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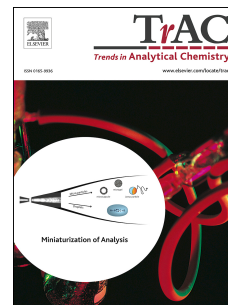
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# 1 **Determination of aminoglycoside antibiotics: current status and future trends**

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## 6 **Abstract**

7 The use of aminoglycoside antibiotics is prevalent in medicine and agriculture. Their overuse  
8 increases their mobility in the environment, resulting in a need for reliable methods for their  
9 determination in a variety of matrices. However, the properties of aminoglycosides, in  
10 particular their high polarity, make the development of such methods a non-trivial task,  
11 inciting researchers to tackle this complex issue from different angles. The necessity to  
12 determine aminoglycosides in complex matrices and at low concentration levels requires the  
13 development of relatively elaborate sample preparation methods and the use of selective and  
14 sensitive detection techniques. Various modes of liquid chromatography coupled with tandem  
15 mass spectrometry are usually the analytical methods of choice. However, the recent  
16 developments in techniques such as bioassays, quantum dot-based colourimetric applications  
17 and various aptasensors point towards the development of more easily accessible and user-  
18 friendly point-of-need tests for screening applications in food control and environmental  
19 monitoring. This review summarizes the state-of-the-art in sample preparation protocols and  
20 the determination of aminoglycosides using various techniques and outlines the future trends  
21 with an emphasis placed on the novel and emerging solutions in this area.

22 **Keywords:** aminoglycoside antibiotics, liquid chromatography, novel trends in sample  
23 preparation, residue analysis.

## 24 **Abbreviations**

25	2D-LC	two-dimensional chromatography
26	ABS	Acid Chrome Black Special
27	AD	amperometric detector
28	AGs	aminoglycoside antibiotics
29	AMI	amikacin
30	APR	apramycin

31	AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
32	AVI	avilamycin
33	BAC	bacitracin
34	BEK	bekanamycin
35	CAD	charged aerosol detection
36	CBX	carboxylic acid sorbent
37	CE	capillary electrophoresis
38	CFSE	6-carboxyfluorescein succinidyl ester
39	C <sup>4</sup> D	contactless conductivity detection
40	DC-ELISA	direct competitive enzyme linked immunosorbent assay
41	DHSTR	dihydrostreptomycin
42	DMIP	dummy molecularly imprinted polymer
43	DSPE	dispersive solid phase extraction
44	ELISA	enzyme linked immunosorbent assay
45	ELSD	evaporative light scattering detection
46	FASS	field-amplified sample stacking
47	FDNB	1-fluoro-2,4-dinitrobenzene
48	FESI	field-enhanced sample injection
49	FIA	fluoroimmunoassay
50	FLD	fluorescence detector
51	FMOC	9-fluorenylmethyloxycarbonyl
52	GCB	graphitized carbon black
53	GEN	gentamycin
54	HFBA	heptafluorobutyric acid
55	HILIC	hydrophilic interaction chromatography
56	HLB	hydrophilic-lipophilic balance
57	HPLC	high performance liquid chromatography
58	HYG	hygromycin
59	IPLC	ion-pairing liquid chromatography
60	IC-ELISA	indirect competitive enzyme linked immunosorbent assay
61	ISE	ion-selective electrode
62	KAN	kanamycin
63	LIF	laser-induced fluorescence detection
64	LIN	lincomycin



65	LIV	lividomycin
66	LLE	liquid-liquid extraction
67	MCX	medium cationic exchangers
68	MIP	molecularly imprinted polymers
69	MS	mass spectrometry
70	MSB	moving substitution boundary
71	MS/MS	tandem mass spectrometry
72	NEO	neomycin
73	NET	netilmicin
74	NITC	1-naphthyl isothiocyanate
75	NMR	nuclear magnetic resonance spectroscopy
76	NP	nanoparticle
77	OPA	o-phthalaldehyde
78	OPD	o-phenylenediamine
79	PAD	pulsed amperometric detection
80	PAR	paromomycin
81	PBS	phosphate-buffered saline
82	PCX	polymeric cation exchanger
83	PFPA	pentafluoropropionic acid
84	PSA	primary-secondary amine
85	PTFE	polytetrafluoroethylene
86	QD	quantum dots
87	RIA	radioimmunoassay
88	RIB	ribostamycin
89	SCX	strong cation exchange
90	SIS	sismocin
91	SPC	spectinomycin
92	SPE	solid phase extraction
93	STR	streptomycin
94	TFA	trifluoroacetic acid
95	TMB	3,3',5,5'-tetramethylbenzidine
96	TOB	tobramycin
97	VAL	validamycin
98	WCX	weak cationic exchangers



99 ZIC-HILIC stationary phases with zwitterionic groups covalently bound to the surface of  
100 silica particles  
101

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## 102 1. Introduction

103 Aminoglycosides antibiotics (AGs) are a group of pharmaceuticals with a broad spectrum  
104 of therapeutic applications [1]. AGs are mainly used against infections caused by Gram-  
105 negative and less often Gram-positive bacteria. The first AG was discovered by A. Schatz and  
106 S. Waksman in 1943. They, for the first time, isolated streptomycin from *Streptomyces*  
107 *griseus* microorganisms and noted its antimicrobial properties. Their discovery was honoured  
108 with the Nobel Prize in 1952. With regard to their origin, AGs are classified into two groups:  
109 natural (e.g. neomycin, gentamycin, kanamycin, streptomycin, tobramycin, sisomicin) and  
110 semi-synthetic (e.g. amikacin, dibekacin, isepamycin, netilmycin, arbekacin) antibiotics.

111 Aminoglycoside antibiotics are a group of drugs with very uniform pharmacokinetic  
112 properties. They are used both in human therapy and in veterinary treatment. The spectrum of  
113 therapeutic usages of AGs includes infections of the urinary system, respiratory tract and also  
114 bones, joints and skin infections. AGs are also used in ophthalmology as well as for  
115 sterilization of the gastrointestinal tract prior to surgery. Moreover, they are used to  
116 supplement feed intended for farm animals and in gardening for pest control [2,3]. Due to the  
117 polycationic character of their molecules, AGs are characterized by poor absorption after oral  
118 administration. Therefore, AGs are administered intramuscularly (injections), through the skin  
119 (creams) and directly into the eyes and ears (drops). The mechanism of AGs action consists of  
120 their bonding with A-site of bacterial ribosome (or protein ribosome) and disrupting protein  
121 translation which ultimately leads to bacteria's cell death [1].

122 Despite their high antimicrobial efficiency, aminoglycosides are also classified as toxic  
123 substances with low therapeutic indices. For example, the ratio of tobramycin concentration in  
124 plasma causing toxic effects to the therapeutic range is approximately 50% [4]. Furthermore,  
125 AGs are able to accumulate in parenchymal tissues e.g. in the renal cortex (by bonding to  
126 glycoproteins, like megalin), which negatively affects the urinary tract function. Side effects  
127 of AGs include oto- and nephrotoxicity, and damage to the digestive and nervous system.  
128 Additionally, AGs may cause fetal damage due to their ability to pass through the placenta.  
129 Therefore, the kind of AG and its dosage must be strictly controlled.

130 Due to their low cost, there is a danger of the overuse of these drugs in commercial  
131 animal farms. AGs are used not only for the animals' treatment but also as preventive  
132 measures and as growth promoters, especially in large-scale farms, with the use of AGs in  
133 veterinary applications amounting to approx. 3.5% of the overall antibiotics use [5]. If not

134 managed properly, this can have an adverse impact on the environment, since AGs are non-  
 135 metabolizable agents and are thus excreted as unchanged molecules [6]. The release of large  
 136 amounts of AGs into the environment leads to the increase of antibiotic resistance in some  
 137 strains of bacteria (especially enteric bacteria) [7]. Half-lives of AGs range from 2 to 3 hours  
 138 in plasma and from 30 to 700 hours in tissues [8]. Since they accumulate in animal tissues,  
 139 AGs may be found in food of animal origin. The awareness of this issue has been raised  
 140 following the publicised issue of agricultural antibiotics overuse in North Carolina (USA),  
 141 where large-scale breeding farms are located, which led to significant amounts of antibiotics  
 142 being found in wastewater from farms, soil and animal tissues. In response, many countries  
 143 have introduced legislation which sets limits on the maximum residue content of antibiotics in  
 144 foods of animal origin (see Table 1). Additionally, the largest restaurant franchises such as  
 145 McDonald's, KFC and Subway are now claiming that the food that they offer does not  
 146 contain antibiotics.

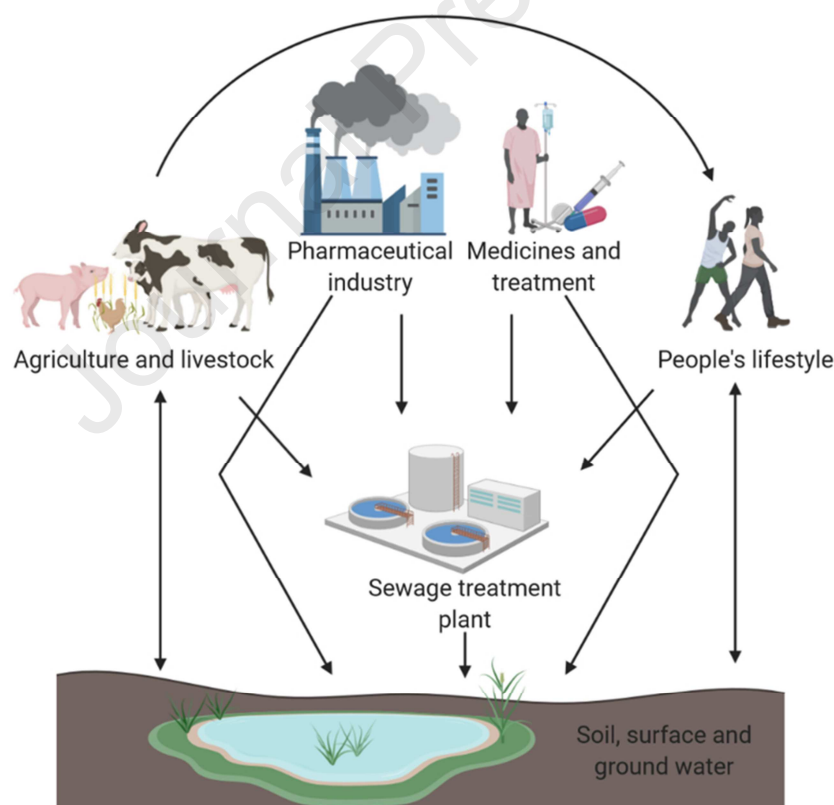
147 Table 1. Maximum residue limits (MRL) of selected aminoglycoside antibiotics in food of  
 148 animal origin set by the EU regulations [9].

Aminoglycoside	Food origin (animal species)	MRL [ $\mu\text{g}/\text{kg}$ ]
APR	Bovine	20000 (kidney)
		10000 (liver)
		1000 (fat, muscle)
STR	Ruminants	1000 (kidney)
	Rabbit	500 (fat, liver, muscle)
	Porcine	200 (ruminants milk)
DHSTR	Ruminants	1000 (kidney)
	Rabbit	500 (fat, liver, muscle)
	Porcine	200 (ruminants milk)
NEO	All species	50000 (kidney)
		500 (fat, liver, muscle, eggs)
		1500 (milk)
GEN (sum of C1, C1a, C2 and C2a)	Bovine	750 (kidney)
	Porcine	50 (muscle, fat)
		200 (liver)
AVI	Porcine	200 (kidney)



	Poultry	100 (fat)
	Rabbit	50 (muscle)
		300 (liver)
		2500 (kidney)
KAN	All species (except finfish)	100 (muscle)
		600 (liver)
		150 (milk)

149 The excretion of AGs in the unchanged forms and inflow of AGs-containing wastewaters  
 150 from the pharmaceutical industry and hospitals facilitates their mobility in the environment  
 151 and increases the levels of surface waters and soil pollution. Appropriate handling of this type  
 152 of pollutants is a critical problem and requires special both dedicated legislation and  
 153 specialised treatment facilities. Schematic illustration of AGs' mobility in the environment is  
 154 shown in Fig. 1.



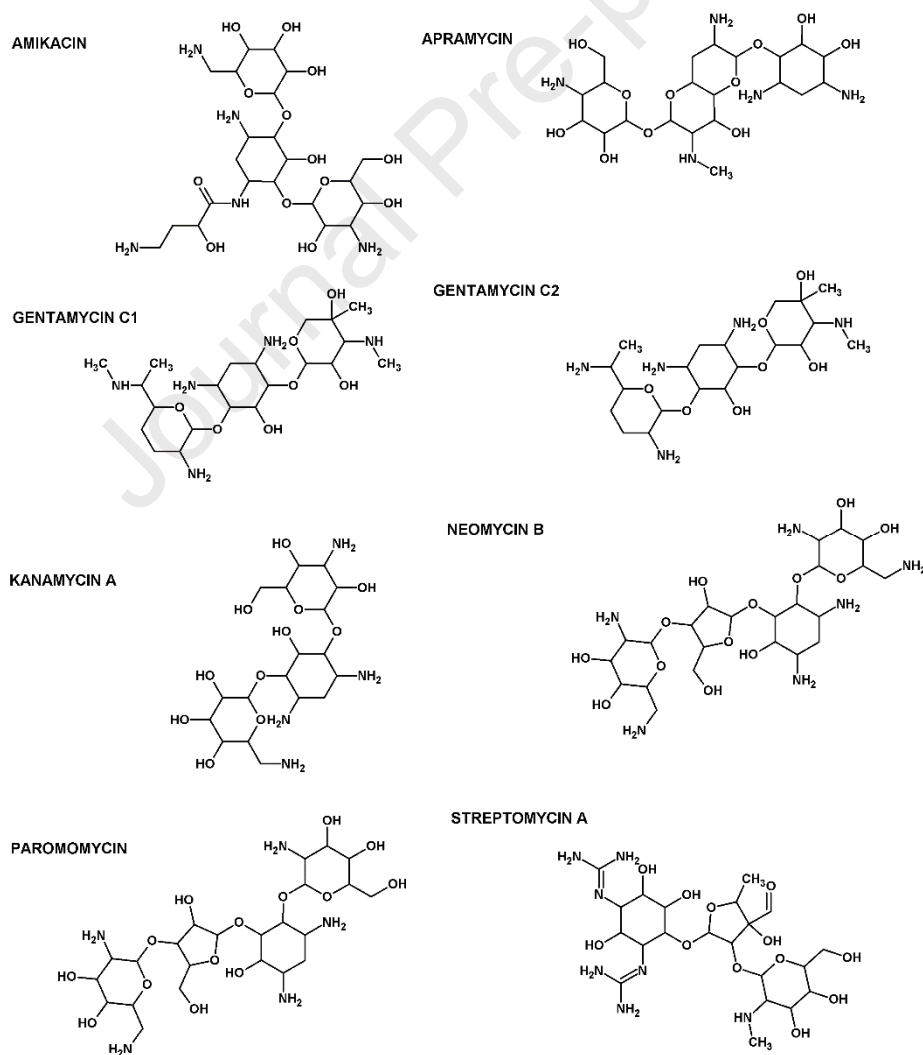
155  
 156 Fig. 1. Mobility of aminoglycoside antibiotics in the environment. Created with  
 157 BioRender.com.

