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# **A liquid chromatography coupled to tandem mass spectrometry method for the quantification of spiramycin and its active metabolite neospiramycin in milk of major and minor species: validation using the accuracy profile**

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## **1. Introduction**

Spiramycin is a macrolide antibiotic mainly used in veterinary medicine for the treatment of respiratory infections in food producing animals and mastitis in lactating cows. This drug can also be used off-label on so-called minor species such as lactating ewes and goats, within the cascade principle in the European Union [1].

Several studies have already been published on the pharmacokinetic of spiramycin in lactating cows after parenteral administrations [2],[3] showing a metabolization into a polar derivative named neospiramycin I, with similar antibiotic activity (Fig. S1 in supplementary materials) [4]. The concentrations of both compounds are higher in milk than in plasma, as shown by the milk-to-plasma ratio between 50 to 60 [2],[4]. However, much less is known about the distribution of spiramycin and neospiramycin in the milk of minor species.

The Maximum Residue Limit (MRL) for spiramycin is set at 200  $\mu\text{g.kg}^{-1}$  for the sum of spiramycin and neospiramycin as marker residues in milk of cows in European Union [5]. The withdrawal period, defined as the time required for the sum of spiramycin and neospiramycin concentrations in milk to fall below the MRL, is set at 13.5 days for the standard dosing regimen (against mastitis) of 30 000 UI. $\text{kg}^{-1}$  for 2 days for cows. For the off-label use of spiramycin in minor species, for which no MRL has been defined in milk, a standard withdrawal period of at least 7 days must be respected. This arbitrary establishment of withdrawal periods for minor species may present a risk. Indeed, the presence of antibiotic residues in milk can favor the emergence of bacterial resistance within the intestinal microbiota of the consumer [6].

Determination of the concentrations of spiramycin and its active metabolite, neospiramycin, in goat and ewe milk is therefore important to assess the suitability of withdrawal times for these species. To be able to quantify concentration of the antibiotic and its active metabolite in the milk of the three species, it is necessary to develop a specific and selective analytical method, able to quantify a wide range of concentrations of both analytes, from high concentrations of the first post-administration milking to concentrations below the MRL after several days, useful for residue depletion studies.

For the assay of this antibiotic and its active metabolite in plasma of cow, in milk of goat, cow and sheep, and in animal feeding products, various analytical methods are described in the literature, using microbiological assay or liquid chromatography with tandem mass spectrometry (LC-MS/MS) [3], [7]–[11]. The microbiological method measures only unbound analyte (spiramycin and neospiramycin) which is the active fraction, whereas the LC-MS/MS method is more sensitive and include an extraction step that allow the measure of both free and bound analyte to milk components, which is necessary to control the level of residues [12]. However,

the currently validated analytical methods did not allow the quantification of both spiramycin and its active metabolite in all three milks simultaneously.

Having a single assay method for multiple analytes and matrices simplifies routine analysis because there are fewer standards to prepare for each analyte and they are prepared in a single matrix. A previous article published by our lab [13] used the accuracy profile to develop a single regression model for the quantification of ceftiofur in muscle, kidney and plasma from poultry. To our knowledge, it is the first paper reporting a unique validated method for the determination of a contaminant in different matrices of the same animal species. The main objective of the present work was to use the same “accuracy profile” approach to develop and validate a single LC-MS/MS method for the assay of spiramycin and neospiramycin, in the same matrix (milk) of three milk-producing species, either major (*i.e.*, cow) or minor (*i.e.*, goat and ewe) ones. Finally, to demonstrate the applicability of the method in routine use, milk samples from lactating ewes, goats and cows treated with spiramycin were assayed using the developed method.

## **2. Materials and methods**

### **2.1. Chemical, reagents and solutions**

#### *2.1.1. Analytical reference standards and standard solutions*

Spiramycin is composed mostly of spiramycin I (85%) and a minority of spiramycin II and III (<5% and 10%, respectively) [14], therefore only spiramycin I was considered (Fig. S1). Spiramycin I was obtained from Sigma-Aldrich (St Louis, USA), isotopically labelled internal standard (spiramycin I-d3) and neospiramycin I (spiramycin metabolite) were purchased from Cluzeau (Sainte Foy la Grande, France). Individual stock solutions at 500 µg.ml<sup>-1</sup> were prepared

in methanol for spiramycin I and for neospiramycin I. Spiramycin I-d3 was prepared at a concentration of 100  $\mu\text{g}.\text{ml}^{-1}$  in methanol. All these solutions were stored at  $-18\text{ }^{\circ}\text{C}$  during one year for spiramycin I and six months for neospiramycin I [15]. To ensure a good preparation of the individual stock solution, they were compared with compliant individual stock solution. One mixed intermediate standard solution of spiramycin I and neospiramycin I at 200  $\mu\text{g}.\text{ml}^{-1}$  (WIS<sub>1</sub>) was prepared by diluting stock standard solution in methanol and was stable for at least 24 days at  $-18\text{ }^{\circ}\text{C}$ . Intermediate solution of deuterated standards at 10  $\mu\text{g}.\text{ml}^{-1}$  (WIS<sub>2</sub>) was prepared by diluting stock standard solution in methanol. These intermediate solutions (WIS<sub>1</sub> et WIS<sub>2</sub>) were diluted in water to prepare working standard mixed solutions at concentration of 2, 20  $\mu\text{g}.\text{ml}^{-1}$  for mixed spiramycin – neospiramycin (WSS<sub>1</sub> and WSS<sub>2</sub>, respectively) and a working deuterated internal standard solution (WSS<sub>3</sub>) at 1  $\mu\text{g}.\text{ml}^{-1}$ . These solutions were prepared fresh daily.

#### 2.1.2. Chemicals and reagents

Acetonitrile HPLC grade, acetonitrile optima® LC/MS grade, methanol analytical reagent and disodium ethylenediaminetetraacetate dihydrate (Na<sub>2</sub>EDTA) were supplied by Fisher Scientific (Leicestershire, England). Formic acid (98-100%) and ammonium acetate were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide was obtained from VWR International (Leuven, Belgium). Deionized water was prepared using a Milli-Q-system (Millipore, Bedford, MA, USA).

A Na<sub>2</sub>EDTA 20 mM solution was prepared by dissolving 0.744 g of Na<sub>2</sub>EDTA dihydrate in 100 ml volumetric flask with deionized water. An ammonium acetate buffer (2 M) was prepared by dissolving 15.4 g of ammonium acetate in 100 ml volumetric flask with deionized

water. A dilution of 1/10<sup>th</sup> in deionized water was done to obtain an ammonium acetate buffer at 0.2 M.

