Studies Regarding the Detection of Sulfonamide Residues and Evaluation of the Level of Contamination in Poultry Meat

Gabriela Valentina VESA¹, Anette Iudit GHEIŢI-HEGHEDUŞ¹,* , Marian MIHAIU¹, Liora Mihaela COLOBĂŢIU², Dariana BRATFELAN¹ and Ana-Andreea CIOCA¹

¹Department of Animal Husbandry and Food Safety, University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, 3-5 Mănăştur Street, Cluj Napoca, Romania
²Department of Medical Devices, Iuliu Hatieganu University of Medicine and Pharmacy, 8 Babeş Street, 400012, Cluj-Napoca, Romania
*Corresponding author: anette.heghedus@usamvcluj.ro

RESEARCH ARTICLE

Abstract
The excessive or uncontrolled use of antibiotics in poultry farming can lead to contaminated food products. Subsequently, the human consumption of products contaminated with these substances increases the global phenomenon of antibiotic resistance. The aim of this study was to develop and validate a high-performance liquid chromatography (HPLC) method suitable for the determination of sulfonamide residues in poultry meat and to check the presence of residues in random samples. The level of antimicrobial resistance was identified in order to evaluate the current stage and to estimate the tendency of this phenomenon. The HPLC method validation was performed in accordance with Commission Regulation (EC) No 657/2002 of 14 August 2002 Samples of fresh meat collected from a poultry slaughterhouse were analysed using the validated method in order to reveal the level of contamination. The data from EFSA was collected and analysed following the antimicrobial resistance for isolates of Salmonella spp., E. coli and Campylobacter spp. The method had good selectivity, linearity (R² ≥ 0.99), precision (<6%), recovery between 97.7-109.6% and low limits of detection (LOD) and quantification (LOQ) Sulfadiazine residues were present in 2 samples and the level of contamination did not exceed the Maximum Residue Level (31.98 ± 5.18 µg/kg and 23.70 ± 3.84 µg/kg). The analysis of data from EFSA highlighted the general presence of antimicrobial resistance especially for the following antibiotics: ciprofloxacin, tetracycline, nalidixic acid, ampicillin, sulfamethoxazole and trimethoprim. The present study brings a contribution to the process of stopping antibiotic resistance through new methods of monitoring of sulfonamide residues. The statistical data shows that there is a direct correlation between the market availability of antibiotics used in poultry farming and the occurrence of antibiotic resistance.

Keywords: contamination; EFSA; HPLC; sulfonamide residues; validation.

INTRODUCTION
Sulfonamides are a class of synthetic antimicrobials, which are characterized by a wide spectrum of action, high efficiency and low-cost price. They are currently used in cattle breeding, pigs and birds for both therapeutic and preventive purposes. They act especially upon bacteria and protozoa (Huertas-Pérez et al., 2016). With regards to the frequency of use, they belong to the third class of antimicrobial substances used in meat-producing animals. In industry, they are frequently used in the therapy of specific diseases in different species. In calves, sulfamethazine is administered orally for diarrhea control and for the treatment of bacterial pneumonia and sepsis in pigs. It is also used in birds, in the treatment of diseases caused by Escherichia coli and Pasteurella multocida and for the control of coccidiosis (Baynes et al., 2016). The detection of sulfonamide residues in meat products intended for human consumption raises important human
health concerns, given by their known adverse effects. In this context, it is necessary to implement efficient analytical procedures for the determination of such antibiotic residues. In order to protect public health, the European Union (EU) has imposed Maximum Residue Limits (MRLs) on sulfonamides in meat and other animal products (Huertas-Pérez et al., 2016). In order to identify sulfonamide residues in foodstuffs, in particular meat, the most commonly used analytical technique is High-Performance Liquid Chromatography (HPLC) coupled with various detectors such as mass spectrometry (HPLC-MS), diode array detector (HPLC-DAD) with ultraviolet or fluorescent light (UV or UV-VIS). In addition to this technique, satisfactory results have been obtained in recent years with capillary electrophoresis, a technique with a sensitivity and selectivity similar to that of liquid chromatography. Dai et al., 2017 successfully identified sulfonamide (sulfadiazine and sulfathiazole) residues from poultry and pork meat samples using chemiluminescent detection coupled with capillary electrophoresis (CE-CL).

