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1 Aminoglycosides analysis optimization using Ion pairing Liquid Chromatography coupled to  
2 tandem Mass Spectrometry and application on wastewater samples

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11

12 Abstract

13 Aminoglycosides are mostly used as veterinary antibiotics. In France, their consumption  
14 accounts for about 10% of all prescribed animal medicine. Due to their high polarity nature (log  
15  $K_{ow} < -3$ ), they require chromatographic separation by hydrophilic interaction liquid  
16 chromatography or ion-pairing chromatography. This study presents the development of an ion  
17 pairing liquid chromatography with alkanesulfonates coupled to tandem mass spectrometry for  
18 the analysis of 10 aminoglycosides (spectinomycin, streptomycin, dihydrostreptomycin,  
19 kanamycin, apramycin, gentamicin, neomycin and sisomicin) in wastewater samples. The  
20 novelty of this method lies in the addition of the ion pairing salt directly and only into the sample  
21 vial and not in the mobile phase, lowering the amount of salt added and consequently reducing  
22 signal inhibition. The optimized method was validated and showed satisfactory resolution,  
23 performances suitable with the analysis of aminoglycosides in wastewater samples, with limits  
24 of quantifications less than 10 ng/mL for most of the compounds, low matrix effects, high

25 accuracy (85%-115% recoveries) and reproducibility (2%-12%RSD). It was then applied  
26 successfully to raw and treated wastewater samples.

27

28 Keywords: Aminoglycosides, wastewater, ion-pairing chromatography

29 Highlights:

30 • In vial addition of salt, limiting the presence of salts in the LC-MS/MS system

31 • A sample preparation reduced to a simple addition of salt in the vial

32 • Low matrix effect allowing external calibration with solvent standards

33

## 34 1. Introduction

35 During the past decade, aminoglycosides (AGs) became one of the most widely used veterinary  
36 antibiotics in both bovine and pork herds [1–3], because of their wide action range for both  
37 Gram-negative and Gram-positive bacteria. In France, while the global antibiotic consumption  
38 is decreasing constantly (divided by 3 in 12 years), aminoglycosides portion is increasing with  
39 up to 10% prescription share in 2017 [2,3]. Furthermore, aminoglycosides are poorly absorbed  
40 due to their high polarity nature and are excreted unchanged in urine [4,5]. Aminoglycosides  
41 antibiotics are also used in human medicine formulation [6,7], increasing the probability of their  
42 presence in wastewaters.

43 Despite their increasing consumption, analytical procedures for environmental matrices are  
44 very few and aminoglycosides are still among the least analysed antibiotics today. The main  
45 reason is probably their highly polar nature, with log K<sub>ow</sub> comprised between -3 for  
46 spectinomycin and -9 for neomycin. Indeed, as noted by Reemtsma et al. [8], this kind of very  
47 polar molecules requires specific analytical tools which do not currently exist and which need  
48 to be developed. Analysis of AGs are more frequent in food-related samples as reported by  
49 Glinka et al. [9] such as meat [10–12], milk [12,13] or honey [14–18]. Maximum residues limits  
50 are defined by food regulations and analytical method performances are set to respect these  
51 values. In the environmental field, there is no regulation or monitoring regarding  
52 aminoglycosides. To the best of our knowledge, only a few studies deal with aminoglycosides  
53 analysis [19–22].

54 Whatever the matrix of interest, separation methods for AGs analysis are based on two major  
55 techniques: Hydrophilic Interaction Liquid Chromatography (HILIC) [4,5,11,23] or Ion Pairing  
56 Liquid Chromatography (IPLC) [17,24], often followed by tandem mass spectrometry  
57 detection. When HILIC is used, a wide variety of column chemistry can be employed for the  
58 analysis of aminoglycosides..

59 Alechaga et al. obtained poor peak shapes using bare silica [25], Ianni et al.[26]used a two-  
60 dimensional LC with HILIC in 1<sup>st</sup>, but required between 51 and 70 minutes to obtain  
61 selectivity.Guillarme tested a variety of HILIC columns and only obtained resolution with  
62 zwitterionic ones[27]. Altogether, HILIC separations lacks of robustness, as variations in  
63 mobile phase composition, pH, buffer concentration or temperature can have a very noticeable  
64 effect on selectivity and retention of compounds [28].