## **2.2. Sample extraction and purification**

Concentrations of spiramycin and neospiramycin were quantified in the milk of goat, ewe and cow. The principle of the sample preparation was based on a liquid-liquid extraction followed by a concentration step under a gentle stream of nitrogen.

First, the weighted sample ( $2.00 \pm 0.02$  g) was fortified with internal standard by adding 200  $\mu$ l of deuterated internal standard solution at 1  $\mu$ g.ml<sup>-1</sup>. Then, 500  $\mu$ l of Na<sub>2</sub>EDTA dihydrate at 20 mM was added, the sample was mixed and allowed to stand for 10 minutes before extraction. After adding 8 ml of acetonitrile HPLC grade, the tube was vortex-mixed vigorously for 30 seconds at maximum speed, shaken for 10 minutes with a rotative stirrer at 100 rpm, and then centrifuged at 14 000 g for 5 minutes at 4 °C. Subsequently, 3 ml of the supernatant was transferred into a new clean tube containing 50  $\mu$ l of Dimethylsulfoxyde (DMSO). Tubes were placed into the evaporation compartment at 50 °C, left for 15 minutes without nitrogen and then submitted for 90 minutes under a gentle stream of nitrogen. DMSO was added to avoid dryness evaporation and prevent compounds degradation, the extracts were resuspended in 550  $\mu$ l of acetate ammonium solution at 0.2 M and filtered through a 0.45  $\mu$ m polyvinylidene difluoride (PVDF) filter towards a 650  $\mu$ L polypropylene vial from Interchim prior to LC–MS/MS analysis.

## **2.3. LC-MS/MS analysis**

The LC system was composed of an Accela pump and a Thermo CTC - Pal (Thermo scientific, Villebon sur Yvette, France). The chromatographic separation was performed on a

core shell reversed phase HPLC column Kinetex® C18 from Phenomenex (100 mm × 2.1 mm; 2.6 μm) equipped with a guard column Symmetry C18 from Phenomenex (4 mm × 2 mm). The mobile phase consisted of [A] deionized water containing 0.2% formic acid and [B] acetonitrile containing 0.2% formic acid with a gradient elution at 350 μl.min<sup>-1</sup> flow rate as follows (t in min): t<sub>0</sub>, A = 90%; t<sub>5</sub>, A = 20%; t<sub>5.1</sub>, A = 0%; t<sub>5.5</sub>, A = 0%; t<sub>5.6</sub>, A = 90% and column pressure balancing up to 7.5 minutes. The injection volume was 5 μl and the column oven was maintained at 28 °C.

The LC system was coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo scientific, Villebon sur Yvette, France) equipped with an electrospray ionization source (H-ESI). Data acquisition and integration were performed using Xcalibur™ version 2.2. The mass spectrometer was operated in positive ESI mode. The source parameters were as follows: spray voltage: 2 800 V; sheath gas pressure: 50 (arbitrary unit); ion sweep gas pressure: 3 (arbitrary unit); aux gas pressure: 15 (arbitrary unit); capillary temperature: 275 °C; vaporizer temperature: 250 °C; collision pressure: 1 (arbitrary unit); FWHM: 0.7. Argon was used as the collision gas; nitrogen was used as the nebulization and desolvation gas. The mass spectrometer was operated in a multiple reaction monitoring (MRM) mode selecting one transition for each analyte and one for deuterated internal standard. Individual solutions at 0.5 μg.ml<sup>-1</sup> of spiramycin, neospiramycin and spiramycin-d<sub>3</sub> were infused into the H-ESI source of the mass spectrometer to select the most sensitive and representative transitions. Spiramycin and neospiramycin were ionized in protonated form in electrospray positive ion mode to form doubly charged [M+2H]<sup>2+</sup> ions and spiramycin-d<sub>3</sub> [M+2H]<sup>2+</sup> <sup>13</sup>C ion was chosen as the internal standard (IS) for both analytes. The <sup>13</sup>C isotope was used for spiramycin d<sub>3</sub> because of the presence of interferences related to the isotopic profile of spiramycin which is a large molecule. The MRM

acquisition parameters: retention time , S-lens, collision energy, m/z precursor ion, m/z product ion was shown in Table 1.

## **2.4. Selectivity and stability**

The following two parameters were evaluated before the assessment of the performance of the analytical method. (i) Selectivity: four different batches of goat, cow and ewe milks (blank matrices), respectively, were analyzed to evaluate potential interference peaks at the retention time (RT) of spiramycin, neospiramycin and spiramycin-d3. The area of peaks attributable to interfering components must not exceed 20% of the peak area of analytes (spiramycin, neospiramycin) at the lower limit of quantification (LLOQ, determined with the global accuracy profile described in section 2.5.2) and not exceed 5% of the peak area of internal standard (spiramycin-d3) used in calibration standard and quality controls (defined in sections 2.5.1 and 2.7), for each matrix. (ii) Stability: spiramycin and neospiramycin stability were assayed in several conditions. First, twelve independent samples of spiramycin and neospiramycin spiked at 200  $\mu\text{g.kg}^{-1}$  (equal to the MRL) in milk were prepared without IS. Three of these samples were spiked with IS and then extracted and injected while the others (n=9, without IS) were frozen. At each freeze-thaw cycle, all the samples were unfrozen and only 3 samples were spiked with IS and then extracted and injected while the others (without IS) were frozen again.

Secondly, stability of three extracts of spiramycin and neospiramycin spiked at the MRL level was determine at time zero and after 24 h of storage at room temperature. For both stability studies, the average ratios (area of analyte peak over the area of internal standard peak) were compared before and after the stability conditions. A tolerance of  $\pm 15\%$  of the nominal value was considered acceptable.



## 2.5. Validation concept

The aim of the article was to develop a unique method to assay the two analytes in the three milks. Similarly to the study of Mompelat *et al.*, [13] two experimental steps were used during the validation process: (i) determination of the most relevant response function using calibration standards; (ii) assessment of the precision and the trueness of the analytical method thanks to the use of validation standards, as defined below. All the calculations were carried out by using the E-NOVAL software Version V4.1c PROD (Pharmalex, Mont-Saint-Guibert, Belgium).