Muscle, liver and kidney samples are complex matrices, so they require an extraction procedure prior to the actual detection. For this purpose, techniques such as pressure fluid extraction (PLE) and ultrasound-assisted extraction (UAE) are usually used, followed by liquid chromatography analysis coupled with mass spectrometry (LC-MS). These steps allowed the detection of 16 sulfonamides and metabolites from tissue samples of sheep, birds, horses and fish organs following research in Brazil (Hoff et al., 2015). The method was developed and validated for the analysis of sulfonamide residues and it was successfully applied for routine laboratory tests.

In addition to the classical methods of sampling, new techniques have emerged over time, which are easier and more flexible than the previous methods. Solid phase matrix dispersion (MSPD) is a modern technique that combines one-step extraction with sample purification, thus saving time and resources. The MIMIP-MSPD-UPLC method is a sensitive, specific and rapid method that can be used to monitor the presence of residues of the three groups of antimicrobial substances in meat (Wang G. N. et al., 2017).

Analyzing the toxicity of the residual substances, it was concluded that the metabolites of sulfonamides are much more harmful due to their embryotoxic, fetotoxic, teratogenic effect, growth retardation, pre- and postnatal malformations. Sulfonamides have a cytotoxic effect through their hyperactive hydroxylamine metabolites which may spontaneously degrade to "nitro" and "nitroso" derivatives or make covalent bonds with proteins in the body (Hiba A. et al., 2015).

In European countries there are national programmes for the control of residues of veterinary medicinal products, including antibiotics. These programs involve the application of analytical methods for identifying meat residues. Analytical methods include both screening and confirmatory methods (Mungroo N.A. and Neethirajan S., 2014). Of these methods, the most common are liquid chromatography coupled with mass spectrometry (LC-MS) with a percentage of 38% and immunoenzymatic chromatography (ELISA) 18%, as shown in Figure 1.

![Figure 1. Screening methods used in the determination of antibiotic residues, adapted from (Cháfer-Pericá, 2010)](image-url)

Screening methods can be classified into qualitative and quantitative methods, respectively conventional and innovative. Qualitative methods are limited to the detection of a particular residue of the medicinal product, indicating its presence or absence, whereas quantitative methods allow, in addition to their identification and quantitative assessment. Screening methods must meet certain conditions: be simple, fast and sensitive. With regard to the confidentiality of conventional methods, they may include microbiological and immunological methods.

Innovative methods bring new technologies for the detection of waste substances, for example the technique of applying biosensors, into the sights. Screening methods therefore allow the identification of residues of
antimicrobial substances. In the case of the identification of residual molecules, follow the stage of application of confirmatory methods, consisting of physico-chemical methods (Gaudin V., 2017). The aim of the article is to contribute to the development and validation of an analytical method for the identification and determination of sulfonamide residues in poultry meat. The present study focused on the analysis of 13 representative compounds from the sulfonamide class of antibiotics. Several poultry meat samples were analyzed using high-performance liquid chromatography (HPLC). The 13 residues were quantified accordingly and the results were compared with the MRLs established for each substance (COMMISSION REGULATION (EU) No. 37/2010 of 22 December 2009). The level of antimicrobial resistance was identified in order to evaluate the current stage and to estimate the tendency of this phenomenon. For this purpose, the data from EFSA archive was consulted.

MATERIALS AND METHODS

3.1. Samples
The samples consisted in muscle tissue from poultry coming from a slaughterhouse located in Cluj County. Two random samples were selected monthly in a one-year period (a total of 24 samples). The meat was stored under refrigeration until the day of analysis. The samples were homogenized and divided into 2 equal samples, one representing the counter-test. The counter-tests were destroyed 3 days after the results were issued. The positive samples were maintained for 60 days, according to the Order of the President of ANSVSA 2/2010.

3.2. Working technique
The steps of the working method using high performance liquid chromatography can be reproduced by the following schematic representation (Figure 2).

Sample preparation ↓
Extraction ↓
Purification ↓
Derivation-30 minutes ↓
HPLC determination

Figure 2: Steps of high-performance liquid chromatography analysis

3.3. Sample preparation and purification
The preparation of working samples is an important step in achieving correct results, as sulfonamides are amphoteric substances (Blasco C.,2009). Thus, during their extraction from samples it is absolutely necessary to maintain an acid medium (pH 5.0), this being achieved by adding acetic acid in the form of acetonitrile to the sample that is analyzed.

