------------------------------------|
| R > 50%                | ≤ 20%                                       |
| 20% < R ≤ 50%          | ≤ 25%                                       |
| 10% < R ≤ 20%          | ≤ 30%                                       |
| R ≤ 10%                | ≤ 50%                                       |

Trueness, repeatability, selectivity, and within-lab reproducibility

During one day, seven different blank batches of ground chicken feathers were spiked at three levels: 50, 100, and 150 μg kg⁻¹ for tetracyclines, quinolones, macrolides, lincosamides, and pleuromutilins, and 10, 20, and 30 μg kg⁻¹ for sulfonamides. The use of different blank feather batches during validation results in a better understanding of the between-sample variation and will reflect a routine analysis situation better. Therefore, the calculated repeatability will include matrix variation, which will be indicated as RSDr. In order to also determine the true repeatability (RSDr) as described in 2002/657/EC [13] (being the repeatability within a single batch), additionally seven samples of the same batch were spiked at target level.

The entire procedure was carried out on three different occasions, which adds up to a total of 21 feather sample batches obtained over three different days at each of the concentration levels. Response factors were calculated by dividing the area of the most abundant product ion of the compound by the area of the internal standard. Trueness, repeatability, and within-lab reproducibility were calculated using analysis of variance (ANOVA).

The performance criteria were established based on the target values, meaning that in this case trueness must lie between 80 and 110%. According to 2002/657/EC, the relative within-lab reproducibility (RSDR) is considered to be acceptable if below the value calculated from the Horwitz equation [18]. However, as demonstrated by Thompson [19], the Horwitz equation is not applicable to the lower concentration range (<120 μg kg⁻¹) and therefore a complementary model was suggested. We adopted these more stringent criteria. Following the complementary model, the RSDR for the...
levels <120 $\mu$g kg$^{-1}$ should be below 22%. Calculated using the Horwitz equation, the RSD$_{RL}$ for the target level 150 $\mu$g kg$^{-1}$ should be below 21.1%. The RSD$_r$ is found acceptable if below two-thirds of the RSD$_{RL}$, meaning that for levels <120 $\mu$g kg$^{-1}$, RSD should be below 14.7% and below 14.1% at 150 $\mu$g kg$^{-1}$. For RSD$_{r*}$ the same criteria were applied as for RSD$_r$.

**Linearity**

On three different days a matrix matched calibration line was prepared at 0, 0.5, 1.0, 2.0, and 3.0 * target level by adding solutions of the antibiotics to aliquots of a ground blank chicken feather sample (a different batch than the seven that were used for determination of trueness, RSD$_r$, RSD$_{r*}$, and RSD$_{RL}$). Calibration lines were constructed by plotting the response factors versus the added concentration and carrying out least squares linear regression. The linearity was considered acceptable if the coefficient of correlation was at least 0.990.

**Analysis of feathers samples**

Feather samples were collected in the slaughter phase. These were analyzed to assess the presented approach. In this case, the method for detection of freely extractable residues was applied as described above. A matrix matched calibration curve was prepared by spiking at 0, 0.5, 2.5, 5, 15, and 25 ng to 1 g of whole feathers for sulfonamides and 0, 2, 10, 20, 60, and 100 ng to 1 g of whole feathers for tetracyclines, quinolones, macrolides, lincosamides, and pleuromutilins. LC-MS/MS analysis for this study was carried out using UHPLC parameters as described above. Detection was done using an AB Sciex Q-Trap 6500 mass spectrometer in the positive electrospray ionization (ESI) mode. The operating parameters were: capillary voltage, 2.0 kV; cone voltage, 25 V; source temperature, 150 °C; desolvation temperature, 550 °C; cone gas flow, 150 L h$^{-1}$; and desolvation gas, 600 L h$^{-1}$. Data were acquired and processed using MultiQuant 2.02 software (AB Sciex).

During this research, all procedures regarding human and animal rights were followed.