### 2.5.1. Definitions and experimental process

Before describing the methods used to build accuracy profile (see section 2.5.2.), it is necessary to define some concepts to avoid ambiguous meanings.

(i) Calibration standards (CS) were made by addition of known concentrations of the analytes of interest (spiramycin and neospiramycin, diluted from the WSS<sub>2</sub> solution for the higher concentration level, or from the WSS<sub>1</sub> solution for the other concentration levels) to blank matrix prior to extraction. They were prepared in the milk of each species at six concentration levels between 0.20 to 10 times the MRL: 40, 80, 200, 600, 1000 and 2000  $\mu\text{g.kg}^{-1}$ . Regression on a scatterplot between CS duplicates was used to build a response function

(ii) Response function also known as calibration curve, is obtained from the CS. It is a regression model reflecting the relationship between the response (area of the peak of the analytes of interest over area of the peak of the internal standard) and the concentration of the analytes within the assay interval. Several regression models corresponding to linear, linear

through 0, weighted linear, linear with logarithm transformation, linear regression with square root, quadratic and weighted quadratic regressions were tested for each analyte and each matrix.

(iii) Validation standards (VS) were made with the same process, with the same stock solutions and for the same concentrations as for the CS, but separately from the CS. VS were used to assess trueness and precision of the analytical method.

(iv) Trueness refers to the closeness of the average experimental value to the acceptable reference value, and was represented as relative bias (%). It was calculated from VS over the six concentration levels.

(v) Precision refers to the random error of the method and was represented by two parameters. The repeatability, corresponding to within series variance (relative standard deviation, RSD %), and the intermediate precision (RSD %), represented the sum of the within series variance and the between series variance determined during validation series. As trueness, it was calculated from the six levels of VS.

(vi) For each analyte and milk, the validation process of the analytical method was carried out on three validation series. In a validation series, the 6 levels of CS (0.2 to 10 times the MRL) and the 6 levels of VS (0.2 to 10 times the MRL, prepared separately from the CS) were prepared in duplicate and then extracted and injected (i.e. 12 CS and 12 VS injected). The scatterplot of CS was used to establish a response function. VS were back calculated from this response function. Three validation series were performed per analyte and per matrix (i.e. 36 CS and 36 VS injected). All in all, counting the 3 matrices (cow's milk, goat's milk, sheep's milk) and the 2 analytes (spiramycin and neospiramycin), a balanced set of 216 CS ( $36 * 3$  matrices  $* 2$  analytes) and 216 VS were injected over 18 validation series ( $2$  analytes  $* 3$  matrices  $* 3$  validation series).

209

### 210 2.5.2. Accuracy study

211 A statistical tools based on total error approach and so called accuracy profile, was used  
 212 to validate the analytical method [16]–[18]. Briefly, it consisted in building a tolerance interval  
 213 (Eq. 1), based on the optimal response function, and considering the trueness (bias) and precision  
 214 (repeatability and intermediate precision) of the method, in which a percentage value ( $\beta$ ), to be  
 215 defined, of the future measurements should fall. In this study, a  $\beta$ -value of 80% was chosen. This  
 216  $\beta$ -expectation tolerance interval ( $\beta$ -TI) should be included within the acceptance limits ( $\lambda$ ), the  
 217 latter being defined in accordance with the future use of the method. The acceptance limits were  
 218 set beforehand at 20% for the LLOQ and 15% for the next levels, a classical choice for  
 219 bioanalytical procedures [6] [16].

$$\beta \text{ TI} = \left[ \begin{array}{l} \text{bias}(\%)_j - Q_t \left( v; \frac{1+\beta}{2} \right) \times \sqrt{1 + \frac{1}{p \times n \times B_j^2}} RSD_{IP,j} ; \\ \text{bias}(\%)_j + Q_t \left( v; \frac{1+\beta}{2} \right) \times \sqrt{1 + \frac{1}{p \times n \times B_j^2}} RSD_{IP,j} \end{array} \right] \quad (1)$$

220 with  $\text{bias}(\%)_j = \frac{\hat{\mu}_j - \hat{\mu}_{Tj}}{\hat{\mu}_{Tj}} \times 100$  ;  $RSD_{IP,j} = \frac{\hat{\sigma}_{IP,j}}{\hat{\mu}_j} \times 100$  ;  $B_j = \sqrt{\frac{r_j + 1}{n \times r_j + 1}}$  ;  $r = \frac{\hat{\sigma}_{B,j}^2}{\hat{\sigma}_{W,j}^2}$

221 where  $p$  is the number of series (3 per analyte and per matrix = 18 series),  $n$  is the number of  
 222 independent duplicates per series (2 per sample level),  $\hat{\mu}_j$  is the estimate of the mean results of  
 223 the  $j$ th concentration level (0.2 to 10 \* MRL),  $\hat{\mu}_{Tj}$  is the theoric mean of the  $j$ th concentration  
 224 level,  $\hat{\sigma}_{IP,j}$  is the estimate of the intermediate precision variance at the  $j$ th concentration level,  
 225 which is the sum of the within series variance ( $\hat{\sigma}_{W,j}^2$ ), and between series variance ( $\hat{\sigma}_{B,j}^2$ ),  $Q_t \times$   
 226  $\left( v; \frac{1+\beta}{2} \right)$  is the  $\beta$  (80%) quantile of the Student t distribution with  $v$  degrees of freedom  
 227 estimated by  $\frac{(r+1)^2}{\frac{(r+\frac{1}{n})^2}{p-1} + \frac{1-\frac{1}{n}}{pn}}$ .

228

229 Several accuracy indexes were used as previously described [19]: (i) the trueness index  
230 ( $I_T$ ) (Eq.2) corresponding to one minus the ratio of the sum of squares of deviations between  
231 observed bias and maximum tolerance bias at each concentration level included in the dosing  
232 range; (ii) the precision index ( $I_P$ ) (Eq.3) defined as the ratio between the area defined within the  
233 acceptance limits and the limits of the  $\beta$ -TI, and the area defined by the upper and lower  
234 acceptance limits, for which the method is considered as valid; (iii) the dosing range index ( $I_{DR}$ )  
235 (Eq.4) representing the extent of the validated range according to the lowest and highest defined  
236 point; (iv) accuracy index ( $I_A$ ) (Eq.5) calculated as the geometric mean of the three-following  
237 indexes.