65 In case of very polar compounds as aminoglycosides, Mokh et al. [20] reported that ion-pairing  
66 liquid chromatography could represent a more suitable and powerful technique, with better  
67 retention time consistency. One of the first and most critical steps of IPLC optimization is the  
68 selection of the counter ion. In most reported works in IPLC, heptafluorobutyric acid (HFBA)  
69 [19,29–31], perfluoropentanoic acid (PFPeA) [32], trichloroacetic acid (TCA) or trifluoroacetic  
70 acid (TFA) [29] were used, added in the mobile phase. To match chromatographic conditions,  
71 ion-pairing reagents were also added in the sample vial. Nevertheless, those additives are  
72 known to cause signal inhibition in mass spectrometry [33], increasing detection and  
73 quantification limits. A comparison of HILIC and IPLC with HFBA as counter ion was realised  
74 by Gremilogianni et al. [30] and concluded to greater performances of the HILIC method,  
75 because of high ion suppression caused by IPLC. Moreover, introduction of high salt content  
76 mobile phases in the mass spectrometer source also leads to more frequent instrument  
77 maintenance. To circumvent this problem, Lehotay et al. [34] proposed an IPLC method for the  
78 analysis of drug residues, adding ion-pairing reagents only into the injection vial, thus reducing  
79 the amount injected in the LC column and in the mass spectrometer, preventing some down-  
80 time of the instrument for cleaning and maintenance. The ion suppression caused by the IP  
81 reagent was consequently reduced, allowing to achieve better quantifications limits. Wang et  
82 al. and Amelin et al. [31,35] also proposed this “in vial only addition” method, using HFBA as  
83 ion pair whereas Lehotay used sodium heptanesulfonate, known as more volatile reagent, in

84 order to further reduce signal suppression. However, in all studies with only in vial addition,  
85 little to no resolution was obtained, with AG separation spread only over 0.5 to 1 min, which  
86 may cause analytical difficulties in complex and charged matrices.

87 Based on these observations, the objective of this work was to develop and optimize an “only  
88 in vial addition” IPLC-MS/MS method with satisfactory resolution of 10 AGs and to evaluate  
89 its suitability for their surveillance in environmental waters. First, various alkanesulfonate  
90 counter-ions with different carbon chain length were tested to evaluate retention and separation.  
91 Chromatographic conditions such as organic mobile phase, gradient or isocratic elution,  
92 mixture of IP reagents were developed to obtain good separation, and minimize matrix effects.  
93 The final method was validated and applied on water samples from both wastewater treatment  
94 plants around Lyon and from the Rhône river (France). This is then the first method reporting  
95 resolution of aminoglycosides with only “In vial addition” IPLC.

96

## 97 2. Experimental

### 98 2.1. Chemicals and reagents

99 Apramycin (APR), dihydrostreptomycin (DHSTREP), gentamicin (GEN), kanamycin (KAN),  
100 neomycin (NEO), spectinomycin (SPEC), streptomycin (STREP) and sisomicin (SISO) were  
101 purchased from Sigma Aldrich (Saint Quentin Fallavier, France) in VETRANAL quality or  
102 equivalent purity (>98%). The GEN standard contained three distinct molecules: Gentamicin  
103 C1 (GEN C1); Gentamicin C1A (GEN C1A) and Gentamicin C2 (GEN C2) (Figure S1). Stock  
104 solutions (1 mg.mL<sup>-1</sup>) of each aminoglycoside were prepared by dissolving about 10 mg  
105 powder, accurately weighted in 10 mL of water/methanol (1/1, v/v) in high density polyethylene  
106 Wheaton vials were stored at -18°C. Accurate concentration was then calculated taking each  
107 standard purity in account. Autosampler vials and centrifuge tubes in polypropylene (PP) were

108 used to prevent adsorption of the analytes on glass. Calibration solutions of each analyte (500  
109 ng.mL<sup>-1</sup>) were prepared by diluting individual stock solutions in water/methanol (1/1, v/v).  
110 Alkanesulfonate salts ranging from butanesulfonate to decanesulfonate (Figure S1) were  
111 acquired from Tokyo Chemical Industry, Belgium to be used as ion-pairing salts. Individual  
112 salt solutions were realised at 75 mM in water and stored in the fridge at 4°C during one month.  
113 Water (LC-MS grade) was obtained from Fisher Scientific (Illkirch, France), methanol (MeOH)  
114 and acetonitrile (ACN) (LC-MS grade) from Honeywell (Seelze, Germany) and formic acid  
115 (UPLC-MS grade) from Biosolve (Dieuze, France).

116

## 117 2.2. Real sample collection and preparation

118 Three kinds of water matrices were collected: river water for the optimization and raw and  
119 treated wastewaters (WW) for application of the optimized method. River water was grab  
120 sampled in the Rhône river (France). WW were collected at 3 different WW treatment plants in  
121 the region of Lyon (France).

122 Samples were stored at -20°C before analysis. With the optimized method, after thawing, a 2  
123 mL aliquot was sampled and transferred to a PP centrifuge tube, centrifuged at 10000 rpm for  
124 5 min (3K3OH, Sigma, Germany). Then 200 µL of supernatant was transferred into a 1 mL PP  
125 vial together with 200 µL of sodium hexanesulfonate solution and 200 µL of sodium  
126 heptanesulfonate solution. The vial was then capped and agitated on a rotor mixer for 20  
127 seconds.