158 AGs are weak bases consisting of two or more molecules of aminosugars (D-glucosamine,  
 159 D-kanosamine) connected by glycosidic bond with cyclitol in the form of (i) streptidine (e.g



160 streptomycin), (ii) 4,5-di-substituted deoxystreptamine (e.g. neomycin) or (iii) 4,6-di-  
 161 substituted deoxystreptamine (e.g. kanamycin) (see Fig. 2). They are characterized by high  
 162 polarity and hydrophilicity (logP values in the range from -4 to -9), very soluble in water,  
 163 slightly soluble in methanol and insoluble in non-polar organic solvents. Some of AGs occur  
 164 in the form of complexes, composed of several different chemical compounds. For example,  
 165 gentamycin consists of 4 main compounds, such as gentamycin C1 (477.6 g/mol), gentamycin  
 166 C1A (449.5 g/mol) and gentamycin C2 in the form of two stereoisomers A and B (463 g/mol).  
 167 Another example is neomycin, which consists of two stereoisomers B and C, where only  
 168 neomycin B has found therapeutical usage [8]. Due to their high polarity, polycationic  
 169 character and lack of chromophores, the analysis of aminoglycosides is a challenging task  
 170 both at the sample preparation and final determination stages.

171



172

173

Fig. 2. Structures of selected aminoglycoside antibiotics.

## 174 **2. Determination of aminoglycosides using liquid chromatography coupled to various** 175 **detectors**

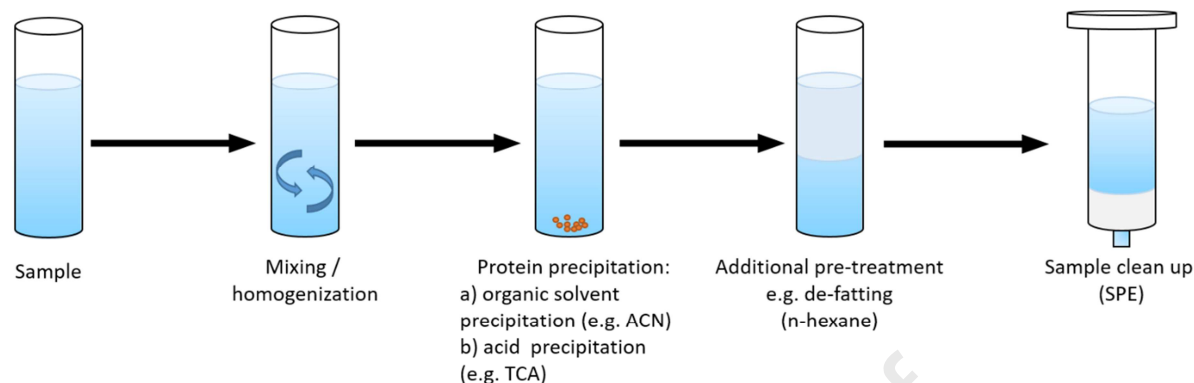
176 Liquid chromatography (LC) remains the gold standard in the separation of AGs prior to their  
177 determination. This is not only due to the fact that it is a well-established technique with  
178 plenty of literature to fall upon when confronted with the issue of analysing a particular type  
179 of samples but also due to the possibility to analyse several AGs at the same time – a trait  
180 common to perhaps only capillary electrophoresis in the context of the analysis of  
181 aminoglycosides in complex matrices. While the choice of the most suitable method  
182 necessarily depends on the analytical task at hand and the type of the sample, LC-based  
183 techniques seem to be the most commonly used due to their high resolution, selectivity and  
184 sensitivity.

### 185 *2.1. Sample preparation and clean-up methods for LC analysis*

186 The physicochemical properties of aminoglycosides and the complexity of the usual matrices  
187 is the source of numerous issues with their determination, leading to irreproducible and  
188 inaccurate results, and so sample preparation is a crucial step in the analytical process.

189 In the case of relatively simple matrices such as pharmaceutical formulations, the  
190 sample preparation protocol is usually limited to the sample dissolution with deionized water,  
191 or with the mobile phase used in further LC investigation. In particular, such procedures are  
192 used for e.g. eye drops, tablets (after grinding) and some types of medicated animal feed [10–  
193 13]. In the case of creams and ointments, additional de-fatting step is usually required.  
194 Various non-polar organic solvents (e.g. DCM) are used for this purpose [11]. When it comes  
195 to matrices such as foods of animal origin, the sample preparation procedures tend to be more  
196 complicated. For instance, honey, consisting mainly of sugars, may cause problems during  
197 sample clean-up and analysis due to the presence of enzymes as well as polyphenols [14]. In  
198 the case of milk or animal material foods, the substantial amounts of proteins, fats, salts,  
199 vitamins and minerals may also interfere with isolation and determination of AGs. The  
200 established and emerging sample preparation methods used for determination of AGs in a  
201 variety of matrices are summarized in Table 2, while a generalised sample preparation scheme  
202 is shown in Fig. 3. It should be noted, however, that the optimal approach to sample treatment  
203 for the determination of AGs necessarily depends on numerous factors such as the desired  
204 application, complexity of the sample matrix, concentration of analytes, etc. As such, there is

205 no one-approach-fits-all solution, and the particular considerations which should be made  
 206 when selecting the sample treatment approach are outlined in the following sub-sections.



208 Fig. 3. Generalised scheme of sample treatment for subsequent determination of  
 209 aminoglycosides.

### 210 *Sample pre-treatment*

211 The first step in the sample preparation protocol is usually sample mixing and  
 212 homogenization or grinding. If large amounts of proteins are present, they are removed by  
 213 precipitation, either with the use of organic solvents such as methanol, or acetonitrile [15–18].  
 214 It has to be noted, however, that AGs are poorly soluble in mixtures containing high amounts  
 215 of organic solvents which can lead to their losses during protein precipitation. On the other  
 216 hand, AGs show high stability and good solubility in acidic aqueous solutions. Acid  
 217 precipitation seems therefore to be a safer alternative for protein removal in the context of  
 218 AGs determination. Chlorinated or fluorinated organic acids such as trichloroacetic acid  
 219 (TCA), trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA) [18–27] are commonly  
 220 used for this purpose due to their high efficiency at relatively low concentrations. It seems  
 221 that TCA is the most frequently used precipitating agent owing to its low price and fast action.  
 222 Typically, 2–5% TCA solutions are used [28]. Precipitating solutions may contain other  
 223 components, such as chelating agents (EDTA) to break down AGs complexes with polyvalent  
 224 ions [22,24,27,29], pH control compounds (NH<sub>4</sub>Ac, KH<sub>2</sub>PO<sub>4</sub>) [22,28–30] or ionic strength  
 225 fixating substances (NaCl) [22,26,29]). After protein precipitation, samples are  
 226 centrifuged/filtered and defatted when necessary [23,26]. Finally, the pH of the sample  
 227 solutions may be set to the desired value [25,28,29].

### 228 *Sample purification and enrichment*

229 The pre-treatment of the sample is followed by a purification and enrichment stage using  
230 a variety of techniques, chief among them SPE. Cationic exchangers (mostly weak cationic  
231 exchangers - WCX), C18 sorbents (in combination with ion-pairing reagents) or hydrophilic-  
232 lipophilic balance mode (HLB) SPE sorbents are commonly used. The selection of  
233 appropriate sorbent type can be challenging, particularly in the case of multiple analytes that  
234 need to be processed simultaneously, due to different values of acid/base dissociation  
235 constants for various AGs.

236 Ion exchange sorbents are frequently employed due to the poly-cationic character of AGs  
237 and their affinity to the functional groups (Table 2). This type of sorbents can be used for  
238 purification of many sample types including animal tissues (kidney, liver, muscle), milk, eggs,  
239 honey, royal jelly and animal feeds [14,24,29,31]. Extraction protocols with WCX cartridges  
240 require pH in the range of 6÷8. Under such conditions, functional groups of sorbent are  
241 negatively charged and attract protonated AGs molecules. At lower pH values (< 3), the  
242 sorbent surface becomes neutral, facilitating AGs elution. The ionic strength of the pre-  
243 extraction mixture may have a notable impact on the recoveries, at least in the case of some  
244 analytes [29]. Additional issues might occur when several AGs are to be extracted at the same  
245 time since in certain matrices the signal for particular AGs might be suppressed depending on  
246 the type of the cartridge used [31]. However, it was shown that polymeric cation exchangers  
247 with strong cation exchange functionality (PCX) provide better results in the case of select  
248 AGs compared to standard WCX cartridges [14], while in the case of the extraction of weakly  
249 basic compounds such as gentamycin, strong cation exchange (SCX) cartridges perform better  
250 altogether [24].

251 Similarly to ion exchange sorbents, hydrophilic-lipophilic balance (HLB)-based sorbents  
252 show AGs extraction efficiency varying with the pH of sample extracts (Table 2). This issue  
253 could be resolved e.g. through developing multi-step extraction protocols [21] or by  
254 synthesizing novel sorbents. The latter path was taken in a study in which urea-formaldehyde  
255 resin has been synthesized inside a small internal diameter PTFE tube [25]. The resulting  
256 monolithic microcolumn was used as a sorbent for determination of streptomycin, neomycin  
257 and tobramycin in fish meat extracts. In a different approach, four monolithic  
258 poly(methacrylic acid-co-ethylene methacrylate) fibers were bunched together and used for  
259 extraction of 6 AGs from honey and milk samples. Due to polymeric nature and carboxyl  
260 functionality present on the surface of the fibers, two types of sorption mechanisms were

261 observed: hydrophobic interactions and cation-exchange mechanism [19], and thus the  
262 extraction process was greatly simplified.

263 Sample clean-up can also be carried out using non-polar octadecyl (C18) cartridges (see  
264 Table 2). Since AGs, due to their hydrophilic nature, interact very weakly with hydrophobic  
265 C18 sorbents, the latter may be used to remove non-polar components of sample extracts.  
266 Such clean-up may be performed e.g. using dispersive SPE (DSPE) [3] and, since it is  
267 relatively fast, incorporated into QuEChERS protocols [32]. Conversely, the polycationic  
268 character of AGs makes it is possible to increase their hydrophobicity by the creation of ion  
269 pairs with reagents such as perfluorinated organic acids (e.g. heptafluorobutyric acid, HFBA).  
270 Such ion pairs may be isolated from extracts using techniques commonly used for other non-  
271 polar substances. In particular, both SPE and liquid chromatography based on reversed-phase  
272 principle can be used [33].

273 The latest trends in AGs extraction/sample clean-up take advantage of molecularly  
274 imprinted polymers (MIPs) and magnetically active sorbents (Table 2). MIPs are tailored,  
275 highly selective sorbents fabricated in the process of spatially constrained polymerization at  
276 the molecular level. The template (analyte) molecule interacts with the monomer functional  
277 groups (e.g. by ionic, hydrogen or covalent bonds) forming complexes. After cross-linking,  
278 the shape of such a complex is trapped/imprinted in the three-dimensional polymer structure.  
279 The template/analyte is then purged from the polymer leaving a molecular imprint. The  
280 resulting material shows high affinity to molecules shaped similarly to the template (analyte)  
281 molecules. In the case of MIPs, the analyte-sorbent interactions are relatively strong, therefore  
282 it is possible to use 2- or 3-step washing procedures (with MeOH or DCM) during sample  
283 preparation. Thorough washing helps reduce interferences caused by both polar and non-polar  
284 matrix components. Unfortunately, only one type of such sorbent is commercially available at  
285 the time of writing (SupelMIP<sup>®</sup> SPE-AG from Supelco). Sample clean-up procedures  
286 employing MIPs were used for AGs determination in animal tissues, fish, eggs, processed  
287 food, honey, milk and milk-based food products [27,34]. The MIP sorbents can be re-used  
288 dozens of times without the use of sorption efficiency [35], and enable overall better  
289 recoveries compared to conventional SPE.

290 Magnetically active sorbents consist of some sort of ferromagnetic particles (most  
291 commonly Fe<sub>3</sub>O<sub>4</sub>) covered with the layer of the actual sorptive material. The sorbents of that  
292 type are used for dispersive solid-phase extraction (DSPE). The small particle size of such

293 sorbents makes it easier to achieve higher extraction efficiencies mostly due to the high  
294 specific surface area. The magnetic activity of sorbent particles facilitates its separation from  
295 the sample matrix. Unfortunately, until now sorbents of this type are not commercially  
296 available and the literature concerning their applications is scarce. However, some promising  
297 applications include the development of a poly(vinyl alcohol)-coated core-shell magnetic  
298 nanoparticles ( $\text{Fe}_3\text{O}_4$ ) for the DSPE extraction of three AGs from honey [36] and the use of a  
299 similar approach in which the  $\text{Fe}_3\text{O}_4$  nanoparticles modified with carbohydrates with  
300 functional groups chosen to mimic AGs structure and properties [37]. The relatively high  
301 recovery values obtained in both scenarios (83% to 101% and 94% to 109%, respectively)  
302 highlight the potential of this approach to the extraction of AGs from various matrices.

303

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304 Table 2. Sample preparation and clean-up procedures for determination of aminoglycoside antibiotics in a variety of matrices.