238 The lower and upper limits of quantification, LLOQ and ULOQ respectively, were  
239 defined as the concentrations for which the  $\beta$ -TI crossed the acceptance limits. The LOD was  
240 defined at one third of the LLOQ [20] .

$$I_T = \frac{SSB_{Max} - SSB_{Obs}}{SSB_{Max}} \quad (2)$$

$$I_P = \frac{U_{Area} + L_{Area}}{AREA_{Max}} \quad (3)$$

$$I_{DR} = \frac{U_{LOQ} - L_{LOQ}}{DR_{Max}} \quad (4)$$

$$I_A = \sqrt[3]{I_T \times I_{DR} \times I_P} \quad (5)$$

241

242 with  $SSB_{Max}$  is the sum of the square of the maximum bias at each concentration level  
243 investigated by the VS included in the dosing range,  $SSB_{Obs}$  is the sum of the square of the  
244 observed bias at each concentration level also included in the dosing range,  $U_{Area}$  is the area  
245 defined by the upper  $\beta$ -expectation tolerance limits and the upper acceptance limits  $+\lambda$  and  
246 included between the lower limit of quantification ( $L_{LOQ}$ ) and the upper limit of quantification  
247 ( $U_{LOQ}$ ),  $L_{Area}$  is the area between the lower  $\beta$ -expectation tolerance limits and the lower

acceptance limits  $-\lambda$  and included between the  $L_{LOQ}$  and the  $U_{LOQ}$ ,  $AREA_{Max}$  is the area defined by the upper and lower acceptance limits  $\pm\lambda$  and the  $L_{LOQ}$  and the  $U_{LOQ}$ ,  $DR_{Max}$  is the difference between the highest and the lowest concentration levels investigated during the validation,.

The aim of our work was to look for a global accuracy profile, associated to a unique and optimal response function, for the two analytes and the three matrices and was conducted in three steps: (i) the best individual profiles (associated to the best response functions and minimizing the impact of this function on its uncertainty) for each analyte and each milk were selected according to the accuracy indexes (the higher, the better) and to the inclusion of the  $\beta$ -TI within the acceptance limits. (ii) Intermediary profiles were established for spiramycin and neospiramycin by pooling CS and VS results obtained for the 3 different milks, as described in the first step. (iii) A global (and unique) profile was established for the 2 analytes and the 3 matrices by pooling all data as described in previous steps.

## **2.6. Relative Expanded Uncertainty**

Relative uncertainty is based on the variability of relative bias (%) and intermediate precision variance expressed by RSD % (Eq.6). A 95% confidence interval around the VS measured value is built by considering a cover factor ( $k=2$ ), allowing to calculate the relative expanded uncertainty (REU). REU of the method was measured at each concentration level and used to establish an uncertainty function to determine the uncertainty of any result within the validation concentration range [21] [22]. Several models were tested as linear, exponential, logarithm and second order polynomial regression and the one associated to the best determination coefficient was chosen for the uncertainty function.

$$REU(\%) = 100 \times \frac{2 \times \sqrt{\hat{\sigma}_{IP}^2 \times \left(1 + \frac{1}{p \times n \times B_j^2}\right)}}{\hat{\mu}} \quad (6)$$

270 with  $B_j = \sqrt{\frac{r_j + 1}{n \times r_j + 1}}$  and  $r = \frac{\hat{\sigma}_{B,j}^2}{\hat{\sigma}_{W,j}^2}$

271 where  $\mu$  is the mean introduced concentration,  $n$  is the number of repetitions per series (2 per  
272 sample level) and  $p$  is the number of series (3 per analyte and per matrix = 18 series).

273

## 274 2.7. Applicability of the method

275 The aim of this applicability study was to check that the validation procedure using the  
276 global accuracy profile allows to reliably predict that 80% ( $\beta$ -value) of future measurements will  
277 be within the acceptance limits [23]. For this purpose, an experimental study, approved by the  
278 Animal Research Ethics Committee, was carried out on the three animal species (cow, ewe,  
279 goat). A total of six healthy individuals of each species received a single dose of 30,000 UI.kg<sup>-1</sup>  
280 of spiramycin (Spirovet®, Ceva) by intramuscular injection in the neck. Milk samples were  
281 taken during morning and evening milkings, separated by 8 hours, for fifteen days following the  
282 administration. The developed method was then used in routine to assay the milk samples of the  
283 6 animals per species, 18 animals in total. The 17 samples per animal (0h to 192h post-  
284 administration) were analyzed over 18 runs (1 per animal). Each analytical run included quality  
285 control (QC) samples containing both spiramycin and neospiramycin at three concentration  
286 levels (50, 1000 and 1600  $\mu$ g.kg<sup>-1</sup>) prepared in duplicate, in milk of goat, ewe and cow.  
287 Concentration of the two analytes in the QC were then back calculated according to the  
288 calibration curve of the global profile obtained from the validation study and accepted according  
289 to the bioanalytical procedures described above. According to the FDA guidelines [24], the run

was considered compliant when 4/6<sup>th</sup> of all QC was accepted and the concentration of unknown samples was reported.

### **3. Results and discussions**

#### **3.1. Selectivity and stability**

For selectivity, an interfering peak was observed in all batches of each milk species and also without matrix (water injection) at the same retention time of spiramycin  $[M+2H]^{2+} > 174.1$  m/z, neospiramycin  $[M+2H]^{2+} > 174.1$  m/z and spiramycine-d3  $[M+2H]^{2+} > 174.1$  m/z. The reason for the presence of this interference was due to a low carry over related to the injection seat of the HPLC system. However, it never exceeds 2.5% of the peak area of spiramycin, neospiramycin and spiramycin-d3 compared to the LLOQ of each analyte and to internal standard. Thus, the selectivity of the method was validated. Chromatograms of spiramycin, neospiramycin and spiramycin-d3 in each milk species are shown in Fig. 1.

Spiramycin and neospiramycin were stable after three freeze-thaw cycles in milk spiked at the MRL level ( $200 \mu\text{g.kg}^{-1}$ ) and also after 24 hours at room temperature in the extract. Previous results had already highlighted the good stability of both analytes at  $100 \mu\text{g.kg}^{-1}$  in milk ( $\frac{1}{2}$  MRL) for 360 days at  $-18^\circ\text{C}$  [15], the same storage temperature than for experimental samples (see section 3.4.)