128

## 129 2.3. Ion Pair Liquid chromatography coupled to tandem mass spectrometry (IPLC-MS/MS)

130 The system used was an Agilent (Massy, France) 1200 Series High-Performance Liquid  
131 Chromatography system with a binary pump. The column was a Kinetex XB-C18, 100\*2.1mm,  
132 1.7 µm from Phenomenex (Le Pecq, France). Optimized IPLC conditions were as followed: a

133 binary mobile phase was used with a flow-rate set to 300  $\mu\text{L}\cdot\text{min}^{-1}$  for a total run time of 15  
134 min, with the column maintained at 40°C. Mobile phase A was an aqueous solution of 0.1%  
135 formic acid, and B was a mixture of ACN/MeOH (1/1) with 0.1% formic acid. The separation  
136 was performed with an isocratic mobile phase at 10% B for 7 min. B was then increased to 90%  
137 for 5 min and then decreased back to 10% for 3 min starting re-equilibration of the column. An  
138 equilibration time of 5 min (i.e. 6 column volumes) was realised before each injection, leading  
139 to a total run time of 20 min. The sample injection volume was 10  $\mu\text{L}$ . The final injection  
140 solvent was composed of 90/10  $\text{H}_2\text{O}/(\text{MeOH}/\text{ACN})$  containing 50 mM of both hexanesulfonate  
141 and heptanesulfonate sodium salts.

142 A 5500 QTrap from Sciex® (Les Ulis, France) was used in Multiple Reaction Monitoring  
143 (MRM) mode with positive electrospray ionization. Source parameters are detailed in Table S1.  
144 MS/MS detection was optimized by infusion of individual standard solutions at 100  $\text{ng}\cdot\text{mL}^{-1}$   
145 via syringe pump at a flow of 10  $\mu\text{L}\cdot\text{min}^{-1}$  and are presented in Table S2. For SPEC, both  
146 protonated and water adducts showed a similar sensitivity; we chose to monitor both ions, and  
147 use the  $\text{H}_2\text{O}$  ion for quantification and the  $[\text{M}+\text{H}]^+$  adduct for confirmation [36]. NEO and SISO  
148 also formed a doubly charged ion in the ionisation source, it was decided to follow both ions.

149

#### 150 2.4. Method validation

151 Limits of quantification (LOQs) were evaluated as the concentrations leading to a signal-to-  
152 noise ratio of 10. The method linearity for each molecule was determined by injection of  
153 standards mixtures from 0.5\*LOQ to 50\*LOQ. Intra day repeatability and intermediate  
154 precision were both evaluated during three days. Each day, a calibration curve for each  
155 component was freshly prepared and injected, followed by three standard solutions spiked at  
156 three concentration levels: LOQ, 2\*LOQ and 10\*LOQ, also freshly prepared. For each analyte  
157 and each level, the concentration was computed with the calibration curve and the accuracy

158 calculated with the mean of the 3 replicates versus the nominal concentration. Intraday  
159 repeatability was determined by calculating the relative standard deviation (RSD) of replicates  
160 injected the 2<sup>nd</sup> day and interday precision was determined by calculating the RSD on the three  
161 days means.

162 Matrix effects were evaluated for each analyte by comparing a calibration curve prepared in  
163 pure LC-MS grade water and in river water, after centrifugation. The ratio of the slopes was  
164 considered as matrix effect indicator.

165

### 166 3. Results and discussion

#### 167 3.1. Adsorption of GEN on container material

168 During the first experiments, decreases appeared on several signals, especially for GEN. A  
169 comparison between glass and PP vial was carried out to test for adsorption of the three distinct  
170 molecules composing GEN. Figure 1 illustrates the significant signal diminution, of about 50%  
171 for each component of the GEN mix when using glass vials. Moreover, the variations in-  
172 between vials were also increased in glass container. PP vials were hence used for all the study.  
173 No adsorption difference was noted between the three gentamicin components as their  
174 distribution was unchanged between glass and PP vials: 29% GEN C1; 34% GEN C1A and  
175 37% GEN C2.