An amount of 2 g of sample was weight into a 50 ml centrifuge tube over which 10 μl of the sulfamer solution (internal standard) of 10 μg/ml concentration is added.

The extraction was carried out by adding 10 ml acetonitrile, then homogenized with an agitator for 15 minutes at maximum speed, ensuring complete dissolution of the sulfonamides. Centrifugation was made for 10 minutes, speed 4000 rpm. The supernatant was separated and a new extraction was made with 10 ml acetonitrile. The extracts obtained were reunited and evaporated on the water bath with Nitrogen, at 50°C. In a new stage, the residue was restored with 10 ml acetat swab pH 5.3, stirring for 15 minutes.

The same technique was used to process the fortified sample, except that in addition to the solution of internal standard, the mixed solution of sulfonamide standards of concentration 1 μg/ml was used. In the same time, a blank reagent sample without muscle tissue was also prepared.

In order to avoid contamination and interference of compounds of interest with organic residues, the purification of the samples is necessary. This purification process was carried out on SPE columns. Initially, a pre-conditioning was carried out with 10 ml methanol and 10 ml acetate buffer 0.2 mol/L, pH 5.3(mobile phase), passage of the extract through gravitational flow followed by washing the columns with water and drying under vacuum. Subsequently, sulfonamides were eluted with 10 ml acetonitrile and the eluate was evaporated on water bath at 50°C. The remaining residue was rediluted with 1000 μl 0.1 N Hydrochloric Acid and was shaken for 15 minutes. In a HPLC vial the 500 μl sample was mixed with a 500 μl pH acetate buffer (pH 3) and 200 μl fluram.
A vial was placed into the autosampler of the HPLC system and it was injected at the 30th minute after the addition of the fluram.

The injection sequence was: standard with concentration at MRL level 100 ng/ml (initial), blanc de reagents, blanc matrix, blanc matrix fortified at the level of the maximum permissible limit (recovery sample), samples to be analyzed, duplicate sample, standard of concentration with MRL level 100 ng/ml (final).

3.3. HPLC determination

The system used for the determination of residues is the Liquid-Chromatograph HPLC VARIAN type ProStar, equipped with autosampler. The work parameters have been set as follows:

- The temperature of the injector was 50 degrees Celsius and the injection volume was 100 μl,
- The injection rate was 0.8 ml/minute,
- Running time of 55 minutes,
- Detectors set to λex = 405 nm, λex = 495 nm to measure fluorescence.

After the pressure was stabilized in the liquid-chromatograph system, 100 μl from the initial standard was injected. The correction of the reading was evaluated (i.e. the measured concentration varies ±10% from the theoretical value) and the same amount was injected from the samples in the injection sequence. At the end of the determination, the final standard was injected at the same injection volume. The identification and determination of the compounds of interest was carried out automatically by the integrated system, which directly displays the retention time compared to the standard but also the area of chromatographic peaks compared to those in the fortified matrix blank.

3.4. Quantification

A linear curve was constructed based on the ratio between concentrations of compounds of interest and internal standard. Concentrations were expressed in μg/kg and unknown concentrations are expressed by interpolation. Concentrations were expressed using an appropriate number of significant figures. The number of significant figures must be sufficient to be entered in the calculations. Thus, if an additional significant figure is less than 5, it is deleted, and if it is more than 5, it will be removed and the previous figure is increased by 1. Only the final results of the analytical operations are subjects of rounding, so the values that are intermediate will be included as such. The final results must be expressed with the same number of significant figures as the values regulated by legislation and using the same unit of expression.

Maximum Residue Limits (MRL) for sulfonamides in meat samples are expressed as MRL amounts and equal to 100 μg/kg. To see if a sample is compliant, according to the SANCO Guide, the sum of the concentration of the substance of interest found in the sample (ci) and the sum of the decision limit (CCα) were calculated, according to the following formula:

\[
\text{Sum } ci = c_1 + c_2 + \ldots + \text{but} \\
\text{Sum } CCα = 100 + 1.64 \times (w_1 \times s_{d1}^2 + w_2 \times s_{d2}^2 + \ldots + w_i \times s_{di}^2)
\]

where: 100 = Sum MRL
1.64 = safety factor for MRL substances
wi = measurement factor for substance i, calculated as the ratio between ci and Sum ci
sdi = standard deviation derived from intralaboratory reproducibility, for substance i, at the concentration closest to the concentration found ci.