#### **3.2. Validation concept**

##### *3.2.1. Response function*

As explained in section 2.5.2, several steps were carried out to choose the optimal response function of the global profile: (i) The results of all regression models for each

individual analyte and milk are detailed in supplementary materials (Table S1). Accuracy indexes ( $I_A$ ) of related accuracy profiles were of the same order of magnitude ( $>0.70$ ) for all models. For all individual profiles, the linear model with logarithm transformation ranked in first or second place (comparing  $I_A$ ) except for neospiramycin in ewe milk but with an  $I_A$  close to the top model (0.83 vs 0.87, respectively). Parameters of linear model with logarithm transformation are presented in supplementary material (Table S2) for all analytes and milks. Determination coefficients ( $R^2$ ) were above 0.998 for each curve of validation series, confirming the adequacy of this regression model. (ii) Combining the results of the three milk species for each analyte in intermediary profiles, the linear model with logarithm transformation also appeared in the top two places. These results confirmed the possibility of using a single regression model as a response function to assay each of the two analytes in the different milks. (iii) Finally, after pooling the two analytes and the three milks in the global profile (Table S1), the linear model with logarithm transformation, with an  $I_A$  of 0.88, was kept to get a unique (and reliable) regression model ( $R^2 = 0.9991$ ) according to the parsimony principle. All parameters (slope, intercept) of the final response function are presented in Table 2.

These results, obtained only from CS analysis, confirmed that both spiramycin and neospiramycin could be quantified in the milk of all three species using the same response function.

### 3.2.2. Accuracy study

#### 3.2.2.1. Trueness and precision

For each individual profiles, trueness and precision measurements were within defined acceptance criteria of  $\pm 15\%$  (Table S3). The widest range of relative bias was -5.5% to 6.8% for



the accuracy profile of spiramycin in cow milk. Regarding the repeatability and the intermediate precision, the widest range was 0.5% to 3.9% for spiramycin in ewe milk and 1.5% to 5.9% for neospiramycin also in ewe milk, respectively.

Main results of trueness and precision of the global profile are presented in Table 2. Ranges of relative bias, relative repeatability and relative intermediate precision were -1.6% to 5.7%, 1.1% to 2.7% and 2.5% to 4.2%, respectively, showing that trueness and precision were adequately included within the acceptance criteria of  $\pm 15\%$ . This highlighted that both the extraction method and the use of the spiramycin-d3 as internal standard for the two analytes gave satisfactory results to validate the method.

#### *3.2.2.2. Accuracy profile*

Individual accuracy profiles for each analyte in the milk of cow, goat and ewe (as described in section 2.5.2) and their related indexes are shown in supplementary data (Table S4; Fig. S2). Individual profiles revealed that precision indexes of both analytes in ewe milk were lower than those of other species, as confirmed by lower accuracy indexes in this milk. The variation in milk composition of the different species may explained the observed precision differences. Indeed, ewe's milk is fattier and has a higher concentration of proteins than the milk of the other two species [25] [26]. As the extraction procedure was the same for the 3 milks, it was possible to build intermediary accuracy profiles of spiramycin and neospiramycin (Table S4; Fig. S2) with accuracy indexes ( $I_A$ ) of 0.88 and 0.87, respectively. These profiles confirmed that the accuracy profile is within the acceptance limit and allowed to quantify each analyte in the 3 milks for the whole tested dosing range.

The global accuracy profile (Fig. 2) was built by using the average parameters of the chosen response function (see section 3.2.1), *i.e.*, using all validation series. This construction was possible due to the common range of concentration of both analytes. The global profile was in total agreement with the defined accuracy criteria, as the  $\beta$ -TI was completely included within acceptance limits. All the related indexes of this profile are presented in Table 2. The dosing range index equal to 1 meant that the whole dosing range tested (40 to 2000  $\mu\text{g.kg}^{-1}$ ) could be quantified with the defined accuracy without exceeding the acceptance limits, for spiramycin and neospiramycin in the different milks. The LLOQ and ULOQ were therefore defined at 40 and 2000  $\mu\text{g.kg}^{-1}$ , respectively and the LOD was equal to 13  $\mu\text{g.kg}^{-1}$ . Trueness and precision indices were of 0.71 and 0.96, respectively, showing an analytical method with great performance, as confirmed by the accuracy index of 0.88. Indeed, as some milks are more difficult to obtain, notably goat and ewe milk, the global accuracy profile of the three milks would simplify routine assays by preparing QC samples in the most accessible milk.

Overall, the global response function (Fig. 2) accurately quantified spiramycin and its active metabolite neospiramycin in milk from three different species over a concentration range from 0.2 to 10 times the MRL. Thanks to this unique model, residue depletion studies in milk can be done more easily using only the milk of the major species, and faster with a single series of QC spiked with spiramycin and neospiramycin in a single type of milk.

### 3.3. Relative Expanded Uncertainty

All REU data (%) obtained with the global accuracy profile (Fig. 2) were presented in Table 3. The maximum REU obtained during validation series was estimated at 8.66% for the ULOQ level. The average REU of the whole dosing range was 6.62%, confirming the high

precision of the method. From all tested models, the polynomial (order 2) function was chosen ( $R^2=0.834$ ) to model the REU as a function of time, as shown in Fig.3. The REU is quite low for all concentration levels. As observed by Wang *et al.*, (2009) [7], this can be explained by the choice of transitions, which are specific and sensitive. We can see that the calculated expanded uncertainty is highest for the higher level of concentration (2000  $\mu\text{g.kg}^{-1}$ ). This result is explained by largest deviation of results at this concentration (see Fig. 2) and by an extensive concentration range (40 to 2000  $\mu\text{g.kg}^{-1}$ ) of study that reached a high concentration near the detection saturation. This model was built based on the global accuracy profile; thus, it was adapted to the response function and applicable to experimental concentration levels between two concentrations levels within the validated range of 40 to 2000  $\mu\text{g.kg}^{-1}$ .