Type of SPE sorbents	Aminoglycoside	Matrix	Sample preparation	Sample clean-up	Recovery	Ref.
Ion-exchange SPE sorbents	GEN (C1, C1A, C2/C2A/C2B)	Fish tissue	2 g of sample → 10 mL 3% TCA with 0.4 mM EDTA → repetition of the procedure → dissolving of collected supernatants to 25 mL	LC-SCX ion-exchange SPE cartridges (3 mL/500 mg) - conditioning: 5 mL MeOH, 5 mL H <sub>2</sub> O - sample volume: 12.5 mL - washing: 5 mL H <sub>2</sub> O, 5 mL MeOH - elution: 5 mL MeOH with NH <sub>3</sub> (17:3 v/v)	80 – 110%	[24]
	SPC, STR, DHSTR, AMI, RIB, KAN, PAR, APR, GEN (C1, C1A, C2/C2A/C2B), NEO	Animal tissue (muscle), milk	<i>Sample pretreatment – muscle:</i> 3 g of sample → 15 mL (two steps: 10 + 5 mL) 10 mM NH <sub>4</sub> Ac with 0.4 mM EDTA, 0.5%	Accell Plus CM, WCX SPE cartridges (6 mL/500 mg) - conditioning: 3 mL ACN, 6 mL H <sub>2</sub> O - washing: 6 mL H <sub>2</sub> O - elution: 3 mL 175 mM NH <sub>4</sub> FA	72 – 96%	[29]



			<p>NaCl and 2% TCA → pH adjustment to 6.5 → dilution to 50 mL</p> <p><i>Sample pretreatment – milk:</i></p> <p>2 g of sample → 15 mL (two steps: 10 mL + 5 mL) 0.25% TCA → pH adjustment to 6.5 → dilution to 50 mL</p>			
	STR, DHSTR, HYG, SPC, KAN, APR, GEN (C1, C2/C2A, C1A), NEO, TOB	Honey, royal jelly	<p>5 g or 2 g of sample (respectively honey, royal jelly) → 0.25 mg trypsin → 5 mL H<sub>2</sub>O → repetition of the extraction → collected supernatants filled up to 20 ml → splitting of the solution into 2 portions</p>	<p>Bond Elut Nexus WCX SPE cartridges (3 mL/150 mg) - 1<sup>st</sup> cartridge:</p> <ul style="list-style-type: none"> <li>- pH adjustment of 1 portion of ample to 7.5</li> <li>- conditioning: 5 mL MeOH, 5 mL H<sub>2</sub>O</li> <li>- washing: 7.5 mL H<sub>2</sub>O</li> <li>- elution: 5 mL AA/ H<sub>2</sub>O/ MeOH</li> </ul>	75 – 114%	[14]





				(10:20:70 v/v/v) Bond Elut Plexa PCX SPE cartridges (3 mL/150mg) - 2 <sup>nd</sup> cartage  - conditioning: 5 mL MeOH, 5 mL H <sub>2</sub> O, 3 mL 20 mM HFBA  - washing: 7.5 mL H <sub>2</sub> O, 7.5 mL MeOH  -elution: 5 mL NH <sub>3</sub> / H <sub>2</sub> O/ MeOH (20:20:60 v/v/v)		
	HYG, AMI, KAN, RIB, APR, TOB, GEN, NEO	Animal feeds	1 g of sample → 5 mL 10 mM KH <sub>2</sub> PO <sub>4</sub> with 0.4 mM EDTA and 2% TCA → repetition of the extraction → pH adjustment to 5.5	Oasis MCX SPE cartridges (3 mL/60 mg)  - conditioning: 3 mL MeOH + 2% AA  - washing: 3 mL 2% AA, 3 mL H <sub>2</sub> O  - elution: 5 mL MeOH + 20% NH <sub>3</sub>	61 – 104%	[38]
Non-polar SPE sorbents	SPC, TOB, GEN, KAN, HYG, APR, STR, DHSTR, AMI,	Animal tissue (muscle),	10 g of sample (1 mL of milk)→ 0.25 mL 150 mM EDTA (0.05 mL for	C18 (DSPE)  - mixing of sample and 25 mg of C18	37 – 98%	[3]



	NEO	milk	milk sample) → 10 mL 15% TCA (1 mL for milk sample)	sorbent		
	NEO, STR, DHSTR, GEN, KAN, SPC	Honey	5 g of sample → 15 mL 0.1% HFBA	Strata-XL SPE cartridge (6 mL/200 mg) - conditioning: 6 mL MeOH, 6 mL H <sub>2</sub> O, 6 mL 0.1% HFBA - washing: 6 mL 0.1% HFBA - elution: 5 mL ACN	87 – 127%	[33]
	VAL	Rice (rice straw, brown rice, rice hull)	5 g of sample → 20 mL MeOH/H <sub>2</sub> O (9:1 v/v) (10 mL for brown rice) → 1 g NaCl	C18, GCB, PSA (DSPE) - sample volume: 1.5 ml - tube with sorbent (50 mg C18 – rice hull, 10 mg GCB and 40 mg PSA – rice straw, 10 mg GCB and 30 mg C18 – brown rice)	78 – 94%	[32]
Hydrophilic- lipophilic	APR, AMI, SPC, KAN, NEO, PAR,	Animal tissue (muscle, liver,	5 g of sample → 10 mL 5% TCA → repetition of	Oasis HLB SPE cartridges (3 mL/60 mg) – 2 steps procedure	47 – 93%	[23]



balance SPE sorbents	STR, DHSTR, TOB, GEN (C1, C2/C2A, C1A), HYG, SIS, NET	kidney)	the procedure → 5 mL 0.2 M HFBA → 5 mL n-hexane	<ul style="list-style-type: none"> <li>- conditioning - 1<sup>st</sup> cartridge: 3 mL MeOH, 3 mL H<sub>2</sub>O, 3 mL 0.2 M HFBA</li> <li>- sample volume - 1<sup>st</sup> cartridge: 5 mL</li> <li>- effluent pH adjustment to 8.5</li> <li>- conditioning - 2<sup>nd</sup> cartridge: 3 mL MeOH, 3 mL H<sub>2</sub>O, 3 mL 0.2 M HFBA, 3 mL solution of NaOH (pH 8.5)</li> <li>- sample volume - 2<sup>nd</sup> cartridge: all collected solution after alkalization</li> <li>- connection of the 2 cartridges</li> <li>- washing: 5 mL H<sub>2</sub>O</li> <li>- elution: 6 mL ACN with 0.15 M HFBA (4:1 v/v)</li> </ul>		
	STR, TOB, NEO	Fish tissue	10 g of sample → 0.2 mL 150 mM EDTA → 10 mL 15% TCA → 10	Urea-formaldehyde monolithic cartridge for hydrophilic online in-tube SPME	82 – 97%	[25]



			<p>mL n-hexane → pH adjustment to 7 → filtration → dilution of 5 mL of sample to 20 mL with ACN/H<sub>2</sub>O (50:50 v/v) + 0.2% TFA</p>	<p>- sampling solution: ACN/H<sub>2</sub>O (50:50 v/v) + 0.2% TFA</p> <p>- elution volume: 0.15 mL</p>		
	SPC, DHSTR, AMI, KAN, TOB, APR	Honey, milk	<p><i>Sample pretreatment – honey:</i></p> <p>1 g of sample → 20 mL H<sub>2</sub>O → pH adjustment to 5</p> <p><i>Sample pretreatment – milk:</i></p> <p>20 mL of sample → 1 mL TFA → dilution of 2 mL of sample to 20 mL with H<sub>2</sub>O → pH adjustment to 5</p>	<p>Multiple monolithic poly(methacrylic acid-co- ethylenedimethacrylate) fibers (20 x 0.5 mm):</p> <p>- fibers activation: MeOH, H<sub>2</sub>O</p> <p>- direct immersion of fiber into sample (40 min)</p> <p>- desorption: 0.4 mL H<sub>2</sub>O/ACN/FA (94:5:1 v/v/v)</p>	68 – 110%	[19]



	DHSTR, STR, KAN, SPC	Honey	2 g of sample → 10 mL 5 mM K <sub>2</sub> HPO <sub>4</sub> (pH 11)	PVA-Sil SPE cartridge (3 mL/200 mg) - conditioning: 5 mL MeOH, 5 mL ACN/H <sub>2</sub> O (90:10 v/v) - washing: 5 mL H <sub>2</sub> O - elution: 2 mL H <sub>2</sub> O/ACN/FA (90:9:1 v/v/v)	84 – 112%	[30]
	STR	Apples	5 g of sample → 20 mL of 10 mM KH <sub>2</sub> PO <sub>4</sub> (pH 4) with 0.4 mM EDTA and 2% TCA → pH adjustment to 7.5	Oasis HLB SPE cartridges (6 mL/200 mg) - conditioning: 6 mL MeOH, 6 mL H <sub>2</sub> O, - washing: 3 mL H <sub>2</sub> O - elution: 6 mL MeOH + 3% FA	101 – 105%	[2]
MIP SPE sorbents and functionalized magnetic nanoparticles	AMI, APR, DHSTR, GEN (C1, C2/C2A, C1A), HYG, KAN, NEO, PAR, SIS, SPC, STR, TOB	Animal tissue (muscle, fat), fish, milk, egg – raw and processed	2 g of sample → 0.5 mL 0.5% EDTA → 20 mL 2% TCA → 4 mL 80 mM (NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub>	SupelMIP SPE-AGs cartridges (3 mL/50 mg) - conditioning: 1 mL MeOH, 1 mL 50 mM K <sub>3</sub> PO <sub>4</sub> (pH 7),	Approx. 90 – 100%	[27]



		food products		<p>- sample volume: 3 mL</p> <p>- washing: 3 mL H<sub>2</sub>O, 1 mL H<sub>2</sub>O/ACN (6:4 v/v), 1 mL DCM/MeOH (50:50 v/v)</p> <p>- elution: 1 mL 30 mM HFBA in ACN/H<sub>2</sub>O (25:75 v/v)</p>		
STR, DHSTR, KAN, GEN C1A, SPC, AMI, TOB, SIS, PAR, NET, HYG	Animal tissue (muscle), honey, milk	<p><i>Sample pretreatment – muscle and milk:</i></p> <p>2 g of sample → 5 mL 10 mM KH<sub>2</sub>PO<sub>4</sub> with 0.4 mM EDTA and 2% TCA → repetition of the procedure → dilution (1:1 v/v) with 50 mM K<sub>3</sub>PO<sub>4</sub> → pH adjustment to 7</p> <p><i>Sample pretreatment – honey:</i></p>	<p>SupelMIP SPE-AGs cartridges (3 mL/50 mg)</p> <p>- conditioning: 1 mL MeOH, 1 mL 50 mM K<sub>3</sub>PO<sub>4</sub> (pH 7 ÷ 8.5),</p> <p>- washing: 3 mL H<sub>2</sub>O, 1 mL 0.1% NH<sub>3</sub> solution, 1 mL ACN/H<sub>2</sub>O (40:60 v/v), 1 mL MeOH/DCM (50:50 v/v)</p> <p>- elution: 1 mL MeOH/H<sub>2</sub>O (80:20 v/v) + 0.1% FA</p>	78 – 95%	[28]	



			2 g of sample → 10 mL 50 mM K <sub>3</sub> PO <sub>4</sub> → pH adjustment to 7			
	AMI, APR, DHSTR, GEN (C1, C2/C2A, C1A), KAN, PAR, SPC, STR, TOB	Milk and milk-based food products	2 g of sample → 0.25 mL 15% TCA → 1 mL n-hexane → 3.5 mL 50 mM potassium phosphate (pH 7) → pH adjustment to 7	SupelMIP SPE-AGs cartridges (3 mL/50 mg)  - conditioning: 1 mL MeOH, 1 mL 50 mM K <sub>3</sub> PO <sub>4</sub> (pH 7),  - sample volume: 3 mL  - washing: 3 mL H <sub>2</sub> O, 1 mL DCM/MeOH (50:50 v/v)  - elution: 1 mL ACN/H <sub>2</sub> O (20:80 v/v) + 1% FA with 20 mM HFBA	70 – 106%	[34]
	STR, DHSTR, KAN	Honey	2 g of sample → 10 mL ACN with 5 mM K <sub>2</sub> HPO <sub>4</sub> (pH = 7) (10:90 v/v)	Magnetic Fe <sub>3</sub> O <sub>4</sub> @PVA nanoparticles for DSPE (40 mg)  - conditioning: ultrasonification of sorbent in 5 mL of MeOH and washing with ACN/H <sub>2</sub> O (10:90 v/v)	83 – 101%	[36]



				- washing: H <sub>2</sub> O - elution: 1 mL H <sub>2</sub> O/ACN/FA (80:19:1 v/v/v)		
AMI, DHSTR, TOB, GEN (C1, C2/C2A, C1A)	Honey	0.2 g of sample → 2 mL H <sub>2</sub> O	Magnetic Fe <sub>3</sub> O <sub>4</sub> @SiN- galactitol nanoparticles for DSPE (1 mg) - elution: 0.15 mL 190 mM NH <sub>4</sub> FA (pH 3)	84 – 109%	[37]	
STR, KAN, APR, GEN, TOB, PAR	Environmental water	50 mL of sample	DMIPs SPE cartridges (2 mL/30 mg) - conditioning: 3 mL MeOH, 3 mL H <sub>2</sub> O, - washing: 3 mL H <sub>2</sub> O - elution: 3 mL H <sub>2</sub> O + 1% FA	70.8 – 108.3%	[35]	

305





306 *2.2 Separation and detection of aminoglycosides in LC- and CE-based analysis*

307 High polarity and polycationic character of AGs can cause a variety of problems during their  
308 chromatographic separation in native forms. Additionally, considering possible ways of  
309 detection, the absence of chromophoric or fluorogenic moieties complicates the determination  
310 of these compounds. Direct AGs detection with UV or fluorescence detectors is not preferred,  
311 although it has found some application in pharmaceutical formulations control [39,40]. Wider  
312 use of this kind of detection for underivatized AGs does not seem to be possible due to the  
313 high risk of possible matrix-related interferences. Furthermore, these methods cannot be used  
314 for every AG. For example, it was shown that using direct UV detection amikacin and  
315 tobramycin can be analysed, but in the case of gentamycin, it is not possible [40].