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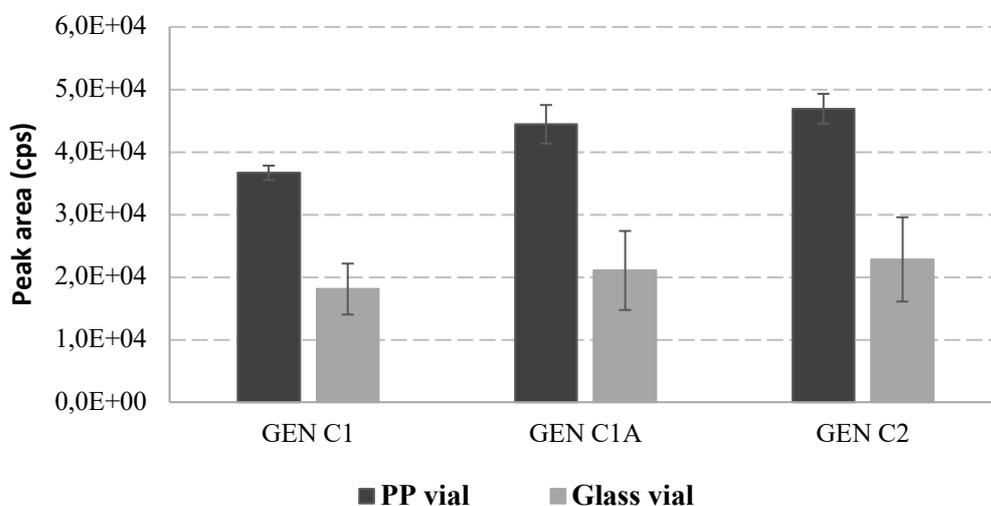
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183 **Figure 1: Comparison of the LC-MS/MS signals of GEN C1, GEN C1A and GEN C2**  
184 **solutions at 200 ng.ml<sup>-1</sup>, left for 24h at 4°C in PP or glass vials (n = 3).**



185

186

### 187 3.2. IPLC-MS/MS method optimization

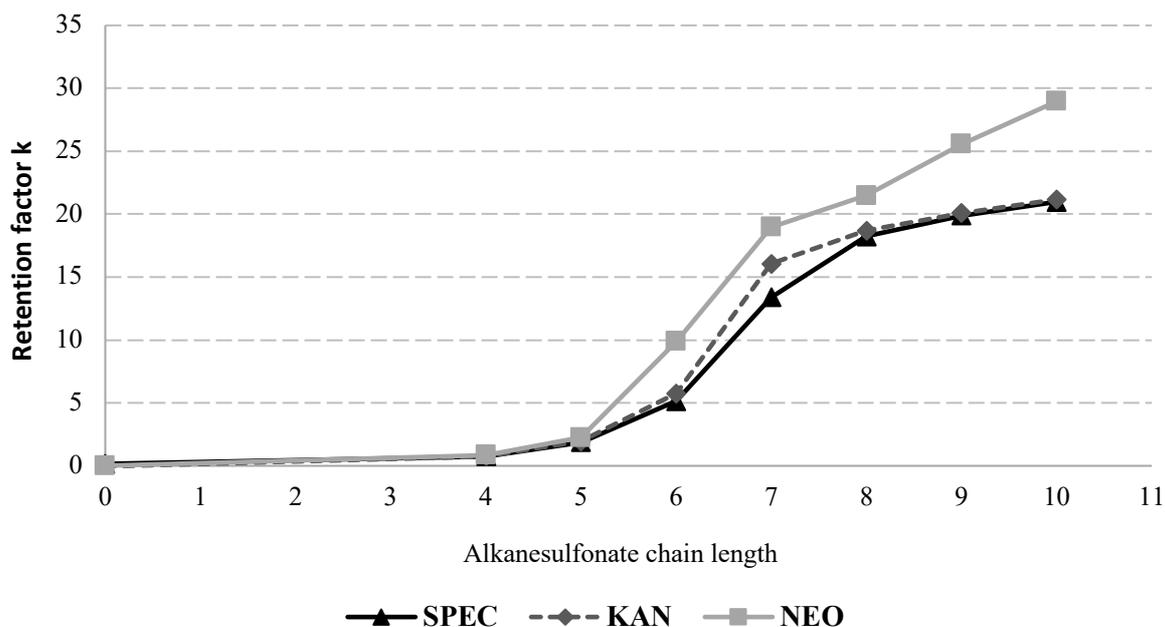
#### 188 3.2.1. IP salts choice

189 Alkanesulfonates ion-pairing salts from sodium butanesulfonate to sodium decanesulfonate  
190 were tested for the separation of AGs. Each salt was evaluated individually at 50 mM, by mixing  
191 300  $\mu$ L of standard solution with 600  $\mu$ L of IP salt at 75 mM in the injection vial, except for  
192 nonanesulfonate and decanesulfonate, tested at 25 mM due to their lower critical micellar  
193 concentration (estimated from [37] at 65 mM and 32 mM for nonanesulfonate and  
194 decanesulfonate, respectively). An injection was also realised without addition of IP salt. The  
195 separation was evaluated by considering the retention factor (k). An illustration of the results  
196 for three aminoglycosides with dispersed retention times (SPEC, low; KAN, middle, NEO,  
197 high) are presented in Figure 2. It can be observed that when no salt was added, all compounds  
198 were eluted in the column dead volume, without separation. On the other hand, a separation  
199 was possible in the presence of salts, with k increase with the carbon chain length. Same  
200 behaviour was observed for all targeted AGs. Satisfactory separation was obtained when using

201 ion pairing alkanesulfonate salt between n=6 and n=7. For n>7, similar retention was observed  
202 for compounds that were previously separated with heptanesulfonate salt.