### 3.4. Applicability to real samples

Several individual milk samples of cows, goats and ewes containing unknown concentrations of spiramycin and neospiramycin were assayed in routine with the developed method. Results of this depletion study of spiramycin and neospiramycin in the milk of one representative animal of each species are shown in Fig. 4. For samples with spiramycin concentrations above the ULOQ, a dilution factor of 10 was evaluated and then applied. Relative bias(%) was calculated for the six QC levels obtained over the 18 runs of routine (see Fig. S2). As observed with the figure S2 [A] and [B], 102 out of 108 QC (*i.e.*, 94.4%) were within the acceptance limits and none of the 18 series were rejected. According to the observed data (Fig. 4), spiramycin was slowly eliminated in the milk of all three species and was detected above the LLOQ for up to 6 days (144h) for minor species and 8 days (192h) for cow. The spiramycin profile for goat was in good agreement with results from a recent publication using the same

dose in lactating goats [27]. The sum of spiramycin and neospiramycin concentrations fell below the MRL ( $200 \mu\text{g}\cdot\text{kg}^{-1}$ ) from day 6 onwards for cow and ewe and for day 4 for goat. The analytical method developed in this paper allows the quantification of spiramycin and its metabolite in the milk of the three species from the high concentration present in the first milking post-administration (8h) to concentrations at 0.20 times the MRL several days post-administration. In further studies, it will be interesting to assess how animal physiology impacts the antibiotic distribution in milk of each species. To this purpose, physiologically based pharmacokinetic (PBPK) modeling, considering animal physiology, milk composition and physico-chemical properties of the drug, will be discussed in a future paper.

#### **4. Conclusion**

The present LC-MS/MS method allowed the simultaneous quantification of residues of spiramycin and its active metabolite neospiramycin in cow's milk as well as in the milk of minor species, goat and ewe. A unique linear regression model after logarithmic transformation of the data permitted to determine the concentrations of both analytes in the three milks, in a wide range from 0.2 to 10 times the MRL. The global accuracy profile, using the results of the two analytes in the milk of the 3 species, demonstrated that 80% of future measurements in routine assays will be within the acceptance limits set at 20% for the LLOQ and 15% for the other concentration levels. The use of spiramycin d3 as internal standard was relevant and allowed a high precision and accuracy of the quantification method. The unique accuracy profile was of great interest for studying antibiotic elimination in food products of animal origin and has been confirmed by the determination of both analytes in milk samples of cow, goat and ewe milk treated with spiramycin. Thanks to this unique assay method, it will now be possible to quantify

the whole kinetic of spiramycin and neospiramycin in milk of minor species by using only the most accessible milk spiked with both analytes at 3 concentrations levels in the validated range (in duplicate).

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## Conflict of Interest

Authors do not declare any conflicts of interest for this particular work.

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## Figure Captions

**Figure 1.** Chromatograms of spiramycin [A], neospiramycin [B] and spiramycin-d3 [C] spiked at 40  $\mu\text{g.kg}^{-1}$  in goat, cow and ewe milk.

**Figure 2.** Global accuracy profile of spiramycin and neospiramycin in the milk of goat, cow and ewe. These profiles were obtained by considering a linear regression model after logarithmic

transformation, in a range of 0.2 to 10 times the maximal residue limit. The plain red line is the relative bias, the dashed blue lines are the  $\beta$ -expectation tolerance limits (with  $\beta$  set at 80%), and the dashed black lines represent the acceptance limits (with  $\lambda$  set at 20% for LOQ and 15% for other levels). The dots represent the relative error of the back-calculated concentrations of validation standards and are plotted with respect to their targeted concentration.

**Figure 3.** Polynomial (order 2) regression curve for determination of relative expanded uncertainty (%). The function was located in the range of 40 – 2000  $\mu\text{g.kg}^{-1}$  of spiramycin and neospiramycin in the three milks.

**Figure 4.** Semi-logarithmic plots of concentrations of spiramycin (rhombus) and neospiramycin (circle) residues in milk of cow [A], goat [B] and ewe [C] after one intramuscular injection of 30000 UI.kg<sup>-1</sup> at t=0h. Maximum residues limits (MRL), equals to 200  $\mu\text{g.kg}^{-1}$ , is the sum of spiramycin and neospiramycin residues allowed in marketed milk after 3 injections. The lower limit of quantification (LLOQ) was 40  $\mu\text{g.kg}^{-1}$ .

#### Table captions

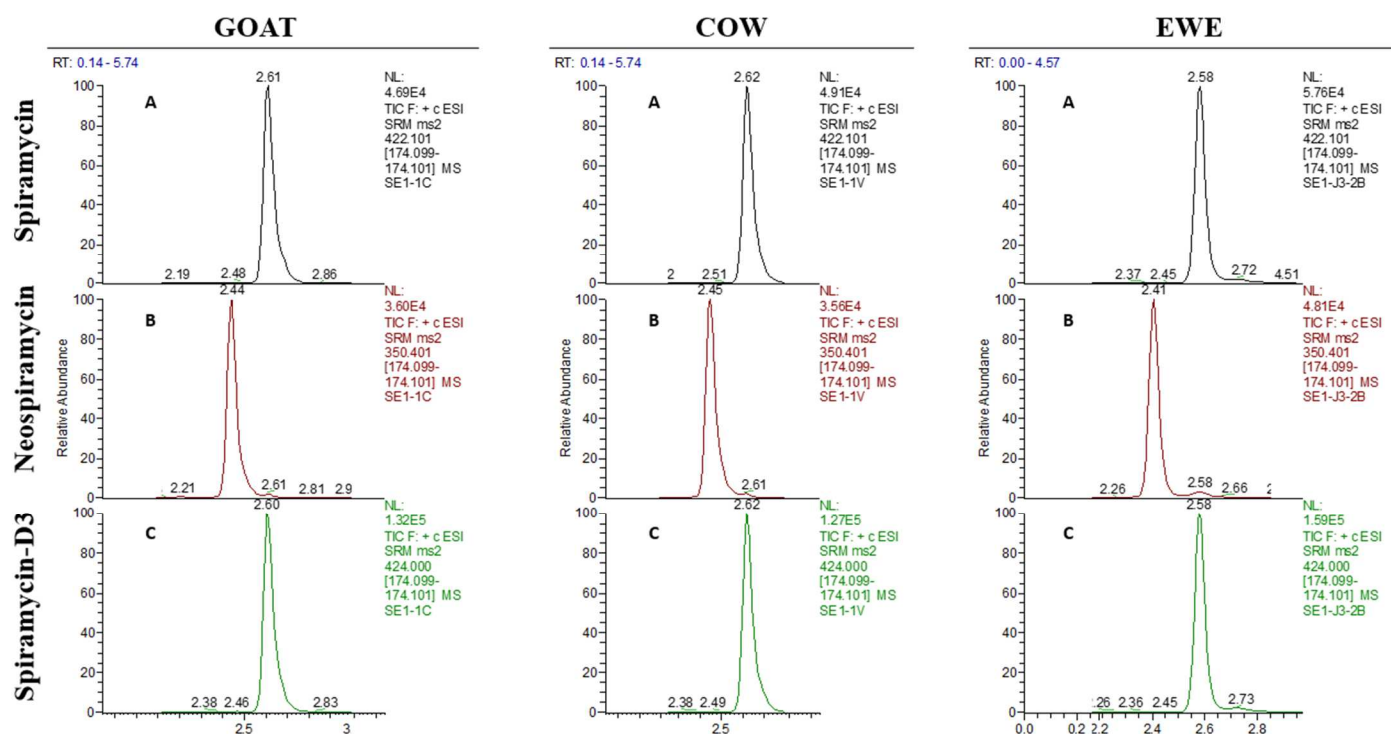
**Table 1.** Specific MS settings and chromatographic characteristics of spiramycin I, neospiramycin I and spiramycin I-d3 acquired with Kinetex C18 (Phenomenex).