316 One way to deal with this problem is derivatization. Not only does it make detection  
317 easier, but also facilitates chromatographic separation of analytes by lowering their polarity.  
318 Derivatization products usually can be easily separated under reversed-phase conditions. The  
319 most popular derivatization agents for AGs are: 1-naphthyl isothiocyanate (NITC), 9-  
320 fluorenylmethyloxycarbonyl (FMOC), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate  
321 (AQC), o-phthalaldehyde (OPA), and 1-fluoro-2,4-dinitrobenzene (FDNB) [15,16,20,41,42].  
322 It has to be stressed however, that the selection of derivatization agent can be a difficult task.  
323 Some derivatizing agents (e.g. FMOC) can react with primary and secondary amino groups of  
324 AGs with different selectivity and efficiency, thus producing multiple derivatives from a  
325 single analyte (Fig. 4). This, in turn, will impair quantitation and increase matrix effects levels.  
326 The stability of derivatization products is another source of potential issues. For instance,  
327 OPA is recommended to be used in post-column mode due to low stability of its derivatives  
328 [43,44]. Certain derivatization agents call for quite harsh reaction conditions, such as high  
329 temperatures (e.g. derivatization with FDNB requires around 85°C) which in some scenarios  
330 may be a limiting factor [42]. What is more, the efficiency of derivatization highly depends  
331 on the composition of the sample matrix. Overall, methods involving derivatization are more  
332 commonly used to analyse samples with less complex matrices (e.g. pharmaceuticals) or  
333 when a single AG is to be determined [15,16,20]. It seems that the application of  
334 derivatization reactions in combination with reversed-phase separation conditions is  
335 becoming less prominent.

336 The latest trends in AGs determination focus on developing protocols employing  
337 hydrophilic interaction (HILIC) or ion-pairing liquid chromatography (IPLC). These

338 approaches allow the determination of AGs in their native forms but also impose the use of a  
 339 different set of detection techniques, like mass spectrometry (single or tandem MS),  
 340 evaporative light scattering detection (ELSD), charged aerosol detection (CAD) or pulsed  
 341 amperometric detection (PAD). Table 4 summarizes exemplary HPLC methodologies for  
 342 AGs analysis. The referenced publications are focused mainly on IPLC and HILIC.

343 When developing a method for the determination of aminoglycosides which involves  
 344 the use of a separation technique such as LC or capillary electrophoresis discussed in Section  
 345 3 of this review, it is also important to carefully consider the choice of the detector. In the  
 346 case of the determination of single analyte and analysis of less complex matrices detectors  
 347 such as ELSD, UV, FLD or CAD could be cost-effective. However, when the opposite is true,  
 348 or when the analytes are present in the sample at trace concentration levels, tandem mass  
 349 spectrometry seems to be the method of choice. The application areas and detection levels of  
 350 various detectors coupled with separation techniques for the determination of AGs are listed  
 351 in Table 3.

352 Table 3. Detectors most commonly used for determination of aminoglycosides in conjunction  
 353 with separation techniques.

Type of detector	Tentative detection levels	Remarks
Refractive index detector (RID)	100 000 ng/mL	<ul style="list-style-type: none"> <li>- determination of aminoglycosides in the native form;</li> <li>- low sensitivity;</li> <li>- nonselective;</li> <li>- response varies with temperature and mobile phase composition - isocratic elution is preferred, which hinders separation of analyte mixtures;</li> <li>- currently, the use for analysis of AGs is marginal;</li> </ul>
Evaporative light scattering detector (ELSD)	100 ng/mL	<ul style="list-style-type: none"> <li>- determination of aminoglycosides in the native form;</li> <li>- higher sensitivity compared to RID and stable baseline during gradient elution;</li> <li>- nonselective;</li> <li>- nonlinear response;</li> <li>- possible problems with detection under HILIC conditions (high concentration of buffers may suppress the signal).</li> </ul>
Charged aerosol detection (CAD)	100 ng/mL	<ul style="list-style-type: none"> <li>- determination of aminoglycosides in the native form;</li> <li>- better sensitivity compared to some universal detectors, such as RID;</li> <li>- narrow range of linearity;</li> <li>- sensitivity dependent on the content of organic solvent in the mobile phase;</li> <li>- possible problems with detection under HILIC conditions (high concentration of buffers may suppress the signal).</li> </ul>
Diode array detection (DAD)	10 000 ng/mL (with	<ul style="list-style-type: none"> <li>- in most cases derivatization of analytes is required;</li> <li>- better selectivity compared to RID and ELSD;</li> </ul>

	derivatization) >10 000 ng/mL (without derivatization)	- wide range of linear response; - high reliability; - used with both LC and CE.
Fluorescence detection (FLD)	100 ng/mL	- derivatization of analytes is required; - improved sensitivity and selectivity compared to DAD); - used with both LC and CE.
Tandem mass spectrometry (MS/MS)	1 ng/mL	- determination of aminoglycosides in the native form; - highly sensitive and selective detection; - the most powerful tool for the simultaneous determination of multi-component mixtures; - high concentration of buffers (HILIC) and some mobile phase additives (IPLC) negatively affects sensitivity; - most commonly used with LC, can be used with CE.
Amperometric detectors	500 ng/mL	- determination of AGs in the native form; - highly sensitive detection, however in the case of samples with complicated matrices problems with repeatability may occur due to deterioration of of electrodes; - highly alkaline pH is necessary (pH > 11) – anion exchange mode; - used with both LC and CE.
Capacitively coupled contactless conductivity detection (C <sup>4</sup> D)	10 ng/mL	- determination of AGs in the native form; - universal type of detection suitable for miniaturization and coupling with CE systems; - good sensitivity; - issues with baseline stability due to changes in the conductivity of the background electrolyte; - most commonly used with CE.
laser-induced fluorescence detection (LIF)	10 ng/mL	- derivatization of aminoglycosides necessary; - high sensitivity and selectivity; - most commonly used with CE.

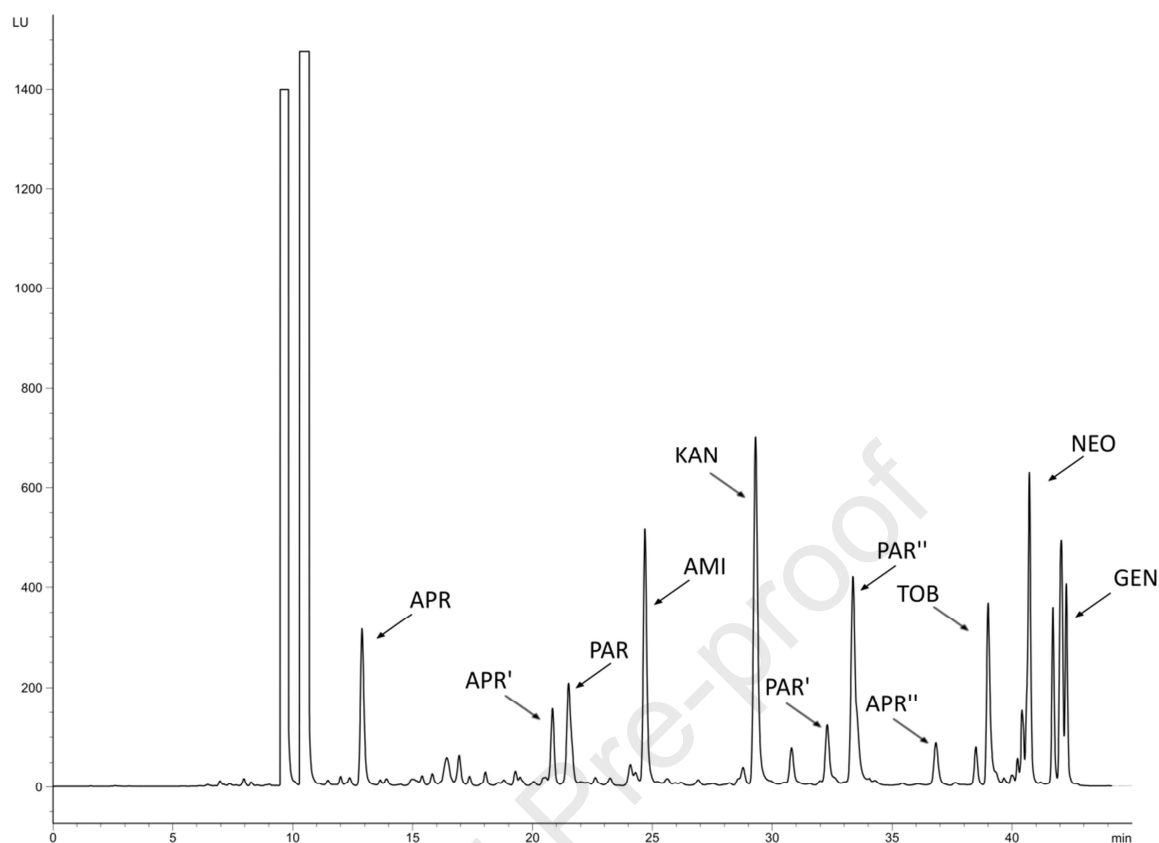
#### 354 *Ion-pairing liquid chromatography (IPLC)*

355 Due to the presence of multiple amino groups in the structures of AGs molecules, they tend to  
 356 form polyvalent cations in the solution. Such cations can interact with negatively charged  
 357 moieties, e.g. anions of perfluorinated organic acids. The resulting ion pairs are notably less  
 358 polar than AGs, as described in section 2.1. This phenomenon can be taken advantage of  
 359 during LC separation to increase AGs retention and separation selectivity using LC columns  
 360 packed with non-polar sorbents (e.g. C8, C18, etc.). Main drawbacks of using IPLC include  
 361 the diminished lifetime of chromatographic equipment and columns as well as detection  
 362 problems when mass spectrometry is employed (ion suppression). Despite some  
 363 disadvantages, IPLC finds widespread application due to good separation of AGs in multi-  
 364 component mixtures (competitive to other LC-based techniques). Problems with column  
 365 lifetime may be alleviated using their dedicated versions showing higher resistance against  
 366 low pH conditions.

367 The most commonly used ion-pairing (IP) reagents which provide the best AGs  
368 separation are volatile perfluorinated organic acids, for example, heptafluorobutyric acid  
369 (HFBA), pentafluoropropionic acid (PFPA) or trifluoroacetic acid (TFA). IP agents are added  
370 to the mobile phase in concentrations ranging from 0.1% to 1% (v/v) [10,11,33,45,46]. Non-  
371 volatile ion-pairing reagents (e.g. alkylsulfonic acids) are not used in modern analytical  
372 chemistry due to their incompatibility with the majority of detectors used for AGs analysis  
373 (MS, evaporative light scattering (ELSD), charged aerosol detectors (CAD)).

374 Depending on the complexity of the sample matrix, different detectors may be used  
375 for AGs determinations using IPCL. Usually, for well-defined and relatively simple matrices  
376 such as pharmaceutical formulations, (ELSD) and (CAD) [10,11,13] are a good choice. Their  
377 advantages are good sensitivity (LODs start from around 10 ng injected on column), a similar  
378 response to all AGs, simple and rugged construction and low running costs. The downsides  
379 are lack of selectivity and non-linear (sigmoidal) response curve. While the non-linearity  
380 problem can be solved quite easily with modern computer technology, the lack of selectivity  
381 calls for the use of more elaborate sample preparation protocols. The performance of IPLC  
382 methods can be significantly improved by employing tandem mass spectrometry at the  
383 detection stage [3,21,23,26,27,46]. Higher sensitivities and sample throughput, better  
384 selectivity and multi-residue analysis capability can be obtained at the expense of higher  
385 instrumentation, maintenance and personnel costs. In many cases, the use of mass  
386 spectrometric detection allows for reliable determination of analytes despite their non-  
387 baseline separation. The choice of the ion-pairing reagent in IPLC-MS/MS should be  
388 carefully considered. While TFA was successfully used to determine 15 AGs [26], it is  
389 commonly regarded as a troublemaker in the context of mass spectrometry.  
390 Pentafluoropropionic acid (PFPA) could be used as an alternative, as it causes less ion  
391 suppression and in comparison to HFBA it has a lower affinity to the stationary phase,  
392 however, its superiority over HFBA is yet to be clearly demonstrated [46].





393

394 Fig. 4. Example of chromatogram after derivatization of AGs mixture with FMOC; APR –  
 395 apramycin, PAR – paromomycin, AMI – amikacin, KAN – kanamycin, TOB – tobramycin,  
 396 NEO – neomycin, GEN – gentamycin.

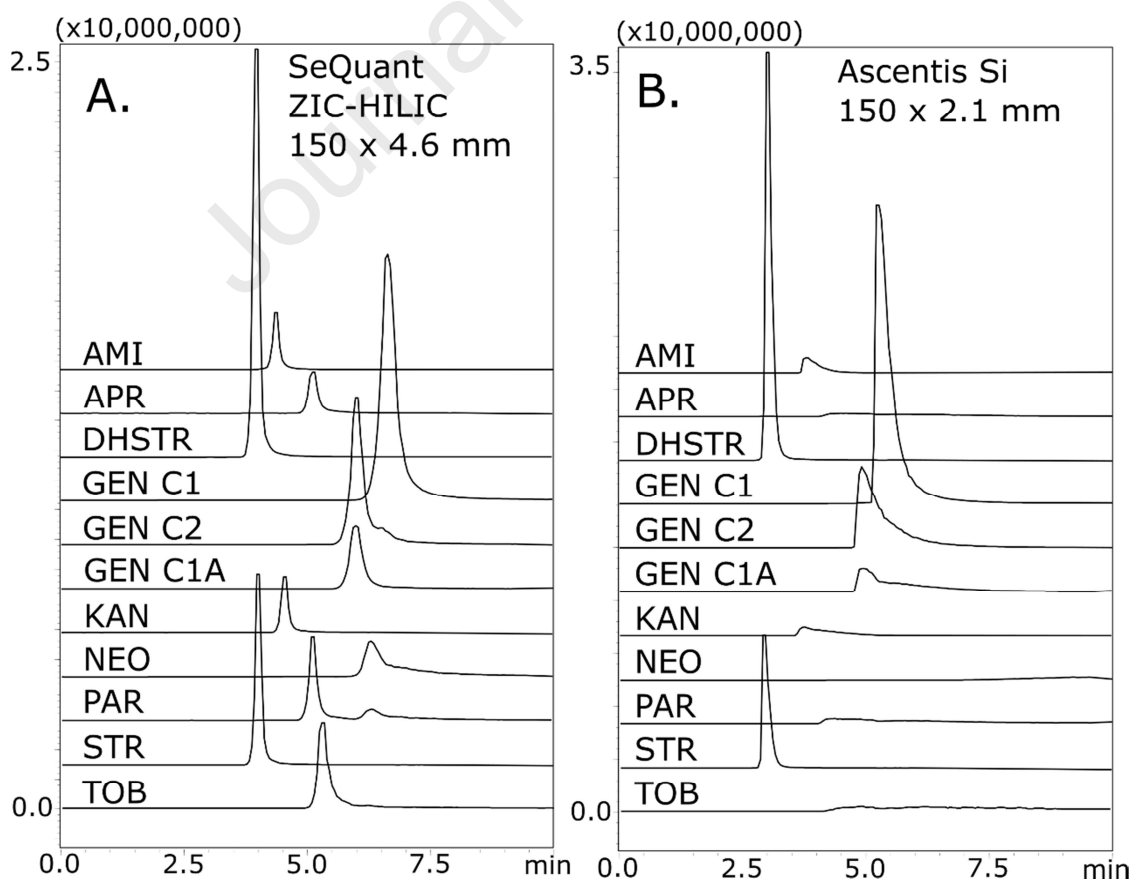
### 397 *Hydrophilic interaction liquid chromatography (HILIC)*

398 Hydrophilic interaction liquid chromatography can be used when the separation of polar,  
 399 water-soluble chemical compounds such as aminoglycoside antibiotics is desired. It is  
 400 believed that the separation mechanism relies on interactions between analytes and quasi-  
 401 stationary phase consisting of water-rich layer surrounding proper stationary phase particles.  
 402 Analyte's retention behaviour is rather difficult to predict since it is dependent on several  
 403 factors including the composition of the mobile phase (percentage and kind of organic  
 404 component), the character of stationary phase, pH, type and concentration of buffer,  
 405 temperature and so on.