203

204 **Figure 2: Evolution of retention factor k for the aminoglycosides SPEC, KAN and NEO**  
205 **as a function of the ion pair carbon chain length**



206

207 To further optimize the separation, different proportions of hexanesulfonate (IP6) and  
208 heptanesulfonate (IP7) salts were tested: 0/100, 25/75, 50/50, 75/25 and 100/0. For all AGs, an  
209 increase of the IP7 percentage resulted in an increase of the retention factor (Figure 3). The  
210 50/50 proportion of hexanesulfonate and heptanesulfonate ion pairing salt was finally chosen,  
211 which allowed an satisfactory separation in 8 minutes . If more retention or faster separation is  
212 required, the method could be easily modulated by modifying IP ratios.

213

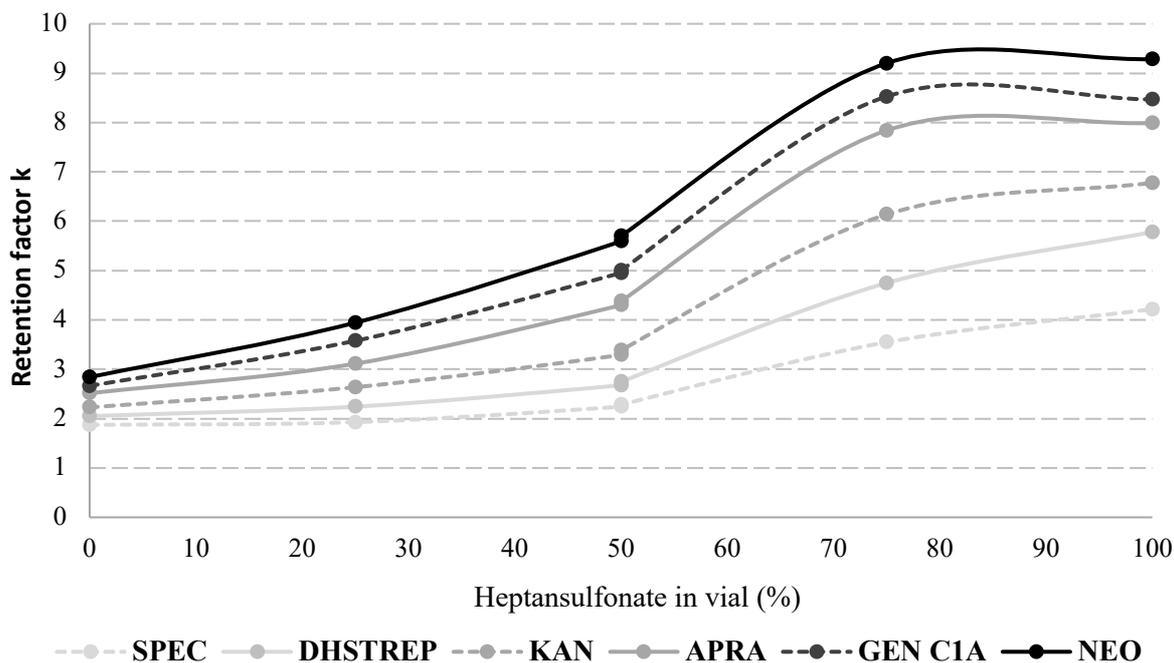
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217

218 **Figure 3 : Evolution of retention factors k with proportion of heptanesulfonate in vial**



219

220

### 221 3.2.2. Organic solvent mobile phase

222 After the selection of the ion pairing salts, interest was moved to the organic solvent used (ACN  
223 or MeOH) to both reduce the analysis run time and improve separation. When ACN was used  
224 in the mobile phase, all compounds eluted earlier, reducing the analytical time, but also  
225 reducing the resolution between compounds that elutes at close retention times i.e. streptomycin  
226 and dihydrostreptomycin or the gentamicin components. A one to one mixture of MeOH/ACN  
227 was finally selected to obtain a satisfactory separation of critical pairs while keeping a shorter  
228 analysis time than using only MeOH. The presence of MeOH also allowed better ionisation of  
229 each molecule in the MS source (data not shown).

230

### 231 3.2.3. Column conditioning and storage

232 To ensure reproducibility of analysis, the column needed to be conditioned with IP salts before  
233 each sequence. To determine the number of injections needed to obtain a repeatable retention

234 times, a serie of six standards injections in IP6/IP7 (50/50, v/v) salt was carried out on a clean  
235 column (with salt removed, with the procedure described thereafter). The evolution of the  
236 retention factors with the number of injections is presented in Figure 4 and it can be noticed  
237 that constant retention factors were obtained after four injections of IP salts. Therefore, in order  
238 to maintain repeatable retention times, six consecutive injections of IP6/IP7 (50/50, v/v) salts  
239 were realized prior to any sequence of analysis. The final chromatogram is presented in  
240 supplementary materials (Figure S2).