**Table 2.** Response function parameters obtained with a linear regression model after logarithmic transformation for the common analysis method of spiramycin and neospiramycin in the milk of cow, goat and ewe. Trueness and precision ranges in the whole range of concentrations are reported, as well as indexes of the related global accuracy profile.

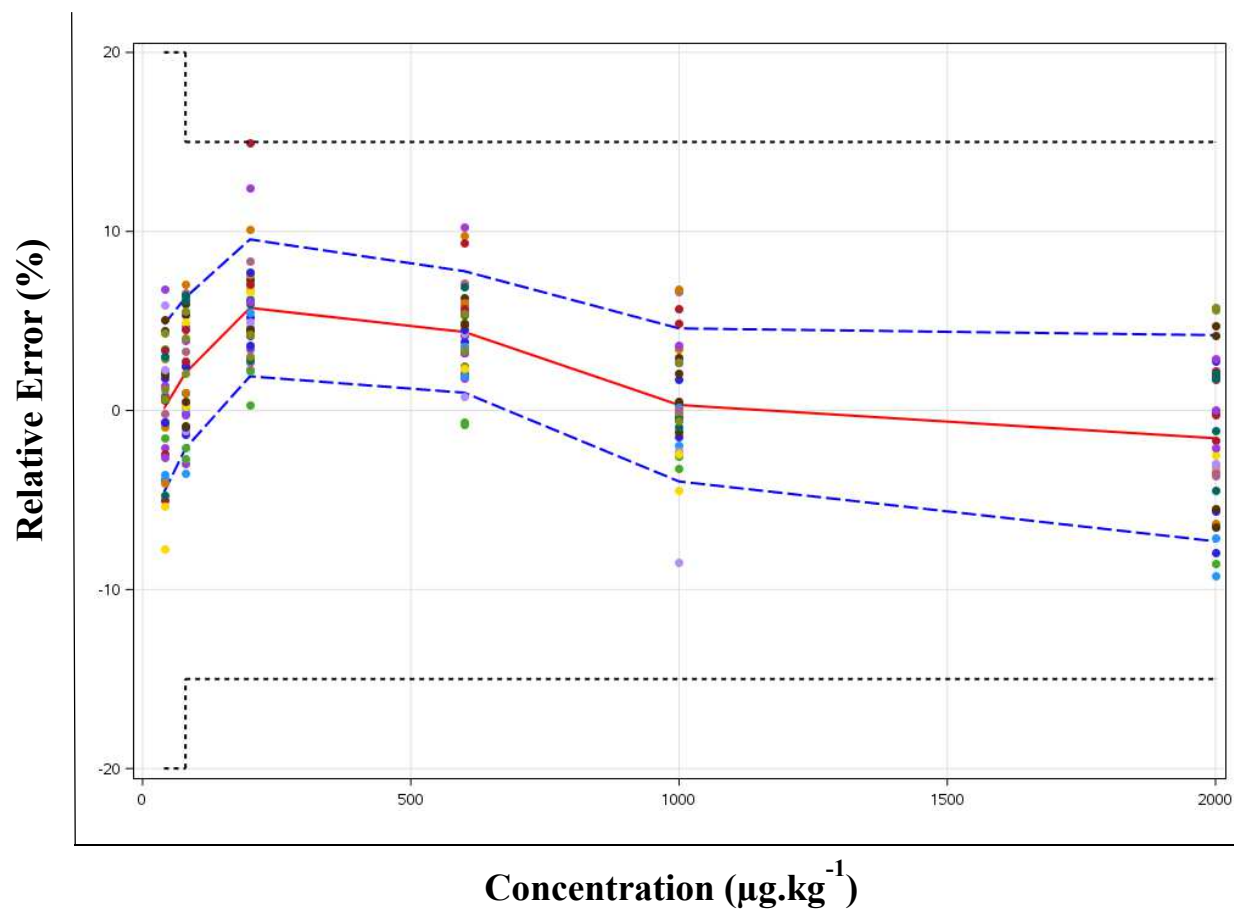
**Table 3.** Relative Expanded Uncertainty (%) obtained from suit data of spiramycin and neospiramycin in the milk of cow, goat and ewe.