406 Stationary phases used in HILIC include bare, diol, pentafluorophenyl, cyanopropyl or  
 407 amino-modified silica gels. These phases can be successfully used for separation of various  
 408 classes of compounds, although for AGs much better results are frequently obtained with

409 novel stationary phases (e.g. ZIC-HILIC and its variants) developed specifically for HILIC  
410 separations (Fig. 5). ZIC-HILIC stationary phases combine hydrophilic partitioning with  
411 weak ionic interactions resulting from the presence of zwitterionic (sulfobetaine,  
412 phosphorylcholine) groups covalently bound to the surface of silica particles.  
413 Phosphorylcholine-modified silica gels seem to be well suited for AGs separations [47].

414 Regarding the aqueous mobile phase composition, typically buffers with a concentration  
415 lower than 60 mM (or water acidified with e.g. formic acid) are preferred for the  
416 determination of no more than 5 AGs in the sample [2,23,30,36]. In these conditions, baseline  
417 separation of AGs is extremely difficult to achieve. The separation resolution increases with  
418 the concentration of the buffer. To obtain satisfactory separation for simultaneous multi-AGs  
419 mixture, buffer concentrations higher than 150 mM are needed [22,28,31,34]. What is more,  
420 most of the protocols include the addition of 0.05 – 2% (v/v) FA to the mobile phase (both to  
421 the buffer and the organic components) [19,22,28,30,31,34] which improves peak shape  
422 (especially their characteristic "tailing" under HILIC conditions) through the weak ionic  
423 interaction with amino groups (affecting slightly reduction of AGs interaction with the  
424 stationary phase).



425

426 Fig. 5. Comparison of chromatographic separation of 11 AGs under HILIC conditions using  
427 SeQuant ZIC-HILIC (A) and Ascentis Si (B) LC columns; AMI – amikacin, APR –  
428 apramycin, DHSTR – dihydrostreptomycin, GEN – gentamycin, KAN – kanamycin, NEO –  
429 neomycin, PAR – paromomycin, STR – streptomycin, TOB – tobramycin.

430 Baseline separation of multiple (more than 8) AGs under the HILIC conditions is difficult if  
431 possible at all, as illustrated by an attempt to simultaneously determine 9 AGs using HILIC-  
432 ELSD [12]. Despite long chromatographic run (120 min), baseline separation of analytes  
433 could not be obtained. Authors tried to resolve overlapping peaks using 2D-LC with IPLC as  
434 a 2<sup>nd</sup> separation dimension but this attempt was only partially successful, and the total  
435 separation time was 240 min.

436 These limitations can be overcome by using HILIC in combination with mass spectrometric  
437 detection since it does not require the use of problematic mobile phase additives (e.g.  
438 perfluorinated IP reagents). Mass spectrometric detection in general, and tandem mass  
439 spectrometry in particular, due to its selectivity allows to overcome the problem of non-  
440 baseline separation of analytes and offers significantly shorter run times (e.g. 9 min when fast  
441 HILIC-MS/MS was applied for the determination of 11 AGs in milk-based food products  
442 [34]). Attention should be devoted to the effect of the composition of the mobile phase on the  
443 peaks shape and the detector's response, with variables such as the impact of the buffer or the  
444 organic modifier (notably, methanol was found to be superior to the commonly used  
445 acetonitrile in certain applications [28]). ZIC-HILIC columns are commonly used to achieve  
446 acceptable separation of AGs and were shown to perform better than another type of  
447 zwitterionic column, namely Obelisc R. The stationary phase of the latter is characterized by  
448 the presence of carboxyl acid functional groups instead of sulfonic groups present in the ZIC-  
449 HILIC columns [28]. Another approach would be to use graphitized LC columns [24],  
450 however, they are characterized by some limitations such as fluctuations in analytes retention  
451 times and time-consuming column deactivation step. In a study in which 3 different HILIC-  
452 ESI conditions for multiresidue drugs determination, including 11 AGs and colistins in animal  
453 muscle and milk samples were compared it was concluded that with ZIC-HILIC column, the  
454 symmetry and retention of neomycin peak weren't acceptable. On the other hand, problems  
455 with retention of spectinomycin and neomycin were observed with a graphitized column. As  
456 the most suitable solution, the authors have chosen bare silica Poroshell HILIC column [29].





457 Table 4. HPLC methods used for determination of aminoglycoside antibiotics in various matrices.

LC conditions	Aminoglycoside	Matrix	LC column	Mobile phase	Detection	LOD/LOQ	Ref.
RP	GEN, NEO	Animal tissues (kidney, liver, muscles)	Hypersil BDS C18 (100 x 4.6 mm), 5 $\mu$ m	ACN/H <sub>2</sub> O (85:15 v/v)	FLD <sub>FMOC-Cl</sub>	LOD: 0.05 – 0.10 $\mu$ g/g	[20]
	STR, DHSTR, HYG, SPC, KAN, APR, GEN (C1, C2/C2A, C1A), NEO, TOB	Honey, royal jelly	Porshell 120 EC-C8 (100 x 2.1 mm), 2.7 $\mu$ m	A: 0.5 mM NH <sub>4</sub> Ac + 0.1% FA  B: ACN + 0.1% FA	ESI-MS/MS	LOD: 0.005 – 0.0125 $\mu$ g/g  LOQ: 0.001 – 0.025 $\mu$ g/g	[14]
IPLC	HYG, AMI, KAN, RIB, APR, TOB, GEN, NEO	Animal feeds	Hypersil BDS C18 (250 x 4.6 mm), 5 $\mu$ m	A: ACN/H <sub>2</sub> O (5:95 v/v) + 20 mM HFBA  B: ACN/H <sub>2</sub> O (50:50 v/v) + 20 mM HFBA	ELSD	LOD: 0.2– 0.7 $\mu$ g/g	[38]
	APR, AMI, SPC, KAN, NEO, PAR,	Animal tissue (muscle, liver,	Atlantis dC18 (150 x 2.1 mm), 5 $\mu$ m	A: ACN + 20 mM HFBA	ESI-MS/MS	LOD: 0.0009 – 0.009 $\mu$ g/g	[23]





STR, DHSTR, TOB, GEN (C1, C2/C2A, C1A HYG, SIS, NET	kidney)		C: ACN/H <sub>2</sub> O (5:95 v/v) + 20 mM HFBA  D: ACN/H <sub>2</sub> O (50:50 v/v) + 20 mM HFBA		LOQ: 0.003 – 0.030 µg/g	
STR, TOB, NEO	Fish tissue	Synchronis C18 (250 x 4.6 mm), 5 µm	H <sub>2</sub> O + 0.2% TFA with ACN (9:1 v/v)	ELSD	LOD: 0.0035 – 0.0052 µg/g	[25]
AMI, APR, DHSTR, GEN (C1, C2/C2A, C1A), HYG, KAN, NEO, PAR, SIS, SPC, STR, TOB	Animal tissue (muscle, fat), fish, milk, egg – raw and processed food products	Kinetex C18 (100 x 2.1 mm), 2.6 µm	A: 20 mM HFBA  B: ACN	MS/MS	-	[27]
SPC, TOB, GEN, KAN, HYG, APR, STR, DHSTR, AMI, NEO	Animal tissue (muscle), milk	Waters X-Terra C18 (100 x 2.1 mm), 5 µm	A: 10 mM NFPA  B: ACN + 10 mM NFPA	ESI-MS/MS	LOD: 0.005 – 0.100 µg/g  LOQ: 0.0125 – 0.250 µg/g	[3]



	NEO, STR, DHSTR, GEN, KAN, SPC	Honey	Kinetex XB C-18 (100 x 3 mm), 2.7 $\mu\text{m}$	A: H <sub>2</sub> O + 0.1% HFBA B: ACN	ESI-MS/MS	LOQ: 0.005 – 0.075 $\mu\text{g/g}$	[33]
2D-LC  HILIC x IPLC	SPC, BAC, DHSTR, STR, GEN, KAN, AMI, APR, PAR, NEO	Tablets	1 <sup>st</sup> dimension:  Grom-Sil 120 Diol (250 x 4.6 mm), 5 $\mu\text{m}$  2 <sup>nd</sup> dimension:  Luna C18 (250 x 2.1 mm), 5 $\mu\text{m}$	A: 20 mM NH <sub>4</sub> FA (pH 2.5) B: ACN  A: 5 mM PFOA/ACN (95:5 v/v) B: 5 mM PFOA/ACN (5:95 v/v)	ELSD	LOD: 130 $\mu\text{g/mL}$  LOQ: 240 $\mu\text{g/mL}$	[12]
HILIC	SPC, STR, DHSTR, AMI, RIB, KAN, PAR, APR, GEN (C1, C1A, C2/C2A/C2B), NEO	Animal tissue (muscle), milk	Poroshell 120 HILIC (100 x 2.1 mm), 2.7 $\mu\text{m}$	A: 1 mM NH <sub>4</sub> FA + 1% FA B: ACN	HESI-II-Q- Orbitrap	LOD: $\leq 0.033$ $\mu\text{g/g}$	[29]



	AMI, APR, DHSTR, GEN (C1, C2/C2A, C1A), KAN, PAR, SPC, STR, TOB	Milk and milk- based food products	Kinetex HILIC (100 x 2.1 mm), 1.7 $\mu$ m	A: 150 mM NH <sub>4</sub> Ac + 0.1% FA  B: ACN	ESI-MS/MS	LOQ: 0.0042 – 0.049 $\mu$ g/g	[34]
	SPC, DHSTR, AMI, KAN, TOB, APR	Honey, milk	ClickXlon HILIC (150 x 3 mm), 5 $\mu$ m	60 mM NH <sub>4</sub> FA and ACN (90:10 v/v) + 1% FA	ESI-MS/MS	LOD: 0.0001 – 0.00059 $\mu$ g/g	[19]
	DHSTR, STR, KAN, SPC	Honey	TE-Cys HILIC (150 x 3 mm), 3 $\mu$ m	A: 30 mM NH <sub>4</sub> FA + 1% FA  B: ACN/H <sub>2</sub> O (80:20 v/v) + 1% FA	ESI-MS/MS	LOQ: 0.0078 – 0.0194 $\mu$ g/mL	[30]
	AMI, DHSTR, TOB, GEN (C1, C2/C2A, C1A)	Honey	Acclaim Mixed-Mode HILIC-1120 Å (150 x 4.6 mm), 5 $\mu$ m	H <sub>2</sub> O/ACN (80:20 v/v) + 0.1% FA	ESI-MS/MS	LOQ: 0.002 – 0.019 $\mu$ g/g	[37]
ZIC-HILIC	SPC, STR, DHSTR, KAN, GEN (C1, C2/C2A, C1A),	Animal tissue (muscle), milk	SeQuant ZIC-cHILIC (100 x 2.1 mm), 3 $\mu$ m	A: 200 mM NH <sub>4</sub> Ac + 2% FA  B: ACN + 2% FA	ESI-MS/MS  *with post- column	LOQ: 0.00019 – 0.0025	[31]



	NEO, AMI				reagent (MeOH and NaAc)	µg/mL	
	STR, DHSTR, KAN, GEN C1A, SPC, AMI, TOB, SIS, PAR, NET, HYG	Animal tissue (muscle), honey, milk	ZIC-HILIC (50 x 2.1 mm), 3.5 µm	A: 175 mM NH <sub>4</sub> FA + 0.3% FA  B: MeOH + 0.3% FA	ESI-MS/MS	LOD: 0.002 – 0.030 µg/g  LOQ: 0.007 – 0.100 µg/g	[28]
HypC(HILIC)	GEN (C1, C1A, C2/C2A/C2B)	Fish tissue	Hypercarb (100 x 2.1 mm), 5 µm	A: 5% NH <sub>4</sub> OH  B: ACN	ESI-MS/MS	LOD: 0.010 – 0.020 µg/g	[24]

458



### 459 3. Alternative analytical techniques used in the determination of aminoglycosides

460 Liquid chromatography remains the gold standard in the determination of aminoglycosides in  
461 a variety of matrices. However, its application often entails the use of relatively expensive  
462 instruments and engagement of highly-trained personnel. While the same is true for certain  
463 capillary electrophoresis-based methods, such as CE-ESI-MS/MS, other emerging techniques,  
464 such as the use of immunoassays and microfluidic devices, could facilitate the development of  
465 less expensive, portable tests. These are unlikely to match the capabilities and versatility of  
466 the established LC-based methods in the near future, they could, however, greatly increase the  
467 access to AGs analyses, particularly in the farming and food processing industry and in  
468 resource-scarce settings.

#### 469 *Capillary electrophoresis*

470 Aminoglycosides tend to form complexes and ionic species in aqueous solutions. This makes  
471 them suitable for electrophoretic separations. Capillary electrophoresis (CE) is a powerful  
472 separation technique which has been successfully used for determination of this class of  
473 compounds. High resolving power is the most important advantage of CE, affordability of  
474 instrumentation and relatively short separation times being less important. The biggest  
475 challenge in the use of CE is improving selectivity and detection sensitivity. In many cases  
476 combining the high resolving power with sensitive and selective detection (e.g. MS) is  
477 difficult or not possible at all due to incompatibility of non-volatile buffers used in CE with  
478 mass spectrometry. Furthermore, due to the very short lifetime of the columns (Huidobro et al.  
479 [48] suggested that the capillary should be changed after just 8 runs to ensure reproducible  
480 results) seriously limits the sample throughput.