By comparing the 2 values, respectively Sum ci and Sum CCα there could be 2 situations:
- Compliant sample, if Sum ci < CCα Sum,
- Non-compliant sample, if Sum ci > Sum CCα

The next step was to determine the percentage of recovery. Two meat samples of 2 g were prepared containing the internal standard solution. They were marked as "P" and "P+E". The mixed sulfonamide standard solution was added to the sample marked as "P+E" in such a way as to contain 100 μg/kg of each sulfonamide. The two samples were analyzed according to the HPLC method described above.

The recovery percentage was calculated by the formula: 

\[
R = \frac{S \times U \times m \times V}{Cw \times V}
\]

where: S – the sulfonamide content of the fortified sample (μg/kg); U – sulfonamide content of unfortified sample (μg/kg); m – the sample mass used for fortification (g); Cw – the concentration of sulfonamide in the working solution (1 μg/ml); V – the volume (μl) of standard sulfonamide solution.
used to fortify the sample.

The recovery percentage (R) calculated for each compound must be in the range of 80 to 120 %.

3.5. Hazard analysis

Hazard analysis can be done by using the cause-effect diagram which leads to the definition and acceptance of a problem and the discovery of the cause.

The main steps of the hazard analysis include: Planning and resources; Establish, implement and maintain a risk assessment system; System performance related to senior management; Provide adequate resources, including trained staff; Evaluation, timing and purpose; Defining the scope, nature and timing; Proactive; Identification of hazards; Hazard evaluation and documentation; Risk assessment and establish appropriate methodologies for risk assessment and recording.

The data regarding antimicrobial resistance was collected and analyzed from the EFSA archive between 2011 and 2019 for the cultures of Salmonella spp., E. coli and Campylobacter (https://www.efsa.europa.eu/en/efsajournal/pub, Accessed on 20 December 2021). This data was included in the annual reports from EFSA entitled “The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food”.

RESULTS AND DISCUSSIONS

4.1. Method validation results

<table>
<thead>
<tr>
<th>Table 1. Analytical Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
</tr>
<tr>
<td>Sulfguanidine</td>
</tr>
<tr>
<td>Sulfanilamide</td>
</tr>
<tr>
<td>Sulfadiazine</td>
</tr>
<tr>
<td>Sulfathiazole</td>
</tr>
<tr>
<td>Sulfamerazine</td>
</tr>
<tr>
<td>Sulfadimidine</td>
</tr>
<tr>
<td>Sulfamethizole</td>
</tr>
<tr>
<td>Sulfachloropyridazine</td>
</tr>
<tr>
<td>Sulfamethoxazol</td>
</tr>
<tr>
<td>Sulfacloropyridazine</td>
</tr>
<tr>
<td>Sulfasulfonamide</td>
</tr>
<tr>
<td>Sulfadimethoxin</td>
</tr>
<tr>
<td>Sulfaquinolactone</td>
</tr>
<tr>
<td>Sulfameter (internal standard)</td>
</tr>
</tbody>
</table>

Table 2: Performance Requirements

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Acceptance Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>Correlation coefficient ≥0.99; RSD&lt;15%</td>
</tr>
<tr>
<td>Detection limit (LOD)</td>
<td>&lt; MRL/10 (µg/kg)</td>
</tr>
<tr>
<td>Quantification limit (LOQ)</td>
<td>&lt; MRL/5 (µg/kg)</td>
</tr>
<tr>
<td>Selectivity and Specificity</td>
<td>N/A, α&gt;1</td>
</tr>
<tr>
<td>Recovery</td>
<td>80% &lt; R &lt; 120 %</td>
</tr>
<tr>
<td>Repeatability and Reproducibility</td>
<td>RSD/R ≤ 20%; r ≤ 0.66xR</td>
</tr>
<tr>
<td>CCα and CCβ</td>
<td>&gt; MRL (µg/kg)</td>
</tr>
</tbody>
</table>