**Results and discussion**

**Washing versus extraction procedure**

In earlier research investigating the distribution of enrofloxacin and ciprofloxacin to feathers [12], it was already shown that in order to discriminate extractable and non-extractable residues, a similar solvent should be used for both washing and extraction, because when washing with a similar solvent as used for extraction, it can be stated that the residues obtained during extraction after washing originate from non-extractable residues, only accessible after grinding. Therefore, based on the results and this assumption, the best washing solvent for this purpose is 0.125% TFA in MeOH.

**LC-MS/MS**

The LC method applied for analysis of the antibiotics in this application is a very generic separation applying common mobile phases and gradient elution. Although our research showed that the selection of the UHPLC column was not very critical [20], a universal column that was especially designed to retain both polar and non-polar compounds and which is compatible with 100% aqueous mobile phases was used. This yielded somewhat higher retention of some polar compounds.

The detection was carried out using tandem MS in Multiple Reaction Monitoring mode. The precursor ions and product ions were determined by continuous infusion of the individual compounds and the ionization setting were optimized. The selection of product ions was done based on their abundance (to allow low detection limits) in combination with their selectivity (in case multiple product ions showing sufficiently high signals were observed) [14].

**Validation**

The aim of the validation was to assess the qualitative confirmatory aspect of the method, and additionally the quantitative aspect was evaluated. The results for trueness, repeatability, within-laboratory reproducibility, confirmation of the identity, CC$\alpha$, and CC$\beta$ are presented in Table 3. Confirmation of the identity is expressed as the percentage of positively confirmed samples out of the 21 spiked samples (seven per validation level on three different occasions). As a result of an outlying result, most probably caused during spiking, most results are based on 20 results instead of 21. In case of tulathromycin, the results are based on a 1-d validation and additional data will be collected during future analysis.

**Qualitative performance**

There were no interfering signals in the blank samples at the retention times corresponding to the product ions of any of the validated compounds. The selectivity of the method is therefore considered to be sufficient. After 28 d of storage at −80 °C, the samples were re-analyzed and results were re-evaluated based on the qualitative validation criteria. For the quantitative compounds, trueness was also re-evaluated. After 28 d, all compounds still complied with the qualitative and/or quantitative validation criteria and therefore it was concluded that the sample extracts are stable for at least 28 d if stored at −80 °C.

At all concentration levels, the identity for all compounds is confirmed in at least 95% of the samples, except for nalidixic acid and norfloxacin. For norfloxacin, the sensitivity of the second
### Table 3