481 Capillary electrophoresis and liquid chromatography share almost the same set of  
482 detectors, and therefore the same detection problems stemming from AGs properties are  
483 reported in the case of CE. The solutions to these problems are also similar. Derivatization  
484 with o-phthalaldehyde (OPA) allows the use of FLD detection [49]. Another reagent, 6-  
485 carboxyfluorescein succinidyl ester (CFSE) can be used to detect kanamycin, bekanamycin  
486 and paromomycin using laser-induced fluorescence detection (LIF) [50]. Direct UV detection  
487 at  $195 \pm 5$  nm without [48] or after complexation with borates [51] as well as indirect UV  
488 detection for the determination of AGs have been described [52]. However, the use of CE  
489 coupled with both direct and indirect UV detection plays a marginal role in the determination

490 of underivatized AGs due to relatively low sensitivity ( $\text{LOD} > 10 \mu\text{g/mL}$ ) of this detection  
491 technique [48,51–53].

492 In order to analyse underivatized AGs with higher sensitivity, other types of detectors have to  
493 be used, such as electrochemical detectors, in which case amperometric detectors (AD) with  
494 transition metal electrodes like Cu and Ni are used [54,55]. The main problem associated with  
495 CE-AD methods in AGs analysis is electrode fouling [56]. In general, due to poor Ni-  
496 electrodes stability, the use of Cu-based electrodes is preferred. Various electrode  
497 modifications have been proposed to further improve CE-AD performance [56–58], e.g. the  
498 use of Chemically modified copper electrode (Cu microparticle-modified carbon fiber  
499 microdisk array electrode, Cu-CFE) which produced repeatable results and LOD values in the  
500 low microgram per millilitre range [59]. Very good results ( $\text{LOD} = 10 \text{ ng/mL}$ ) were also  
501 reported by Mukhtar et al. [60] in a study in which CE was coupled with capacitively coupled  
502 contactless conductivity detection ( $\text{C}^4\text{D}$ ) to determine tobramycin in human plasma. The  
503 popularity of CE- $\text{C}^4\text{D}$  methods is currently growing, mainly due to their flexibility as well as  
504 comprehensive nature towards all ionic analytes [61].

505 The improvement of the capabilities of the analytical instrumentation in the past two  
506 decades drastically increased the application potential of CE in the determination of AGs. One  
507 of the most promising approaches is the coupling of CE with highly-selective and sensitive  
508 mass spectrometric detectors [62]. However, while it is possible in general, this solution does  
509 not seem to be practical, at least at the moment. As already mentioned, bringing out the full  
510 potential of CE requires the use of non-volatile buffers which are incompatible with mass  
511 spectrometry. Although the replacement of the said buffers with their volatile alternatives is  
512 possible, the resulting methodology would suffer either from low resolution or low sensitivity.  
513 Up to now, only two reports describing the successful determination of AGs using CE-  
514 MS/MS instrumentation were published [63,64].

515 Alternatively, CE sensitivity could be improved by using various online pre-  
516 concentration techniques such as field-enhanced sample injection (FESI) or field-amplified  
517 sample stacking (FASS) [49,55]. Long et al. [49] compared results of kanamycin  
518 determination (UV detection) with and without online FASS pre-concentration. The method  
519 sensitivity was twenty times higher using FASS. Ge et al. [55] used hyphenation of transient  
520 moving substitution boundary (MSB) with FESI for streptomycin, neomycin and kanamycin  
521 determination (AD detection). Application of online pre-concentration techniques improved

522 the sensitivity of the method by two to three orders of magnitude over those of previously  
523 reported CE-AD methods. CE methods for the determination of aminoglycoside antibiotics  
524 are summarized in Table 5.

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525 Table 5. CE methods for the determination of aminoglycoside antibiotics.

Method	Aminoglycoside	Matrix	Sample preparation	CE conditions	LOD/LOQ Rec.	Ref.
CE-UV <sub>OPA</sub> / FASS CE- UV <sub>OPA</sub>	KAN	Human serum	WCX SPE; Pre-capillary derivatization with OPA and mercaptoacetic acid	Background buffer: 30 mM borax + 16% v/v MeOH (pH = 10) Capillary: uncoated fused- silica (42.5 cm x 50 µm ID) Separation voltage: 23.5 kV Temperature: 20°C	CE-UV <sub>OPA</sub> LOD: 2 µg/mL Rec. 90% FASS CE- UV <sub>OPA</sub> : LOD: 0.1 µg/mL Rec. 60%	[49]
CE-Argon ion LIF <sub>CFSE</sub>	KAN, BEK, TOB, PAR	Human plasma	Protein precipitation (ACN); Pre-capillary derivatization with CFSE	Background buffer: 30 mM sodium borate buffer (pH = 9) Capillary: fused silica capillary (50 cm x 50 µm ID)	LOD: 7 – 14 ng/mL Rec. 92 – 105%	[50]





				Separation voltage: 8 kV	
				Temperature: N/A	
CE-UV <sub>direct</sub>	STR, DHSTR	Standard solution	-	a) Option 1 – anodic mode:	LOD: 10 µg/mL [51]
				Background buffer: 160 mM sodium tetraborate buffer (pH = 9)	
				Capillary: uncoated fused silica (90 cm x 50 µm ID)	
				Separation voltage: 18 kV	
				Temperature: 34°C	
				b) Option 2 – cathodic mode:	
				Background buffer: 75 mM sodium tetraborate buffer and 0.5 mM myristyltrimethylammonium bromide (TTAB) (pH = 9)	
				Capillary: uncoated fused	



silica (90 cm x 50 µm ID)

Current: – 18 kV

Temperature: 34°C

CZE-UV <sub>direct</sub>	NEO	Pharmaceutical formulations (ointments)	LLE with chloroform	Background buffer: 35 mM orto-phosphoric acid + 15 mM acetic acid (pH = 4.7); Capillary: polyacrylamide (30 cm x 50 µm ID) Separation voltage: 20 kV Temperature: 25°C	Rec. 99.93%	[48]
CZE-UV <sub>indirect</sub>	NEO, DHSTR, LIV, AMI, KAN, TOB, SIS	Pharmaceutical formulations (ear drops), standard solution	Addition of cetyltrimethylammonium bromide	Background buffer: 0.01 M imidazole acetate + Fluorad® FC 135 (pH = 5) Capillary: fused silica (67 cm x 50 µm ID) Separation voltage: 12.5 kV	LOD: 10 – 50 µg/mL	[52]



				Temperature: N/A		
CE-AD	NET, TOB, LIN, KAN, AMI	Pharmaceutical formulation (injections)	-	Background buffer: 125 mM NaOH	LOD: 0.63 – 2.7 µg/mL	[59]
				Capillary: uncoated fused silica capillary (45 cm x 50 µm ID)	Rec. 91 – 99%	
				Separation voltage: 6.2 kV		
				Temperature: N/A		
FESI-MSB with CE-AD	STR, NEO, KAN	River water	C18 SPE Addition of 18-crown-6- tetracarboxylic acid (18C6H4)	Background buffer: 15 mM sodium tetraborate buffer + 55 mM NaOH + 10% ACN	LOD: 0.35 – 4.3 ng/mL	[55]
				Concentration of 18C6H4 in pseudostationary phase: 150 mM	Rec. 87.7 – 106.3%	
				Capillary: fused-silica (75 cm x 25 µm ID)		
				Separation voltage: 17 kV		



				Temperature: 25°C		
CE-C <sup>4</sup> D	TOB	Human plasma	Dynamic mixed matrix membrane tip extraction	Background buffer: 200 mM acetic acid	LOD: 10 ng/mL	[60]
				Capillary: fused base silica (55 cm x 50 µm ID)	Rec. 99.6 – 99.9%	
				Separation voltage: 25 kV		
				Temperature: N/A		
CE-C <sup>4</sup> D	AMI	Bronchial epithelial lining fluid	Addition of urease	Background buffer: 30 mM malic acid + 10 mM 18- Crown-6 + L-arginine (pH = 4.1)	LOD: 0.14 µg/mL	[65]
				Capillary: fused base silica (65 cm x 75 µm ID)	Rec. 100%	
				Separation voltage: 30 kV		
				Temperature: 25°C		
Sheathless CE-ESI-	AMI, PAR, HYG, APR, GEN C1	Milk	Protein precipitation (TCA, NaCl, EDTA,	Background buffer: 10% v/v acetic acid	LOQ: 0.67 µg/kg	[63]
					Rec. 76.2 –	



MS/MS			NH <sub>4</sub> Ac)	Capillary: bare fused silica (90 cm x 30 µm ID)	110.0%	
			PWCX-SPE	Separation voltage: 25 kV		
				Temperature: 25°C		
CE-ESI- MS/MS	GEN (C1, C1A, C2), NEO, APR, PAR, DHSTR, SPC, STR	Honey	MIP-SPE	Background buffer: 200 mM formic acid + 7 mM ammonium hydroxide (pH = 2.2)	LOD: 0.4 – 28.5 µg/kg Rec. 88.2 – 99.8%	[64]
				Capillary: bare fused-silica (90 cm x 50 µm ID)		
				Separation voltage: 25 kV		
				Temperature: 25°C		

527 *Immunological methods*

528 Immunological methods, e.g. fluoroimmunoassay (FIA) [28], radioimmunoassay (RIA) [66],  
529 as well as enzyme-linked immunosorbent assay (ELISA) [67,68], are characterized by high  
530 sensitivity and LOD values in the range of few ng/mL, or even pg/mL when used for the  
531 determination of AGs. However, due to their high sensitivity, false-positive results are  
532 frequently obtained [69]. Additionally, low reproducibility of these methods makes them  
533 suitable only for screening and semi-quantitative testing, which usually needs confirmation by  
534 LC measurements [70]. Currently, the most often used immunological method in AGs  
535 analytics is competitive ELISA, which can be realized as either indirect or direct competitive  
536 ELISA. In both cases, the product of the enzymatic reaction is detected by spectrophotometric,  
537 fluorescence or chemiluminescence measurement.

538 Direct competitive ELISA (DC-ELISA) procedures are usually less labour- and  
539 resource-intensive and time-consuming than their IC-ELISA equivalents, however at the cost  
540 of higher false-negative results and no signal amplification. For example, DC-ELISA method  
541 for neomycin determination proposed by Jin et al. [71] guaranteed LOD = 2.73 ng/mL (for  
542 milk samples). In comparison, the equivalent IC-ELISA method developed by Xu et al. [67]  
543 was characterized by LOD = 0.08 ng/mL. There are cases, however, when DC-ELISA  
544 methods can be improved and perform at the same level as IC-ELISA ones. Jiang et al. [72]  
545 used horseradish peroxidase-modified gold nanoparticles for determination of kanamycin and  
546 tobramycin in milk. The LOD of the method was improved fivefold over the conventional  
547 DC-ELISA.

548 In general, the ELISA procedures are very sensitive and relatively easy to carry out,  
549 even by untrained personnel, while the biggest problem associated with AGs determination  
550 using this technique is the cross-reactivity (CR) phenomenon resulting in poor selectivity of  
551 the assay [73]. To avoid cross-reactivity, highly specific antibodies are required, which makes  
552 the implementation of ELISA more expensive, complicated and time-consuming [74]. At  
553 present researchers working with both IC-ELISA and DC-ELISA are focused on the  
554 preparation of highly-selective antibodies to avoid or attenuate cross-reactivity of AGs.  
555 Examples of ELISA-based methods for determination of AGs are provided in Table 6.

556

557 Table 6. ELISA-based methods for the determination of aminoglycoside antibiotics.

Aminoglycoside	Matrix	Sample preparation	Enzyme	Substrate	Antibody	LOD/LOQ Rec.	Ref.
NEO	Milk	Protein precipitation (TCA)	HRP	TMB	Polyclonal	LOD: 0.08 ng/mL Rec. 85 – 110%	[67]
AMI	Milk	Protein precipitation (TCA)	HRP	TMB/H <sub>2</sub> O <sub>2</sub>	Polyclonal	LOD: 11.3 ng/mL Rec. 69.8 – 93.9%	[70]
KAN	Milk, honey	Milk: centrifugation, addition of Na <sub>2</sub> [Fe(CN) <sub>5</sub> NO]·H <sub>2</sub> O and ZnSO <sub>4</sub> and deproteinization by centrifugation  Honey: fat removing (PBS extraction)	SA-HRP	TMB	Polyclonal	LOD: 0.07 ng/mL Rec. 91.0 – 103.3%	[75]
NEO	Rabbit plasma,	-	HRP	OPD	Monoclonal	LOD: 2.73 – 6.85	[71]



	milk					ng/mL	
						Rec. 87 – 108%	
NEO	Pig muscle, chicken muscle, egg, fish, milk, kidney	PBS extraction	HRP	TMB	Polyclonal	LOD: 0.1 – 20 µg/kg	[68]
						Rec. 75.1 – 105.8%	
KAN, TOB	Milk	-	a) HRP b) AuNPs/ HRP	TMB	Monoclonal	a) 0.13 ng/mL b) 0.022 ng/mL	[72]
						Rec. 81 – 123.9%	

558 HRP – horseradish peroxidase; TMB – 3,39,5,59-tetramethylbenzidine; SA-HPR – streptavidin-horseradish peroxidase; PBS – phosphate-  
 559 buffered saline; OPD – o-phenylenediamine.





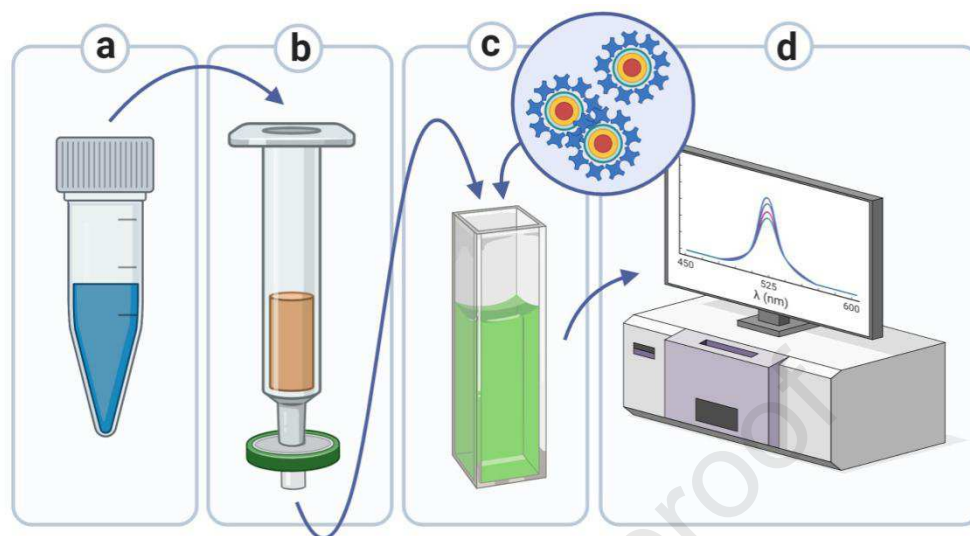
560 *Spectrophotometric & spectrofluorimetric methods*

561 Spectrophotometric and spectrofluorimetric methods can be used for the straightforward and  
562 non-separative analysis of AGs. Due to the lack of selectivity, the application of these  
563 methods is limited to the routine control of a single compound in e.g. pharmaceuticals  
564 formulations. In general, analytical procedures include a derivatization step with various  
565 reagents.