The method used for validation was an in-house method, adapted from Tamosiunas (2007). The aim of validation was to verify if the Performance Requirements were following the limits of acceptability imposed by law. For this purpose, a total number of 89 clean samples were analyzed. In the Laboratory, the Method Validation Protocol was applied in accordance to the experiments contained in Commission Regulation (EC) No 657/2002 of 14 August 2002 laying down detailed rules for the application of Council Directive 96/23/EC on the functioning of methods of analysis and interpretation of results. The results of the method validation are presented in Table 3.
Table 3. Method validation

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Analyte level (µg/kg) (MRL)</th>
<th>Linearity*</th>
<th>Detection limit/Quantification limit (µg/kg)</th>
<th>Recovery (%)</th>
<th>Repeatability (%)</th>
<th>Reproducibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R²≥0,99</td>
<td>RSD&lt;15%</td>
<td>LOD&lt; MRL/10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>100</td>
<td>0,997758</td>
<td>4,515</td>
<td>3,67/11,01</td>
<td>104,73/109,46</td>
<td>103,0 2,67 2,75</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>100</td>
<td>0,998476</td>
<td>3,602</td>
<td>3,27/9,81</td>
<td>104,24/108,49</td>
<td>102,8 2,31 2,48</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>100</td>
<td>0,998174</td>
<td>3,693</td>
<td>3,92/11,76</td>
<td>104,48/108,96</td>
<td>102,9 2,53 2,63</td>
</tr>
<tr>
<td>Sulfathiazole</td>
<td>100</td>
<td>0,997460</td>
<td>4,184</td>
<td>4,32/12,96</td>
<td>106,62/113,25</td>
<td>100,5 3,80 3,91</td>
</tr>
<tr>
<td>Sulfamerazin</td>
<td>100</td>
<td>0,996214</td>
<td>3,939</td>
<td>4,32/12,96</td>
<td>106,96/113,92</td>
<td>102,7 3,89 4,06</td>
</tr>
<tr>
<td>Sulfadimidin</td>
<td>100</td>
<td>0,998688</td>
<td>2,611</td>
<td>4,41/13,23</td>
<td>102,83/105,66</td>
<td>101,4 1,47 1,66</td>
</tr>
<tr>
<td>Sulfamethizole</td>
<td>100</td>
<td>0,997752</td>
<td>4,165</td>
<td>4,55/13,65</td>
<td>110,42/120,85</td>
<td>109,6 5,92 5,96</td>
</tr>
<tr>
<td>Sulfachloropyridazin</td>
<td>100</td>
<td>0,998517</td>
<td>4,149</td>
<td>4,78/14,34</td>
<td>104,74/109,48</td>
<td>98,0 2,51 2,94</td>
</tr>
<tr>
<td>Sulfamethoxazol</td>
<td>100</td>
<td>0,997236</td>
<td>5,378</td>
<td>5,09/15,27</td>
<td>104,06/108,11</td>
<td>97,7 2,49 2,53</td>
</tr>
<tr>
<td>Sulfisoxazol</td>
<td>100</td>
<td>0,997627</td>
<td>5,709</td>
<td>5,12/15,36</td>
<td>104,92/109,83</td>
<td>100,6 2,48 3,02</td>
</tr>
<tr>
<td>Sulfadimethoxin</td>
<td>100</td>
<td>0,997042</td>
<td>6,293</td>
<td>5,01/15,03</td>
<td>104,74/109,49</td>
<td>95,0 2,92 3,08</td>
</tr>
<tr>
<td>Sulfquinoxaline</td>
<td>100</td>
<td>0,991686</td>
<td>8,209</td>
<td>5,19/15,57</td>
<td>103,07/106,15</td>
<td>91,9 2,07 2,07</td>
</tr>
</tbody>
</table>

Selectivity (α) for each substance is greater than 1, as shown in Table 4.