| Analyte          | Levels (µg kg⁻¹) | Trueness (%) | RSDr (%) | RSDα (%) | RSDβ (%) | Identity confirmed (%) | CCα⁺ (µg kg⁻¹) | CCβ⁺ (µg kg⁻¹) |
|------------------|------------------|--------------|----------|----------|----------|------------------------|----------------|----------------|
| **Tetracyclines**|                  |              |          |          |          |                        |                |                |
| Doxycycline      | 50 (n=20)        | 103          | 17.1     | 18.7     | 100      | <50                    | 50             | 50             |
|                  | 100              | 89           | 5.6      | 16.3     | 20.9     | 100                    | <50            | 50             |
|                  | 150 (n=20)       | 85           | 17.4     | 24.6     | 100      |                        |                |                |
| Oxytetracycline  | 50               | 99           | 6.3      | 12.1     | 13.0     | 100                    | <50            | 50             |
|                  | 100              | 95           | 8.0      | 14.2     | 100      |                        |                |                |
| Tetracycline     | 50 (n=20)        | 106          | 5.2      | 11.8     | 21.3     | 100                    | 40             | 50             |
|                  | 100              | 98           | 9.1      | 21.3     | 100      |                        |                |                |
| **Quinolones**   |                  |              |          |          |          |                        |                |                |
| Ciprofloxacin    | 50 (n=20)        | 93           | 8.5      | 8.7      | 100      | 20                     | 50             | 50             |
|                  | 100              | 96           | 6.2      | 3.9      | 5.2      | 100                    | 20             | 50             |
|                  | 150 (n=20)       | 98           | 7.4      | 9.0      | 100      |                        |                |                |
| Danofloxacin     | 50 (n=20)        | 95           | 14.1     | 15       | 100      | 30                     | 50             | 50             |
|                  | 100              | 97           | 4.7      | 7.3      | 7.6      | 100                    | 30             | 50             |
| Difloxacin       | 50 (n=20)        | 99           | 9.1      | 9.4      | 100      |                        |                |                |
|                  | 100              | 104          | 4.8      | 12.5     | 95       | 50                     | 50             | 50             |
| Enrofloxacin     | 50 (n=20)        | 98           | 8.8      | 10       | 100      |                        |                |                |
|                  | 100              | 101          | 3.8      | 6.1      | 6.3      | 100                    | 30             | 50             |
| Flumequin        | 50 (n=20)        | 103          | 8.5      | 11.6     | 100      |                        |                |                |
|                  | 100              | 103          | 12.2     | 3.5      | 4.3      | 100                    | 30             | 50             |
| Marbofloxacin    | 50               | 114          | 29.2     | 38.3     | 100      | <50                    | 50             | 50             |
|                  | 100              | 127          | 6.8      | 28.8     | 37.9     | 100                    | <50            | 50             |
| Difloxacin       | 50 (n=20)        | 107          | 8.7      | 12.1     | 90       |                        |                |                |
| Nalidixic acid   | 50 (n=20)        | 99           | 7.5      | 8.7      | 100      | 20                     | 50             | 50             |
| Norfloxacin      | 50 (n=20)        | 95           | 15.9     | 17.5     | 100      | 40                     | 80             | 50             |
| Oxylic acid      | 50 (n=20)        | 98           | 10.3     | 10.8     | 95       |                        |                |                |
| Sarafloxacin     | 50 (n=20)        | 99           | 11.8     | 14       | 100      |                        |                |                |
|                  | 100              | 104          | 6.9      | 5.7      | 10.2     | 100                    | 30             | 50             |
| Macrolides       |                  |              |          |          |          |                        |                |                |
| Erythromycin     | 50 (n=20)        | 109          | 11.5     | 11.8     | 100      | 30                     | 50             | 50             |
|                  | 100              | 106          | 8.9      | 7.1      | 8        | 100                    | 30             | 50             |
|                  | 150 (n=20)       | 103          | 6.1      | 8.8      | 100      |                        |                |                |
| Gamithromycin    | 50 (n=20)        | 116          | 3.3      | 8.3      | 12.4     | 100                    | <50            | 50             |
|                  | 100              | 108          | 3.3      | 14.6     | 14.6     | 100                    | <50            | 50             |
| Josamycin        | 50 (n=20)        | 134          | 5.13     | 53.8     | 100      |                        |                |                |
|                  | 100              | 120          | 4.9      | 38.1     | 38.7     | 100                    | <50            | 50             |
| Neospiramycin    | 50 (n=20)        | 125          | 38.3     | 38.5     | 100      |                        |                |                |
|                  | 100              | 116          | 6.6      | 31       | 31.8     | 100                    | <50            | 50             |
| Spiramycin       | 50               | 104          | 17.4     | 27.4     | 100      | 40                     | 50             | 50             |
|                  | 100              | 107          | 1.8      | 4.5      | 7.5      | 100                    | 40             | 50             |
| Tildipirosin     | 50 (n=20)        | 105          | 9.1      | 9.1      | 100      |                        |                |                |
| Tilmicosin       | 50 (n=20)        | 77           | 39.8     | 50.8     | 95       |                        |                |                |
|                  | 100              | 73           | 15.2     | 37.5     | 41.1     | 100                    | <50            | 50             |
The analysis of tetracyclines, quinolones, macrolides