566 Although the vast majority of the spectroscopic methods used for AGs determination  
567 relies on derivatization, there are few exceptions, such as the use of direct fluorimetry for  
568 quick determination of apramycin in pharmaceuticals and milk samples [76]. Micelle-  
569 enhanced native apramycin fluorescence allowed to obtain sensitivity comparable with  
570 methods in which derivatization was used. Ghodake et al. [77] used silver nanoparticle  
571 (AgNP) probe coated with gallic acid for colourimetric determination of streptomycin in  
572 water, serum and milk samples with LOD lower than 0.1 ng/mL. Ma et al. [78] determined  
573 the tobramycin in milk and eggs samples with detection limits of 11 ng/mL using golden  
574 nanoparticle (AuNP)/single-stranded DNA-based colourimetric sensors. Recently, methods  
575 employing fluorescent quantum dots (QD) sensing for AGs determination have been  
576 developed. Some applications highlight the potential of QD-based procedures in selective  
577 determination of particular AGs in relatively complex matrices, following MIP SPE  
578 extraction (see Fig. 6) [79,80]. Furthermore, the development of miniaturized, AGs-specific  
579 biosensors could be particularly useful in system automation, enabling on-line detection of  
580 contaminants. Tang et al. [45] developed an evanescent wave aptasensor based on target  
581 binding facilitated fluorescence quenching (FQ-EWA) for such purpose. The selectivity  
582 towards a particular AG (kanamycin) was achieved using a fluorophore-labelled DNA  
583 aptamer. Notably, the FQ-EWA was characterised by relatively high durability, enabling  
584 more than 60 detection-regeneration cycles which showcases the application potential of  
585 AGs-specific biosensors, including electrochemical sensors, as discussed in the following  
586 sub-section on electrochemical methods.

587 The colourimetric methods have potential to be developed into point-of-need methods  
588 owing to the ongoing efforts to use them in conjunction with the ubiquitous smartphones,  
589 which combine a convenient interface, detector (CCD camera), processing power and network  
590 connectivity [81]. Such developments could greatly decrease the cost and increase the

591 availability of in-field AGs analysis. Examples of spectrophotometric and spectrofluorimetric  
592 methods for determination of AGs are shown in Table 7.



593

594 Fig. 6. Determination of kanamycin in vaccine samples based on its effect on the thioglycolic  
595 acid-CdTe quantum dots photoluminescence [79]. Aliquots of reconstituted vaccine samples  
596 (a) were loaded into an SPE cartridge coupled with a syringe filter and packed with  
597 kanamycin-MIP (b). After washing and elution with acidic water, the aliquots were added to a  
598 TGA-CdTe quantum dots dispersion probe (c) and the subsequent photoluminescence  
599 measurements were carried out using a luminescence spectrophotometer (d). Created with  
600 BioRender.com.

601 Table 7. Spectrophotometric and spectrofluorimetric methods for the determination of aminoglycoside antibiotics.

Method	Aminoglycoside	Matrix	Sample preparation	Type of optical reagent	LOD/LOQ Rec.	Ref.
Spectrophotometric	KAN	Pharmaceutical formulations (suspension)	-	Vanillin ( $\lambda = 404$ nm)	LOD: 1.24 $\mu\text{g/mL}$ Rec. 100.13%	[82]
	KAN	Pharmaceutical formulations (suspension)	-	Eosin ( $\lambda = 548$ nm)	LOD: 0.215 $\mu\text{g/mL}$ Rec. 100.34%	[82]
	NEO	Pharmaceutical formulation (tablets)	-	Ninhydrin ( $\lambda = 574$ nm)	LOD: 3.33 $\mu\text{g/mL}$ Rec. 99.2 – 108.9%	[83]
	AMI	Pharmaceutical formulations (injections),	-	Chloranillic acid ( $\lambda = 524$ nm)	LOD: 6.49 $\mu\text{g/mL}$ Rec. 94.44 –	[84]



		standard solution			106.4%	
	KAN	Pharmaceutical formulations (injections), standard solution	-	Ascorbic acid ( $\lambda = 390$ and $530$ nm)	LOD: 8.58 – 9.6 $\mu\text{g/mL}$ Rec. 99.98 – 100.09%	[85]
	STR	Water, serum, milk	-	Colorimetry with AgNP-gallic acid probe  (ratio between peak intensity at $\lambda_1 = 560$ nm and $\lambda_2 = 400$ nm)	LOD: 0.02 – 0.1 ng/mL	[77]
Spectrophotometric/ spectrofluorimetric	SPC	Pharmaceutical formulations (vials), human plasma and urine	Plasma: Protein precipitation (ACN)	Benzofuran  (colorimetry: $\lambda = 410$ nm; fluorimetry: $\lambda_{\text{em}} = 530$ nm; $\lambda_{\text{ex}} = 410$ nm)	LOD:  Colorimetry: 55 ng/mL  Fluorimetry: 4.15 ng/mL	[86]



Rec.

Colorimetry:

97.11%

Fluorimetry:

101.19%

Spectrofluorimetric	NEO, TOB, KAN	Pharmaceutical formulations (tablets, ointments, drops, syrup)	Tablets: grinding and dissolving Ointments: LLE (chloroform)	Acetyloacetone and formaldehyde – Hantzsch condensation $(\lambda_{em} = 471 \text{ nm}; \lambda_{ex} = 410 \text{ nm})$	LOD: 1.6 – 4.93 ng/mL [87] Rec. 99.35 – 100.3%
	AMI, TOB, NEO, GEN, KAN, STR	Pharmaceutical formulations (tablets, ointments, drops, ampoule, vial, syrup), human plasma	LLE (chloroform) Human plasma: protein precipitation (ACN)	Safaranin $(\lambda_{em} = 545 – 570 \text{ nm}; \lambda_{ex} = 519 – 524 \text{ nm})$	LOD: 1.2 – 1.5 pg/mL [88] Rec. 99.2 – 101.0%



NEO, TOB, AMI, KAN	Pharmaceutical formulations (injection tablets), human serum, human urine	Plasma:  Acidic protein precipitation (TCA)  Urine: protein precipitation (MeOH)	2-hydroxyl-1- naphthaldehyde  ( $\lambda_{em} = 434 \text{ nm}$ ; $\lambda_{ex}$ $= 366 \text{ nm}$ )	LOD: 10 ng/mL [89]  Rec. 99.67 – 100.26%
APR	Pharmaceutical formulations (powder), milk	Protein precipitation (ACN)	Inherent native fluorescence  ( $\lambda_{em} = 388 \text{ nm}$ ; $\lambda_{ex}$ $= 335 \text{ nm}$ )	LOD: 50 ng/mL [76]  Rec. 98.03 – 100.7 %
APR	Pharmaceutical formulations (powder), milk	Protein precipitation (ACN)	Micelle-enhanced method (enhancing the native fluorescence intensity using sodium dodecyl sulfate)	LOD: 20 ng/mL [76]  Rec. 98.10 – 101.40%



			( $\lambda_{em} = 398 \text{ nm}$ ; $\lambda_{ex} = 360 \text{ nm}$ )		
AMI	Human urine, river water	-	Molecularly imprinted polymer on fluorescent graphitic carbon nitride quantum dots	LOD: 1.2 – 3 ng/mL Rec. 97.13 – 101.3 %	[80]
			( $\lambda_{em} = 520 \text{ nm}$ ; $\lambda_{ex} = 374 \text{ nm}$ )		

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603 *Electrochemical methods*

604 Aminoglycoside antibiotics can also be determined using a variety of electrochemical  
605 methods. While the reports on the use of more traditional techniques such as potentiometry or  
606 different flavours of voltammetry for AGs determination are rather scarce, a rising trend can  
607 be observed in employing these and other electrochemical sensing techniques in the  
608 construction of aptamer-based AGs biosensors (aptasensors).

609 Potentiometric determination of gentamycin and kanamycin was demonstrated employing an  
610 ion-selective electrode (ISE) constructed using plasticized membranes containing ionophores  
611 based on ion pairs of both aminoglycosides with tetraphenylborate and Acid Chrome Black  
612 Special (ABS) [90]. LODs for gentamycin and kanamycin were in the range of 0.5  $\mu\text{g/mL}$  and  
613 selectivity constants (gentamycin/kanamycin) were close to unity. Such high values of  
614 selectivity constants mean that the electrodes are completely nonselective which limits their  
615 usage either to pharmaceutical formulations containing single AGs or to the measurement of  
616 total gentamycin/kanamycin concentrations. A voltammetric sensor containing reduced  
617 graphene oxide/graphene oxide hybrid  $\square$  modified electrode was used for the electrochemical  
618 detection of tobramycin [91]. The linear response was observed in two concentration ranges:  
619  $3.2 \div 23.4 \mu\text{g/mL}$  and  $23.4 \div 420.3 \mu\text{g/mL}$ . The LOD value was estimated at 0.9  $\mu\text{g/mL}$  and  
620 the sensor was successfully used for determination of tobramycin in human saliva.

621 Electrochemical aptasensors are gaining more and more interest from the researchers working  
622 on easy, selective, quick and reagentless methods for AGs determination, more in line with  
623 the stipulations of Green Analytical Chemistry than the more established methods. Briefly,  
624 such sensors consist of an aptamer specific for the antibiotic of interest, bound to the surface  
625 of the electrode. In the absence of antibiotic molecules, such sensor is in the 'off' state,  
626 meaning that aptamer molecule/chain has some specific conformation and the sensor, as a  
627 whole, has certain electrochemical properties. Introduction of the antibiotic molecules results  
628 in their binding with aptamers leading to changes in their conformation and measurable  
629 changes in electrochemical properties of the sensor as a whole. Numerous reports describing  
630 such aptasensors have been published in recent years. The body of literature on this topic is  
631 too extensive for inclusion in this work, and so the reader interested in this topic is advised to  
632 read one of the excellent reviews available, such as the comprehensive paper by Mehlhorn *et*  
633 *al.* [92].

634 *Qualitative analysis*



635 Quantitative methods involve aminoglycosides identification, confirmation of structures as  
636 well as understanding the mechanisms of interactions, or transformations. The structural  
637 configuration of AGs and characterization of interactions between AGs and RNA can be  
638 obtained using nuclear magnetic resonance spectroscopy (NMR), in particular  $^{15}\text{N}$ -NMR, H-  
639 NMR and  $^{13}\text{C}$ -NMR [93–96]. Attempts to use X-ray diffraction spectroscopy for AGs  
640 characterization have been made as early as the 1960s and 1970s. Due to their amorphous  
641 structure and problems with producing diffraction-quality crystals, it can be realized only for  
642 selected AGs (e.g. fortimicin) [97]. At present, X-ray crystallography is used to define types  
643 of interactions and structures of crystal complexes of AGs with enzymes and RNA [98].

644 Several studies on structural analysis of AGs using mass spectrometry have also been  
645 published. Mass spectrometry was applied to obtain information about interactions between  
646 aminoglycosides and other substances (RNA and enzymes) [99,100], to investigate bacterial  
647 resistance [101] and to study the structure after chemical modifications [102]. NMR, X-ray  
648 diffraction spectroscopy and mass spectrometry provide important support for drug design  
649 and bacterial resistance mechanisms investigations.

#### 650 **4. Conclusions**

651 Aminoglycoside antibiotics are valuable antibacterial drugs employed in many areas of  
652 human activity. They are effective against a number of microorganisms but must be used with  
653 care due to their low therapeutic indices. Since they are relatively inexpensive, cases of  
654 unlawful use of these drugs have been reported. Assuring proper food and drugs quality and  
655 prevention of environmental pollution requires analytical tools suitable for food, drugs and  
656 environmental samples control. Due to high polarity, polycationic character and lack of  
657 chromophores, the determination of aminoglycosides is a challenging task both at the sample  
658 preparation and final determination stages. The sample preparation step in the context of  
659 aminoglycoside antibiotics determination depends heavily on the nature of the sample being  
660 analysed and the final determination technique. Protocols used in the analysis of  
661 pharmaceuticals tend to be relatively straightforward and generally consist of dissolution,  
662 filtration, defatting and derivatization before the actual measurement. The sample preparation  
663 workflows applied for food and environmental samples are usually much more elaborate due  
664 to both complicated matrices and low levels of analytes concentration. Sample clean-up and  
665 analytes preconcentration are conveniently achieved using solid-phase extraction technique. A  
666 variety of sorbents can be used for this purpose, from the “traditional” reversed-phase (e.g.

667 C18 or C8) sorbents through hydrophilic-lipophilic balance, ion exchange, to molecularly  
668 imprinted and magnetically active materials. While the selection of the particular sorbent type  
669 is determined by the nature of analyte(s) and the final determination technique, molecularly  
670 imprinted polymer-based materials seem to be the most promising due to their high selectivity  
671 against aminoglycoside antibiotics.

672 Final determination step may be accomplished using a variety of techniques. In this  
673 context, liquid chromatography seems to be the most powerful tool due to the variety of  
674 separation modes available and compatibility with several types of selective and sensitive  
675 detectors, as evidenced by the body of literature on this topic. Non-separative techniques are  
676 well suited for the determination of a single compound. Recent years have seen developments  
677 in techniques such as bioassays, quantum dot-based colourimetric applications and  
678 aptasensors. They show great potential in the development of low-cost, user-friendly point-of-  
679 need tests which could greatly increase the access to AGs analysis. The demand for such  
680 solutions might come from consumers who are increasingly aware of the dangers associated  
681 with the ubiquitous presence of antibiotics in food and the environment. While the biosensors  
682 for such prospective on-site screening tools show overall good specificity, they can fall short  
683 in this regard compared to the more conventional methods when analysing samples containing  
684 multiple AGs. An interesting development could be the development of an array of different  
685 biosensors, e.g. electrochemical aptasensors, akin to the holistic approaches used in electronic  
686 noses and tongues, thus leveraging their partial selectivity using multivariate statistical  
687 analysis and machine learning models.