241  
242 **Figure 4 : Evolution of the average retention factor of all targeted AGs with injection**  
243 **number**



244  
245  
246 One of the major drawbacks in using ion pairing chromatography is the possible clogging of  
247 the LC column. Removal of the salts after an analysis sequence is important to preserve the  
248 column and avoid increasing pressure, or even column blockage. The cleaning procedure  
249 proposed included three steps. First the LC was disconnected from the mass spectrometer and  
250 the flow was directed to a waste bottle. A 75/25 H<sub>2</sub>O/MeOH mixture was then flowed through

251 the column at  $100 \mu\text{L}\cdot\text{min}^{-1}$  for 15-20 min for optimal salts solubilisation. Then, 50/50  
252  $\text{H}_2\text{O}/\text{MeOH}$  was flowed at the same rate for 15 min, resulting in a higher pressure, allowing  
253 further penetration in the column particles pores and further salts solubilisation. Finally, 25/75  
254  $\text{H}_2\text{O}/\text{MeOH}$  mixture was flowed at  $300 \mu\text{L}\cdot\text{min}^{-1}$  to equilibrate the column for storage and to  
255 allow the C18 chains to be reconditioned.

256

### 257 3.3. Method validation

#### 258 3.3.1. Linearity of the method

259 First, approximate quantification limits of aminoglycosides were determined by injecting  
260 replicates ( $n=3$ ) of standard solutions, based on signal-to-noise ratios of 10. Aminoglycosides  
261 response linearity was then determined from the injection of standards. Each compound  
262 displayed a good linearity over the selected range, with determination coefficients ( $R^2$ ) greater  
263 than 0.99 (Table 1).

	Retention time (min) (%RSD <sup>b</sup> )	LOQ (ng.ml <sup>-1</sup> )	Linearity		Transition ratio (%RSD <sup>b</sup> )	LOQ		2 LOQ		10 LOQ	
			Range (ng.ml <sup>-1</sup> ) (0.5LQ-50LQ)	R <sup>2</sup>		Mean (%RSD <sup>(a/b)</sup> )	Accuracy (%)	Mean (%RSD <sup>(a/b)</sup> )	Accuracy (%)	Mean (%RSD <sup>(a/b)</sup> )	Accuracy (%)
<b>SPEC</b>	3.4 (1.0)	9.6	5-500	0.992	0.58 (1.3)	9.0 (5/33)	94	20.2 (5/4)	105	96.2 (1/6)	101
<b>SPEC+H2O</b>	3.4 (1.0)	9.6	5-500	0.998	0.29 (0.7)	10.2 (5/18)	107	19.7 (4/5)	103	98.2 (4/2)	103
<b>STREP</b>	4.4 (1.3)	5.2	2-260	0.994	0.54 (2.3)	5.4 (4/20)	104	10.3 (3/2)	99	50.7 (1/4)	98
<b>DHSTREP</b>	4.4 (1.1)	0.7	0.3-35	0.995	0.37 (3.0)	0.7(19/11)	96	1.5 (12/10)	104	7.1 (6/5)	101
<b>KANA</b>	5.2 (1.2)	3.1	1-160	0.991	0.65 (1.9)	3.5 (6/8)	111	6.4 (2/4)	101	31.5 (4/0.6)	100
<b>APRA</b>	5.8 (0.3)	17	8-850	0.996	0.86 (1.6)	15.7 (6/23)	92	35 (6/12)	103	170.9 (2/11)	100
<b>GEN C1</b>	6.5 (0.4)	45	22-2230	0.995	0.84 (3.5)	40.0 (14/4)	90	87.8 (7/10)	98	443.8 (7/2)	100
<b>GEN C1A</b>	6.2 (0.5)	28	14-1420	0.999	0.57 (3.3)	30.4 (4/24)	107	55.5 (4/7)	98	285.2 (2/4)	100
<b>GEN C2</b>	6.3 (0.8)	64	30-3200	0.998	0.60 (2.3)	65.5 (6/10)	103	122.3 (6/4)	96	620.3 (1/6)	98
<b>NEO</b>	6.8 (0.1)	465	230-23234	0.995	0,49 (1.4)	577 (13/3)	124	1022.6 (7/5)	110	4671.5 (4/5)	101
<b>NEO 2+</b>	6.8 (0.1)	464	230-23234	0.994	0.76 (6.2)	/	/	941.3 (12/7)	101	4703.9 (9/2)	101
<b>SISO</b>	6.0 (0.5)	111	55-5531	0.995	0.54 (1.5)	120.4 (11/8)	109	22.3 (11/7)	100	1116.8 (7/3)	101
<b>SISO 2+</b>	6.0 (1.5)	111	55-5531	0.994	0.36 (12.9)	111.7 (17/21)	101	222.1 (8/12)	100	1064.4 (3/7)	96