Table 3 (continued)

| Analyt                | Levels (µg kg⁻¹) | Trueness (%) | RSD₁ (%) | RSDᵦ (%) | RSDᵦᵢ (%) | Identity confirmed (%) | CCα² (µg kg⁻¹) | CCβ² (µg kg⁻¹) |
|-----------------------|------------------|--------------|-----------|-----------|------------|-----------------------|----------------|---------------|
| Tulathromycin         | 50₀ (n=7)        | 85           | 45.3      | 72.5      | 100        | <50 50                |                |               |
|                       | 100₀ (n=7)       | 82           | 7.3       | 31.3      | 50.1       | 100                   |                |               |
|                       | 150₀ (n=7)       | 112          | 26.6      | 26.6      | 29.9       | 100                   |                |               |
|                       | 100              | 86           | 25.8      | 26.6      | 26.6       | 100 <50 50            |                |               |
|                       | 150 (n=20)       | 94           | 60.1      | 63.5      | 100        |                       |                |               |
| Tylosin               | 50₀ (n=20)       | 118          | 30.9      | 33.4      | 100        |                       |                |               |
|                       | 100              | 101          | 27        | 23.3      | 23.7       | <50 50                |                |               |
|                       | 150 (n=20)       | 120          | 75.8      | 78.9      | 100        |                       |                |               |
| Lincomycin            | 50₀ (n=20)       | 105          | 7.5       | 7.9       | 100        |                       |                |               |
|                       | 100              | 106          | 3.7       | 3.6       | 100        | 20 50                 |                |               |
|                       | 150 (n=20)       | 105          | 5.3       | 9.4       | 100        |                       |                |               |
| Pirlimycin            | 50₀ (n=20)       | 110          | 22.4      | 30.6      | 100        |                       |                |               |
|                       | 100              | 107          | 26.5      | 31.2      | 100 <50 50 |                       |                |               |
|                       | 150 (n=20)       | 99           | 21.6      | 33.9      | 100        |                       |                |               |
| Tiamulin              | 50₀ (n=20)       | 129          | 35.9      | 36.1      | 100        |                       |                |               |
|                       | 100              | 119          | 9.8       | 22.5      | 22.6       | <50 50                |                |               |
|                       | 150 (n=20)       | 125          | 26.6      | 27.5      | 100        |                       |                |               |
| Valnemulin            | 50₀ (n=20)       | 123          | 34.5      | 39.2      | 100        |                       |                |               |
|                       | 100              | 103          | 10.6      | 19.8      | 25.5       | <50 50                |                |               |
|                       | 150 (n=20)       | 109          | 22.6      | 34.8      | 100        |                       |                |               |
| Dapsone               | 10               | 102          | 16.2      | 17.2      | 100        |                       |                |               |
|                       | 20               | 102          | 2.3       | 4.4       | 7.0        | 100 5 10              |                |               |
|                       | 30₀ (n=20)       | 99           | 3.8       | 10.2      | 100        |                       |                |               |
| Sulfacetamide         | 10               | 101          | 1.7       | 18.1      | 95         |                       |                |               |
|                       | 20               | 104          | 3.7       | 9.0       | 100        | <10 10 <10 10         |                |               |
| Sulfachloropyridazine | 10               | 103          | 16.0      | 16.0      | 100        |                       |                |               |
|                       | 20               | 105          | 5.1       | 5.2       | 100        | 5 10                  |                |               |
| Sulfadiazine          | 10               | 104          | 15.6      | 15.8      | 100        |                       |                |               |
|                       | 20               | 103          | 1.4       | 4.0       | 4.6        | 100 6 10              |                |               |
|                       | 30₀ (n=20)       | 102          | 5.7       | 8.7       | 95         |                       |                |               |
| Sulfadimethoxine      | 10               | 105          | 14.4      | 14.9      | 100        |                       |                |               |
|                       | 20               | 106          | 5.6       | 3.8       | 4.7        | 100 5 9               |                |               |
| Sulfadimidine         | 10               | 103          | 5         | 5         | 8.5        | 100 <10 10 <10 10    |                |               |
|                       | 20               | 105          | 5.2       | 4.4       | 5          | 100 5 10              |                |               |
| Sulfadoxine           | 10               | 103          | 14.6      | 15        | 100        |                       |                |               |
|                       | 20               | 106          | 7.1       | 4.4       | 4.6        | 100 5 10              |                |               |
| Sulfamerazine         | 10               | 104          | 15.0      | 15.5      | 100        |                       |                |               |
|                       | 20               | 105          | 1.7       | 3.5       | 4.2        | 100 5 9               |                |               |
| Sulfamethazine        | 10               | 103          | 4.6       | 6.8       | 100        |                       |                |               |
|                       | 20               | 103          | 3.0       | 5.8       | 5.9        | 100 5 9               |                |               |
| Sulfamethoxazole      | 10               | 107          | 16.5      | 17.4      | 100        |                       |                |               |
|                       | 20               | 108          | 19.5      | 6.5       | 8.7        | 100 6 10              |                |               |
| Sulfamethoxypyridazine| 10               | 105          | 3.1       | 5         | 5.1        | 100 4 9               |                |               |
| Sulfadoxime           | 10               | 101          | 6.1       | 8.9       | 100        |                       |                |               |
| Sulfamoxole           | 10               | 101          | 21.1      | 22.4      | 100        |                       |                |               |
|                       | 20               | 105          | 2.7       | 14.4      | 16.9       | <10 10 <10 10         |                |               |
| Sulfaphenazole        | 10₀ (n=20)       | 93           | 13.5      | 15.2      | 95         |                       |                |               |
|                       | 20               | 93           | 4.7       | 10.3      | 11.7       | <10 10 <10 10         |                |               |
| Sulfapyridine         | 10               | 102          | 14.2      | 14.2      | 100        |                       |                |               |
diagnostic ion was, for unknown reasons, low during the first validation day compared with the other days. This resulted in a higher error and for three out of the seven samples the identity could not be confirmed. For tiamulin the peak shape was suboptimal during the first day of validation. This resulted in an ion ratio deviation that is slightly higher than permitted according to the criteria. On validation day 2 and 3 all additions in all samples satisfy the ion ratio criteria. It is expected that all antibiotics can be detected and their identity can be confirmed using the applied method at the indicated concentration levels.