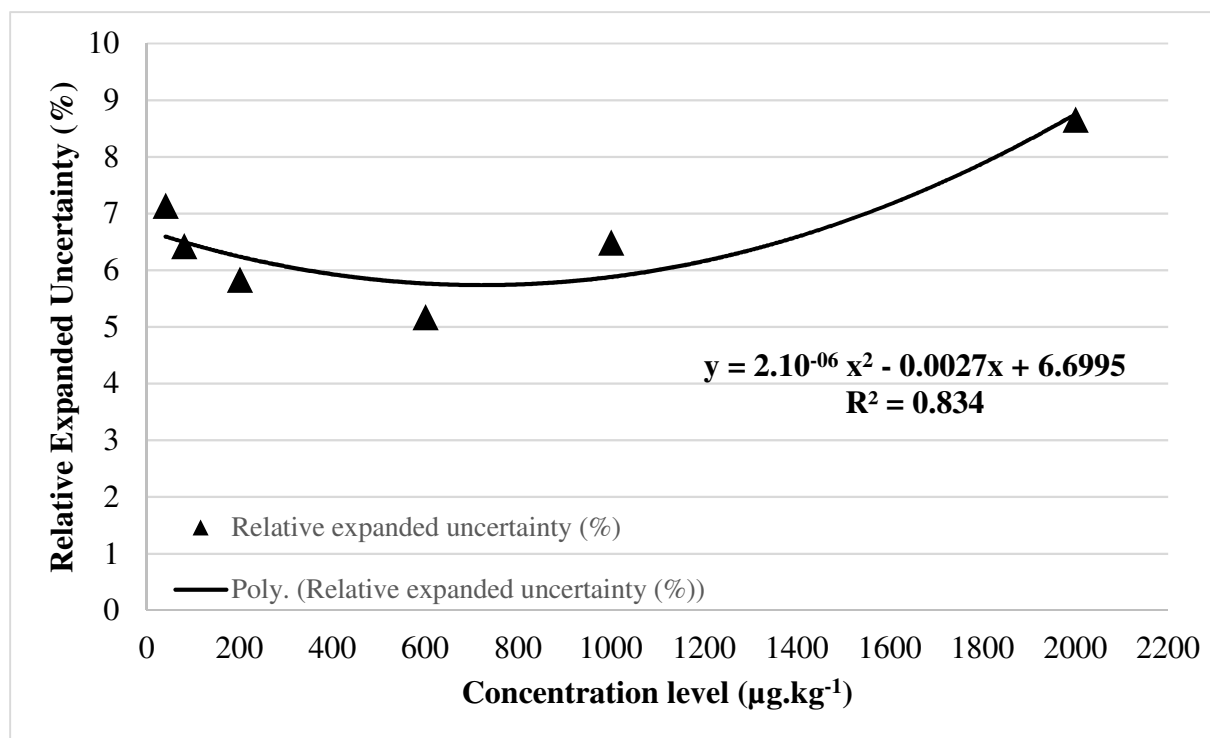
**Fig. 1.**



**Fig. 2.**

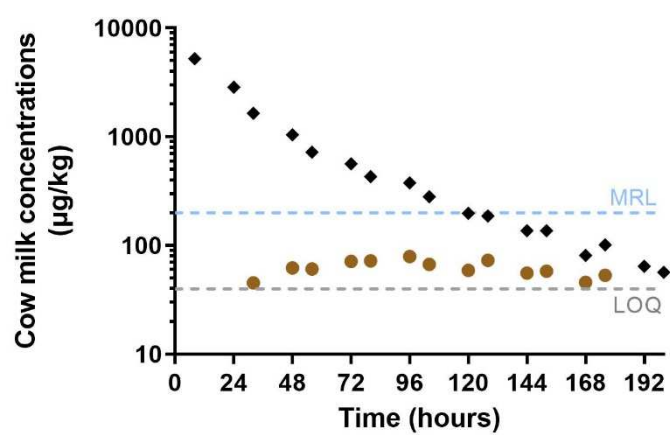


**Fig. 3.**

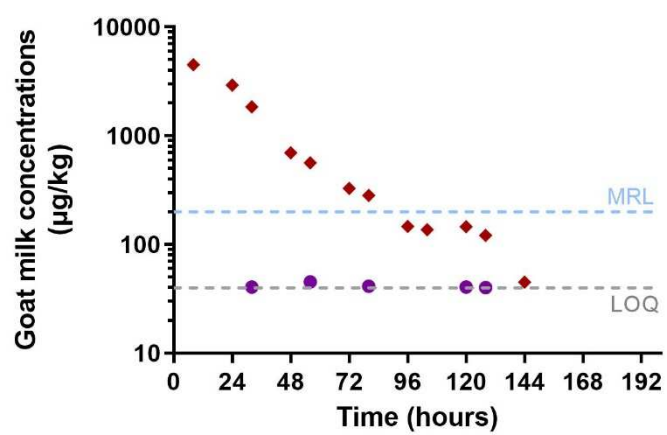


**Fig. 4.**

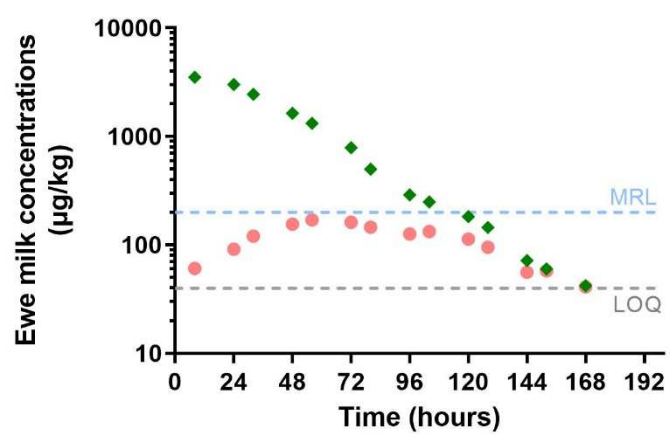
[A]



[B]



[C]



**Table 1**

<b>Parameters</b>	<b>Spiramycin I</b>	<b>Neospiramycin I</b>	<b>Spiramycin I-d3</b>
<b>Elemental composition</b>	C <sub>43</sub> H <sub>74</sub> N <sub>2</sub> O <sub>14</sub>	C <sub>36</sub> H <sub>62</sub> N <sub>2</sub> O <sub>11</sub>	C <sub>43</sub> H <sub>71</sub> D <sub>3</sub> N <sub>2</sub> O <sub>14</sub>
<b>Retention time (min)</b>	2.59	2.44	2.60
<b>Ion nature</b>	[M+2H] <sup>2+</sup>	[M+2H] <sup>2+</sup>	[M+2H] <sup>2+</sup> <sup>13</sup> C
<b>m/z precursor ion</b>	422.1	350.4	424.0
<b>m/z product ion</b>	174.1	174.1	174.1
<b>S-Lens</b>	91	94	101
<b>Collision Energy (volt)</b>	21	16	20

**Table 2**

	Parameters	Values
<b>Response function</b>	Intercept	-1.956
	Slope	0.9325
	R <sup>2</sup>	0.9991
<b>Trueness</b>	Relative bias (%)	[-1.6, 5.7]
<b>Precision</b>	Relative repeatability (RSD %)	[1.1, 2.7]
	Relative intermediate precision (RSD %)	[2.5, 4.2]
<b>Indexes</b>	Accuracy index (I <sub>A</sub> )	0.8795
	Dosing range index (I <sub>Dr</sub> )	1.000
	Precision index (I <sub>P</sub> )	0.7077
	Trueness index (I <sub>T</sub> )	0.9613

**Table 3**

Concentration level ( $\mu\text{g.kg}^{-1}$ )	Relative Expanded Uncertainty (%)
40	7.14
80	6.43
200	5.83
600	5.17
1000	6.49
2000	8.66
<b>Average</b>	6.62