264 **Table 1: Validation results: Linearity and reproducibility at 3 different levels (a: intraday n=3; b: interday n=3 days)**

265

### 266 3.3.2. Limits of quantification

267 Quantification limits of the optimized analytical method were calculated according to the  
268 obtained calibration curves and the coefficients of variation, and were comprised between 0.6  
269 and 460 ng.mL<sup>-1</sup> (Table 1) therefore consistent with the literature for aqueous environmental  
270 matrices (Table 2). Mass spectrometry response factors of NEO and SISO were lower leading  
271 to higher LOQs than for the other analytes [20]. It is worthnoting that previous studies included  
272 a pre-concentration step (solid phase extraction, lyophilisation) whereas the method optimised  
273 in the present work includes a simple addition of salts in the injection vial. It is therefore just  
274 as sensitive while being faster and less solvent consuming.

275 For STREP, DHSTREP and KAN, the method developed in this work resulted in improved  
276 LOQ by a factor of 10, 50 and 5, respectively, regarding the work of Mokh et al. [20], who used  
277 pentafluoropropionic acid as IP in the mobile phases with a 16 min run time. Qiu et al. [21]  
278 reported similar LOQs, after a preconcentration step by a factor of 10.

279

280

<b>Analytes</b>	<b>Matrix</b>	<b>Sample preparation</b>	<b>LC conditons</b>	<b>LOQs (ng.ml<sup>-1</sup>)</b>	<b>Ref.</b>
<b>SPEC, STREP, DHSTREP, GEN, KAN, APRA, SISO, NEO</b>	Wastewaters	SPE	IPLC-MS/MS	15-45	[21]
<b>SPEC, STREP, DHSTREP, GEN, KAN, APRA, SISO,NEO</b>	Wastewaters	Lyophilisation	Mixed mode LC-MS/MS	2-20	[22]
<b>SPEC</b>	Liquid hog manure	SPE	HILIC-MS/MS	6	[23]
<b>SPEC, STREP, DHSTREP, GEN, KAN, APRA, SISO, NEO</b>	Wastewaters	Centrifugation and dilution by salt additions	IPLC-MS/MS	0.3-60 (100-500 for SISO and NEO)	This work

281

**Table 2 : Quantification limits obtained in different environmental matrices**

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### 3.3.3. Intraday repeatability, intermediate precision and recovery

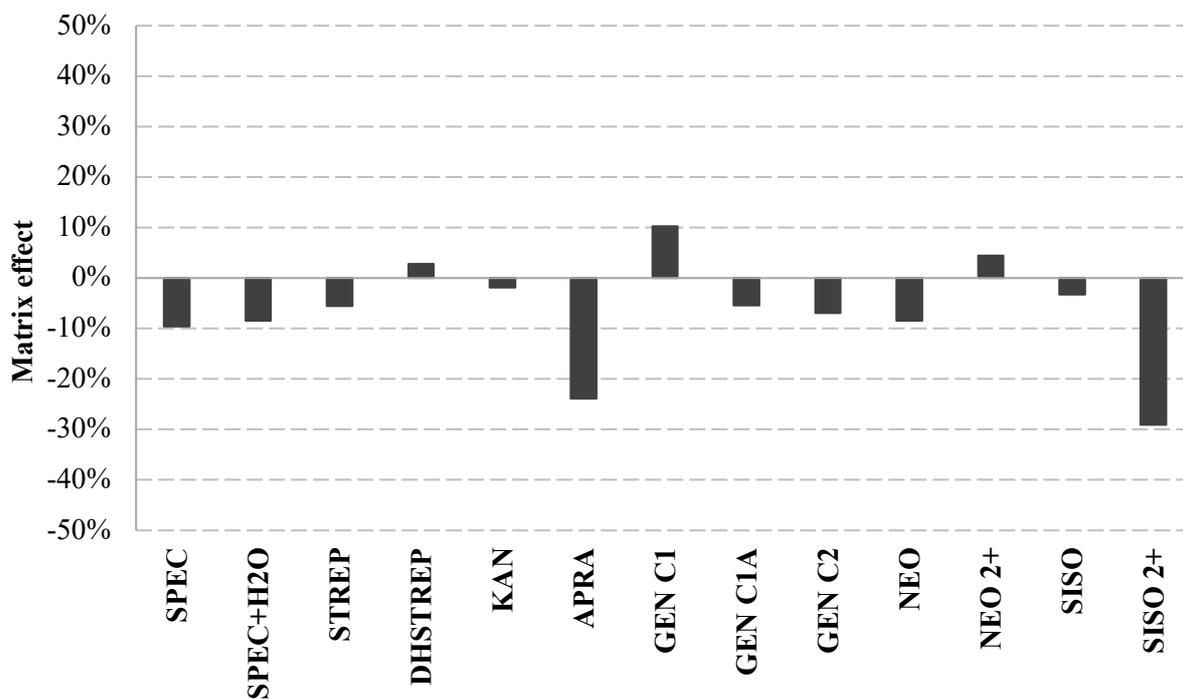
Results of both repeatability and intermediate precision experiments are compiled in Table 1. Concerning the intraday repeatability, RSD were measured inferior to 20% at LOQ level for all compounds, showing good repeatability from one analysis to another. At 10\*LOQ level, RSD are further reduced, below 5% for 80% of the followed molecules. For intermediate precision, at LOQ levels, most RSD were measured below 25%, except for SPEC, evaluated at 33%. As  $[M+H]^+$  ion was only monitored for confirmation, this higher variation had no impact on the validation process. These variations were deemed acceptable based on the paper from Rambla-Alegria et al. [38], stating that, for analyte concentration around the  $ng.g^{-1}$  level, RSD below 30% are reasonable. At levels 2\*LOQ and 10\*LOQ, all RSD were below 12%. For all levels, quantification was accurately realised with calculated values within  $\pm 15\%$  of the nominal concentration. As responses of the MRM transitions corresponding to NEO 2+ were very variable at the LOQ level, it was excluded from the reproducibility results. This exclusion did not impact the results as the singly-charged ion for neomycin was well detected at the LOQ. Both variations on the retention time and the ratio between the two followed transitions were also recorded, and presented in Table 1. Throughout the analysis, retention time variations were all lower than 0.1 min, and ratio variations were all below 5%RSD, except for the doubly charged transitions we followed for NEO and SISO. These higher variations could be explained by the tendency of the molecules to form either the single charged ion or the doubly charged, with no predominant form. Qiu et al. reported recoveries between 66% and 116% of their analytes at 20, 50 and 100  $ng.mL^{-1}$  with inter-day RSD below 16%. The method presented here reduced the recoveries range to 85%-115%. Direct injection of the sample avoids some losses due to lyophilisation step.