Table 4. Level of selectivity for substances of interest

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linearity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfanilamide</td>
<td>1,64</td>
</tr>
<tr>
<td>Sulfaguanidine</td>
<td>-</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>1,35</td>
</tr>
<tr>
<td>Sulfathiazole</td>
<td>1,06</td>
</tr>
<tr>
<td>Sulfamerazin</td>
<td>1,09</td>
</tr>
<tr>
<td>Sulfadimidin</td>
<td>1,11</td>
</tr>
<tr>
<td>Sulfamethizole</td>
<td>1,06</td>
</tr>
<tr>
<td>Sulfameter</td>
<td>1,05</td>
</tr>
<tr>
<td>Sulfachloropyridazin</td>
<td>1,10</td>
</tr>
<tr>
<td>Sulfamethoxazol</td>
<td>1,08</td>
</tr>
<tr>
<td>Sulfisoxazol</td>
<td>1,03</td>
</tr>
<tr>
<td>Sulfadimethoxin</td>
<td>1,08</td>
</tr>
<tr>
<td>Sulfquinoxaline</td>
<td>1,05</td>
</tr>
</tbody>
</table>

4.2. Chromatogram interpretation

In order to identify antibiotic residues, 24 samples of poultry muscle tissue, marked with figures from 1 to 24, were analyzed. Each sample was processed individually. Qualitative results were obtained as chromatograms, generated by the Software of the HPLC VARIAN type ProStar. A number of 13 sulfamic compounds were detected in the above-mentioned samples. The basic standard solutions of these compounds were added to the blank solution, thus obtaining a fortified sample at a concentration of 100 µg/kg, rendered as the chromatogram in Figure 4.

The standards with concentration at MRL level 100 ng/ml required for automatic identification of compounds were successfully read, obtaining comparison-appropriate chromatograms (Figure 5).
Figure 4. Chromatogram for fortified blank at 100 μg/kg

Figure 5. Chromatogram of standard with concentration at MRL level 100 ng/ml

Based on the calibration curves obtained during the validation of the method, the following chromatogram (Figure 6) was built, which was used to identify the compounds.
Following the quantification of residues, positive values were obtained for Sulfadiazine in two samples analyzed, namely sample number 7 (Figure 7) and sample number 8 (Figure 8), which means 8.33% of the samples to be analyzed.

The level of these residues was as follows: Sample No.7: Sulfadiazine: 31.98 ± 5.18 μg/kg; Sample No.8: Sulfadiazine 23.70 ± 3.84 μg/kg (Figure 7 and Figure 8).
Analyzing the previous chromatograms, the presence of 2 compounds in each sample was observed, the first one was present in all samples and it was represented by the internal standard (Sulfameter), the second one was Sulfaquinoxalin. In order to demonstrate that the second sulfamide in the samples is Sulfaquinoxalin, the chromatograms of the positive samples were overlapped on the chromatogram of the fortified control sample (Figure 9.), as follows:

In this case, the red line corresponds to sample No.7, the green line to sample No.8, and the blue line belongs to the fortified sample. Peaks are present in all 3 samples only at the level of Sulfameter and Sulfaquinoxalin.

The results obtained for positive samples did not exceed the Maximum Residue Level (MRL), with values below 100 μg/kg. To see if the evidence is compliant, the amount of the decision limit (CCα) was calculated, which is 102.03. In this case Sum ci < Sum CCα for both samples, respectively sample No.7 and sample No.8, concluding they are compliant. Therefore, 24 samples were analyzed in this paper, two of which were positive and both were...
compliant, without exceeding the Maximum Limit.

The positive samples came from the same batch of slaughtered animals.
Franco et al., 1990, debates the idea of the presence of sulfonamide residues in meat intended for human consumption due to the subtherapeutic use of these substances to promote livestock farming. They argue that both therapeutic and subtherapeutic doses can cause the persistence of residues in animal tissues representing thus a real danger to public health. Given that in this study the positive samples came from the same batch, it can be suspected that the existence of residues was due to a production error, possibly due to non-compliance with the withdrawal period. Compliance with this period is absolutely necessary for obtaining safe food.

Currently, there are surveillance systems that monitor the addition of antimicrobial substances to animal feed, thereby reducing the possibility of their transmission to humans through meat consumption. Residue levels of sulfonamides in meat are increasingly low or even non-existent due to the limitation of their use in animals. However, in order to increase the effectiveness of these surveillance systems reducing the incidence of antibiotic residues in meat, closer cooperation between the parts involved in meat production and the control authorities is recommended.