### Quantitative performance

For tetracyclines, trueness lies between 85 and 106%, for quinolones between 93 and 131%, for macrolides between 73 and 134%, for lincosamides between 99 and 107%, for pleuromutilines between 103 and 129%, and for sulfonamides between 93 and 116%.

Linearity was determined using a calibration curve that was injected before and after the samples. For most compounds, linearity complies with the established criterion. For some compounds (tiamulin, sulfadiazine, tilmicosin, tyllosin, ciprofloxacin, norfloxacin, and natamycin) a coefficient of correlation of <0.990 was found on one occasion; in most cases the calibration line at the end of a sample batch.

For all compounds, the RSD$_r$ complies with the criteria, with the exception of sulfadiazine, pirlimycin, tyllosin, and tyleclosin. This demonstrates that the method yields satisfactory quantitative performance for most compounds within a single batch of feathers. Note that for sulfadiazine, RSD$_r$ does comply with the criteria, indicating that the batch of feathers used for determination of RSD$_r$ is a challenging one. This also demonstrates the importance of including a large number of different feather batches in a validation.

For 26 out of 45 compounds (58%), also the RSD$_{r*}$ and RSD$_{RL}$ comply with the criteria at the validation target level and higher. Most of these compounds also comply at 0.5*target level. Clearly, there is a large batch to batch variation, which is most likely the result of severe matrix effects, resulting in an increase of the analytical variation. Nevertheless, for these 26 compounds the method can be applied, not only for qualitative confirmatory analysis but also for quantification of the level of antibiotics present. Out of these 26 compounds, 25 compounds have an isotopically labeled standard available. Note that because repeatability of the method is sufficient, for the other 19 compounds the method can, besides only for qualitative confirmatory analysis, also be applied for quantitative analysis within a single batch as is appropriate for feather segmentation analysis [7, 12].

Quantitative performance could be further improved by using additional isotopically labeled internal standards, if commercially available.