307 3.3.4. Matrix effects

308 The matrix effects, expressed in %, are presented in Figure 5. For most molecules, matrix effects  
309 were comprised within -20% and 20% and was considered negligible. Therefore the  
310 quantification could be performed from a calibration realised in solvent. Only APRA and the  
311 doubly-charged ion for SISO presented higher matrix effect (a signal inhibition) and would  
312 require a matrix match calibration or the use of isotope labeled standards. Further dilution of  
313 the sample could reduce those matrix effect to enable only calibration in solvent.

314

315 **Figure 5 : Evaluation of matrix effects for aminoglycosides in river water (calibration**  
316 **curve : LQ-50\*LQ)**



317

318

319 3.4. Application to WW samples

320 Six samples of raw and treated WW were analysed unspiked and spiked at 10\*LOQ with all  
321 the aminoglycoside standards. Blank controls composed of H<sub>2</sub>O/ACN/MeOH (90/5/5) +0.1%  
322 formic acid, corresponding to the chromatographic initial conditions, were injected during the

323 analytical batch, in the same way as the standard solutions and the samples No aminoglycoside  
324 was detected in unspiked samples neither raw nor treated wastewaters. Chromatograms are  
325 supplied in supplementary materials (Figure S2). In similar studies, Löffler et al.[19] and Mokh  
326 et al.[20] only detected GEN (average 4 ng.mL<sup>-1</sup> and 0.3 ng.mL<sup>-1</sup> , respectively) in hospital  
327 effluents, as GEN can also be used in human medicine. Regarding spiked samples, accuracy at  
328 10\*LOQ was calculated and was comprised between 75% and 125%, as reported in Table 1.  
329 These results demonstrate again the negligible matrix effects induced by this method, even  
330 when analysing more complex environmental water samples, such as wastewaters.

331

#### 332 4. Conclusion

333 The analytical method developed in this study allows the simultaneous determination of 10  
334 aminoglycosides in environmental water samples, with simple, fast, and eco-friendly sample  
335 preparation. Ion pairing liquid chromatography was developed with a novelty : adding the ion-  
336 pairing salt directly in the sample vial instead of the mobile phases reservoirs. This allowed the  
337 use of lower quantity of ion-pairing salt per analysis, therefore limiting their negative impact in  
338 mass spectrometry such as signal inhibitions, while still obtaining a good and consistent  
339 separation. The method was found sensitive and reliable in environmental analysis with almost  
340 non existent matrix effects, recoveries comprised between 85% and 115%, with low variations  
341 across intra and inter-day analyses. This work is the 1<sup>st</sup> to demonstrate the ability of “in vial  
342 addition only” IPLC-MS/MS methods to monitor accurately, in environmental waters, highly  
343 polar molecules and could be adapted to other substances of this type, with modulation of IP  
344 reagents ratios or chain length.

345

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