Given that antibiotics are used in many livestock breeding units for the purpose of increasing productivity, Muaz et al., 2018 recommends that this habit be placed with more effective farm management practices. This can prevent detectable residue levels in animal products.

4.3. Statistical data

The data regarding the antimicrobial resistance (AMR) phenomenon collected from EFSA shows that the following antibiotics were mainly present in poultry meat: ciprofloxacin, nalidixic acid, tetracycline, ampicillin (both in Europe and Romania), as shown in Figure 10 and Figure 11.

![Figure 10](image1.png)  
**Figure 10.** AMR phenomenon in EU and other European countries in poultry, between 2011-2019 for *Salmonella* spp., *E. coli* and *Campylobacter* spp.

![Figure 11](image2.png)  
**Figure 11.** AMR phenomenon in Romania in poultry meat, between 2011-2019 for *Salmonella* spp., *E. coli* and *Campylobacter* spp.

![Figure 12](image3.png)  
**Figure 12.** AMR phenomenon in EU and other European countries for *Salmonella* spp. between 2011-2019, in poultry meat

![Figure 13](image4.png)  
**Figure 13.** AMR phenomenon in Romania for *Salmonella* spp. between 2011-2019, in poultry meat.
The AMR phenomenon of Salmonella spp., Campylobacter jejuni and Campylobacter coli were present for quinolones and tetracycline in poultry meat (both in Europe and also in Romania), as shown in Figures 12 - 15.

**Figure 14.** AMR phenomenon in EU and other European countries for *Campylobacter jejuni* and *Campylobacter coli* between 2011-2019, in poultry meat

The AMR phenomenon of *E.coli* was present for quinolones, sulfamethoxazole and tetracycline in poultry meat (both in Europe and also in Romania), as shown in Figure 16 and Figure 17.

**Figure 15.** AMR phenomenon in Romania and other European countries for *Campylobacter jejuni* and *Campylobacter coli* between 2011-2019, in poultry meat

**Figure 16.** AMR phenomenon in EU and other European countries for *E.coli* between 2011-2019, in poultry meat

**Figure 17.** AMR phenomenon in Romania countries for *E.coli* between 2011-2019, in poultry meat

**CONCLUSIONS**

The objectives set have been successfully achieved and the conclusions to be drawn are as follows:

- The studies regarding the validation of the method shows that the values obtained for each Performance Requirement (linearity, repeatability, reproducibility and limits of detection (CCα and CCβ) are within the limits of acceptance imposed by the law, thus the method is appropriate to the intended purpose.
- Following the development and validation of the method, the samples of muscle tissue from poultry were analyzed during one year, identifying 13 sulfonamides of which quantifiable residues of Sulfamethoxazole were present at an incidence of 8.33% from the analyzed samples.
- Residue levels found in the two positive samples do not exceed the Maximum Permitted Limits laid down by the European legal rules in force, the samples being considered compliant.
- Considering the statistical data from the archive of EFSA, it can be concluded that the AMR phenomenon is present for different classes of antibiotics: tetracycline, ciprofloxacin, nalidixic acid, ampicillin, sulfamethoxazole and trimethoprim for Salmonella spp, E. coli and Campylobacter spp.
The study shows that quantifiable levels of sulfonamide residues can be detected when using therapeutic or subtherapeutic doses of these compounds, and their most rational use by livestock farmers is recommended.

**Author Contributions:** G.V.V. Conceived and designed the analysis; A.I.G.H. Collected the data; G.V.V.; M.M.; A.I.G.H., L.M.C. Contributed data or analysis tools; G.V.V. Performed the analysis; G.V.V.; A.I.G.H.; D.B. and A.-A.C. Wrote the paper.

**Funding Source:** Not applicable.

**Conflicts of Interest**
The authors declare that they do not have any conflict of interest.

**REFERENCES**


8. Hoff RB, Pizzolato TM, Peralba MCR, Díaz-Cruz S, Barceló D. Determination of sulfonamide antibiotics and metabolites in liver, muscle and kidney samples by pressurized liquid extraction and ultrasound-assisted extraction followed by liquid chromatography– quadrupole linear ion trap- tandem mass spectrometry (HPLC–QqLT-IT-MS/MS), Talanta 2015; 134: 768 – 778. DOI: 10.1016/j.talanta.2014.10.045.


Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Veterinary Medicine 65