### LOD and LOC for freely-extractable residues

In the analysis of freely extractable residues, approximately 1 g of feather sample is taken into account. This results in a significantly lower LOD and LOC compared with the analysis of ground feathers (sample intake 100 mg). Therefore, additionally the
Table 4  An overview showing the comparison between the administrated treatments according to registration of the farmer in the food chain information and the results of the feather analysis of freely extractable residues

| Chicken # | Treatment | Active compound(s) | Start of treatment (days before slaughter) | No. of days treated | Concentration (mg kg\(^{-1}\) body weight/d) | Detected freely extractable residues | Component | Amount (ng g\(^{-1}\)) |
|-----------|-----------|--------------------|--------------------------------------------|--------------------|-----------------------------------------------|-------------------------------------|-----------|----------------------|
| 1         | Spectron, Laboratorios Hipra S.A. (Zul.-Nr. 401356.00.00) | Enrofloxacin | 42 | 3 | 10 | Enrofloxacin | 49 |
| 2         | Pyanosid Pulver, Bela-Pharm (Zul.-Nr. 13076.00.00) | Lincomycin/Spectinomycin | 42 | 3 | 16.6 / 33.3 | Ciprofloxacin/Lincomycin | 5 / 4 |
| 3         | Lanflox, Dopharma Research (Zul.-Nr. 401347.00.00) | Enrofloxacin | 34 | 3 | 10 | - | - |
| 4         | Lanflox, Dopharma Research (Zul.-Nr. 401347.00.00) | Sulfadimidine | 27 | 6 | 100 | Sulfadimidine | 5300 |
| 5         | Sulfadimidine Na, Eurovet Animal Health BV (Zul.-Nr. 6093467.00.00) | Lincomycin | 30 | 3 | 16.6 / 33.3 | Lincomycin | 3 |
| 6         | Lanflox, Dopharma Research (Zul.-Nr. 401347.00.00) | Sulfadimidine | 27 | 6 | 100 | Sulfadimidine | 4400 |
| 7         | Methoxasol-T, Dechra Veterinary Products (Zul.-Nr. 401190.00.00) | Sulfamethoxazole/Trimethoprim | 45 | 4 | 33 | Sulfamethoxazole | 15 |
| 8         | T.S.-Sol 20/100, Dopharma Research (REG NL 7611) | Sulfamethoxazole/Trimethoprim | 21 | 3 | 37.5 | Sulfamethoxazole | 3 |
| 9         | T.S.-Sol 20/100, Dopharma Research (REG NL 7611) | Sulfamethoxazole/Trimethoprim | 21 | 3 | 37.5 | Sulfamethoxazole | 3 |
| 10        | Tylo-Suscit, Bela-Pharm (Zul.-Nr. 6933163.00.00) | Tylosin | 11 | 3 | 100 | Tylosin | 234* |
| 11        | T.S.-Sol 20/100, Dopharma Research (REG NL 7611) | Sulfamethoxazole/Trimethoprim | 38 | 3 | 37.5 | Sulfamethoxazole | 76 |
| 12        | Spectron, Laboratorios Hipra S.A. (Zul.-Nr. 401356.00.00) | Enrofloxacin | 34 | 3 | 10 | Enrofloxacin | 17 |
| 13        | Cosumix plus, Elanco Europe (REG NL 5388) | Sulfachloropyridazine/Trimethoprim | 8 | 3 | 30 | Sulfachloropyridazine | 1858 |
| 14        | Methoxasol-T, Dechra Veterinary Products (Zul.-Nr. 401190.00.00) | Sulfamethoxazole/Trimethoprim | 46 | 4 | 33 | Sulfamethoxazole | 7 |
| 15        | Tylan W.O., Elanco Europe (REG NL 9984) | Tylosin | 16 | 3 | 20-100 | Tylosin | 237* |
| 16        | Enro Sleecol, Eurovet Animal Health BV (Zul.-Nr. 401098) | Enrofloxacin | 40 | 3 | 5 | Enrofloxacin | 29 |
| 17        | Enro Sleecol, Eurovet Animal Health BV (Zul.-Nr. 401098) | Tylosin | 30 | 2 | 100 | Ciprofloxacin/Tylosin | 4 / - |
| 18        | T.S.-Sol 20/100, Dopharma Research (REG NL 7611) | Sulfamethoxazole/Trimethoprim | 42 | 3 | 37.5 | Lincomycin/Sulfamethoxazole | 3 / 17 |
| 19        | Doxylin 50% WSP, Dopharma Research (REG NL 8753) | Doxycycline | 10 | 3 | 25 | Doxycycline | 535* |
| 20        | Doxylin 50% WSP, Dopharma Research (REG NL 8753) | Doxycycline | 10 | 3 | 25 | Doxycycline | 715* |

*Estimated amount for qualitative compounds.
LOD and LOC were determined for this efficient, effective qualitative approach.

Most compounds show a LOD of 2.0 ng per portion of feathers (approximately 1 g) or lower. The LOD was higher for sulfachloropyridazine (2.5 ng), tylosin (10 ng), and tylvalosin (20 ng). Most compounds have a LOC of 2.0 ng or lower. The LOC was higher for sulfachloropyridazine (2.5 ng), neospiramycin (5 ng), oxytetracycline, erythromycin, tylosin, valnemulin (10 ng), and tulathromycin, tylvalosin (60 ng). Because data was unavailable, it was unknown if these detection limits were adequate for effective analysis of antibiotics on feathers. Therefore, this procedure was applied to real samples.

Analysis of feather samples

A total of 20 feather samples of chickens for which antibiotic treatment was registered in the food chain information (FCI) were obtained from a slaughterhouse. In total, 26 different medicines (of which the active compounds are within the scope of this paper) were administered. The feather samples were analyzed for freely extractable residues and compared with the registered treatments according to the FCI. An overview of this comparison is presented in Table 4. In 23 out of the 26 treatments, the active compounds were successfully detected. Note that some of the treatments that were detected during slaughter occurred, according to registration, over a month before slaughter.

In three cases, antibiotics were administered but could not be detected. Two relate to a Lanflox treatment (active compound is enrofloxacin, samples 3 and 4) that was carried out 31 d before slaughter. These samples were obtained from the same farmer, but from different stables. Note that in sample 1, enrofloxacin was detected 39 d after the end of the treatment. For sample 1, Spectron was used and for samples 3 and 4, this is Lanflox. However, this does not explain the difference because both are applied at the same dosage and for 3 d. The other relates to the use of Tylo-Suscit (active compound is tylosin), 29 d before slaughter (sample 16). Note that another treatment with Tylo-Suscit (sample 17), also 29 d before slaughter, was detected with a concentration just above the detection limit of tylosin. Therefore, it could be possible that the concentration of tylosin in sample 16 was just below the detection limit and as a result not detected.

In two cases, ciprofloxacin was detected. These animals were treated with enrofloxacin and as a result its metabolite ciprofloxacin is present in low concentrations as well. Furthermore, in two cases, antibiotics were detected in the feathers even though, according to the registration, they were not administered. This regards sample 14, in which sulfadimidine was detected in addition to sulfamethoxazole, and sample 18, in which a low concentration of lincomycin was detected next to tylosin.

These samples demonstrate that the presented method successfully detects most antibiotics that are administered to poultry, even over a month after treatment. The method proves to be a strong tool in the enforcement of the correct registration of antibiotic administration in the poultry sector.

Conclusion

A qualitative confirmatory method was validated for the analysis of tetracyclines, sulfonamides, quinolones, macrolides, lincosamides, and pleuromutilines in chicken feathers. The method is applicable for qualitative confirmatory analysis for all compounds included and, additionally, 58% of the compounds can also be analyzed quantitatively showing trueness, repeatability, and within-laboratory reproducibility within the criteria established in CD 2002/657/EC. Additionally, to analyzing the total residue concentration in feathers, we propose a more cost-efficient method for analyzing of freely extractable residues only. With this approach, antibiotic use in the poultry sector can effectively be monitored; in many cases even if the animals were treated in the first week of their lives and samples were taken at slaughter.

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Compliance with ethical standards

Disclosure statement No potential conflict of interest was reported by the authors.

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