688 Liquid chromatography coupled to a variety of detectors will likely remain the  
689 mainstay of qualitative and quantitative determination of AGs, especially in complex matrices  
690 and samples containing multiple antibiotics, and the avenues of research in this area are far  
691 from exhausted. However, in the view of the recent trends in which an emphasis is placed on  
692 the development of green and equitable (more ubiquitous and affordable) analytical  
693 techniques, the coming years will likely see exciting developments in the application of  
694 microfluidic devices and biosensors.

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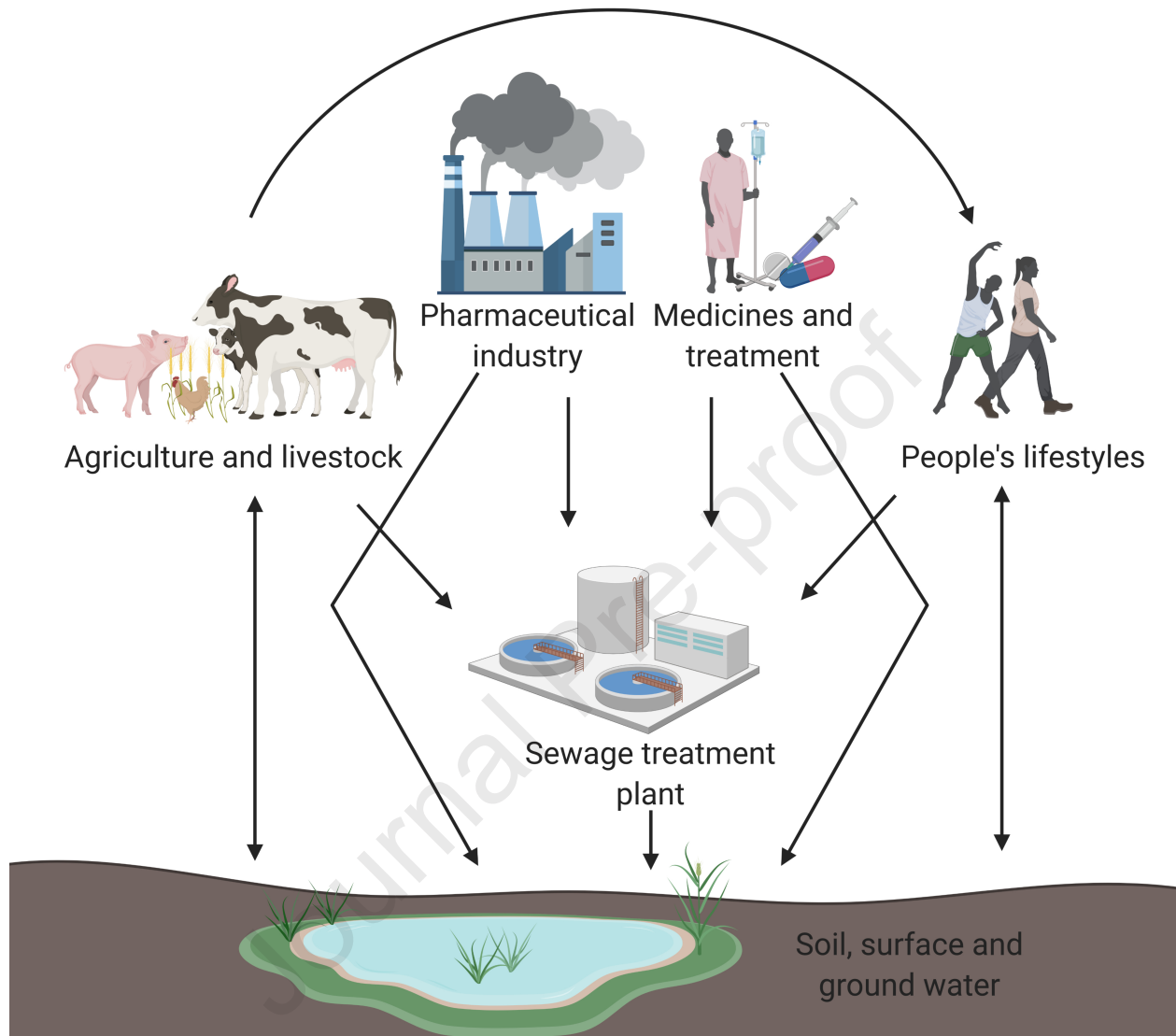
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#### Figure captions

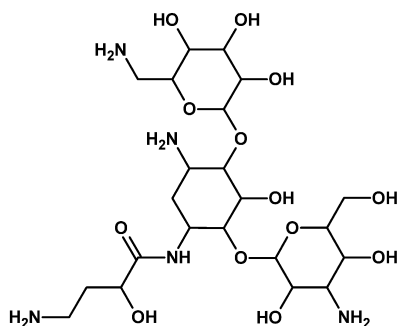
- Fig. 1. Mobility of aminoglycoside antibiotics in the environment. Created with BioRender.com.
- Fig. 2. Structures of selected aminoglycoside antibiotics.
- Fig. 3. Generalised scheme of sample treatment for subsequent determination of aminoglycosides.
- Fig. 4. Example of a chromatogram after derivatization of AGs mixture with FMOC; APR – apramycin, PAR – paromomycin, AMI – amikacin, KAN – kanamycin, TOB – tobramycin, NEO – neomycin, GEN – gentamycin.
- Fig. 5. Comparison of chromatographic separation of 11 AGs under HILIC conditions using SeQuant ZIC-HILIC (A) and Ascentis Si (B) LC columns; AMI – amikacin, APR – apramycin, DHSTR – dihydrostreptomycin, GEN – gentamycin, KAN – kanamycin, NEO – neomycin, PAR – paromomycin, STR – streptomycin, TOB – tobramycin.
- Fig. 6. Determination of kanamycin in vaccine samples based on its effect on the thioglycolic acid-CdTe quantum dots photoluminescence [79]. Aliquots of reconstituted vaccine samples (a) were loaded into a SPE cartridge coupled with a syringe filter and packed with kanamycin-MIP (b). After washing and elution with acidic water, the aliquots were added to a TGA-CdTe quantum dots dispersion probe (c) and the subsequent photoluminescence measurements were carried out using a luminescence spectrophotometer (d). Created with BioRender.com.



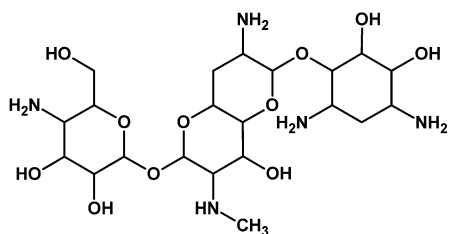




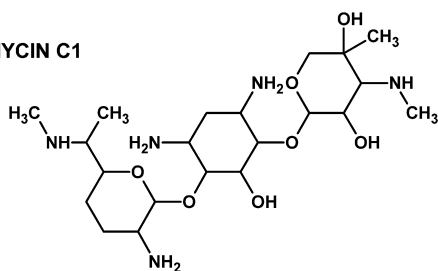
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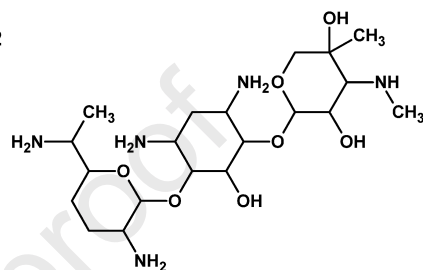
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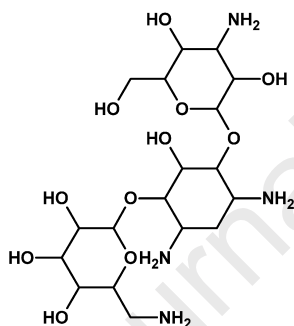
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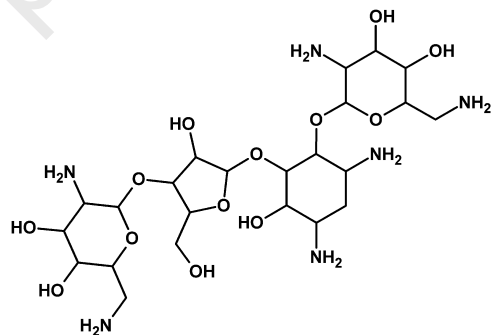
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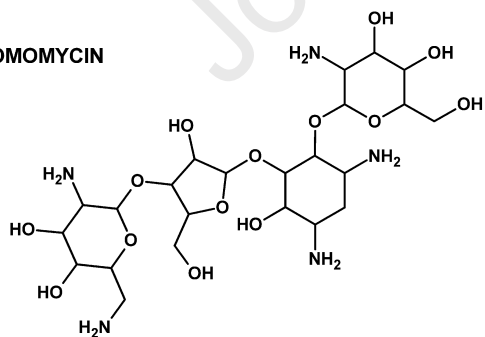
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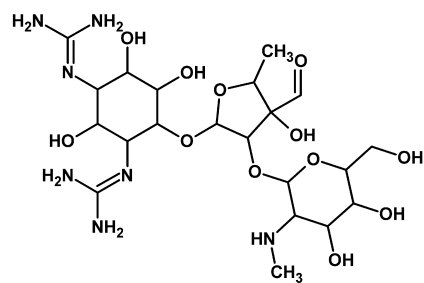
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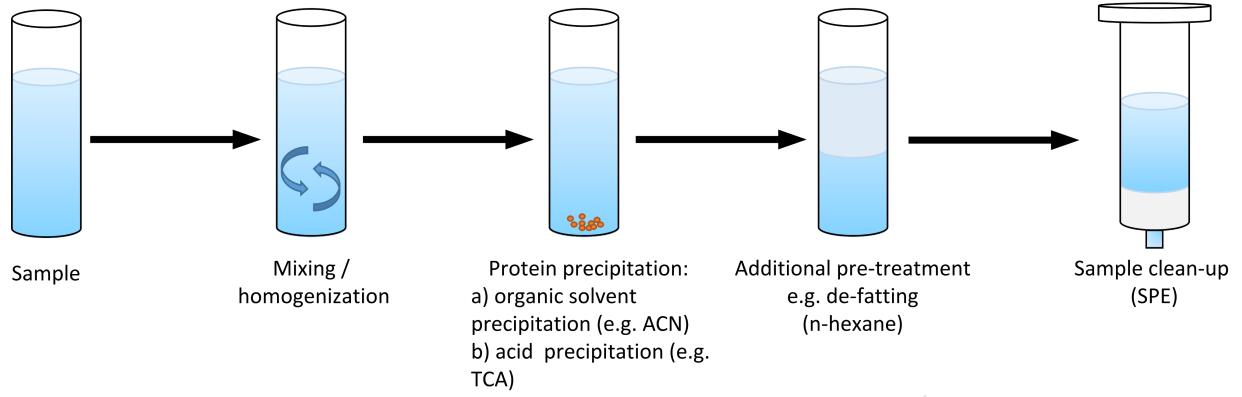


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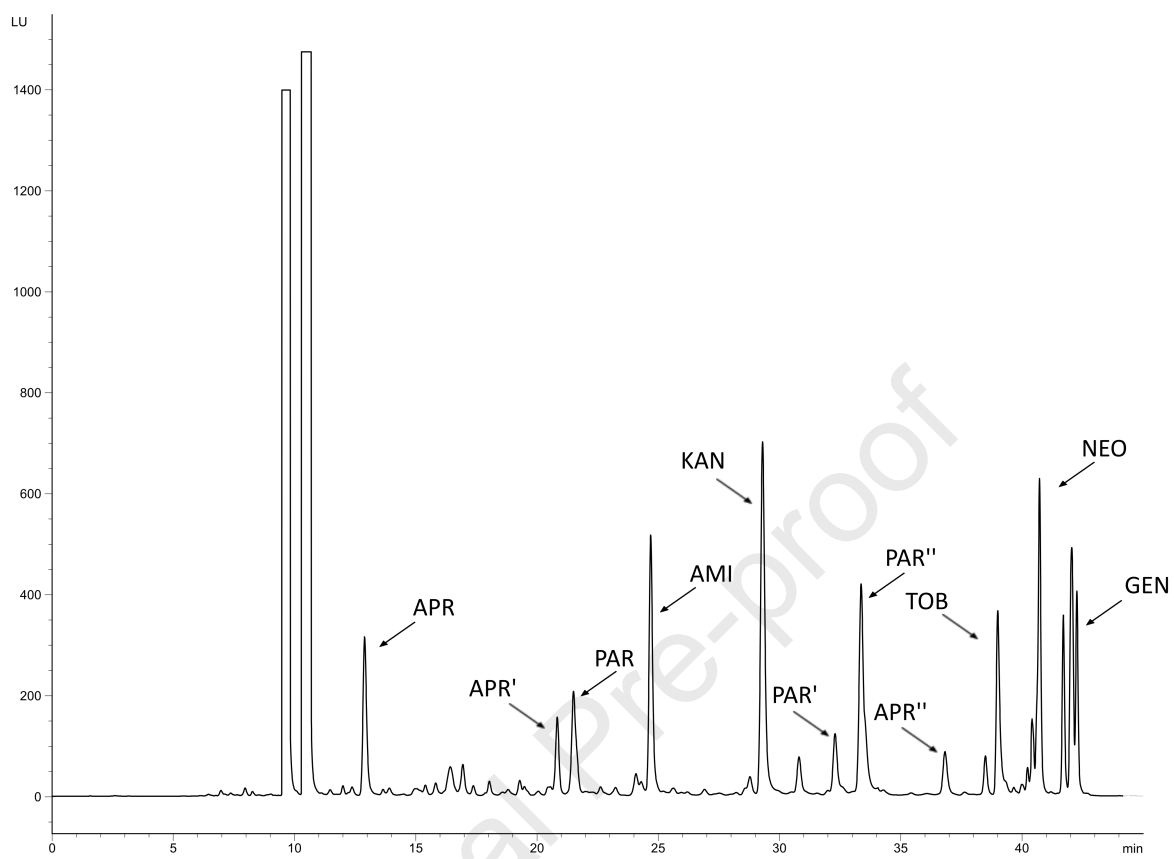


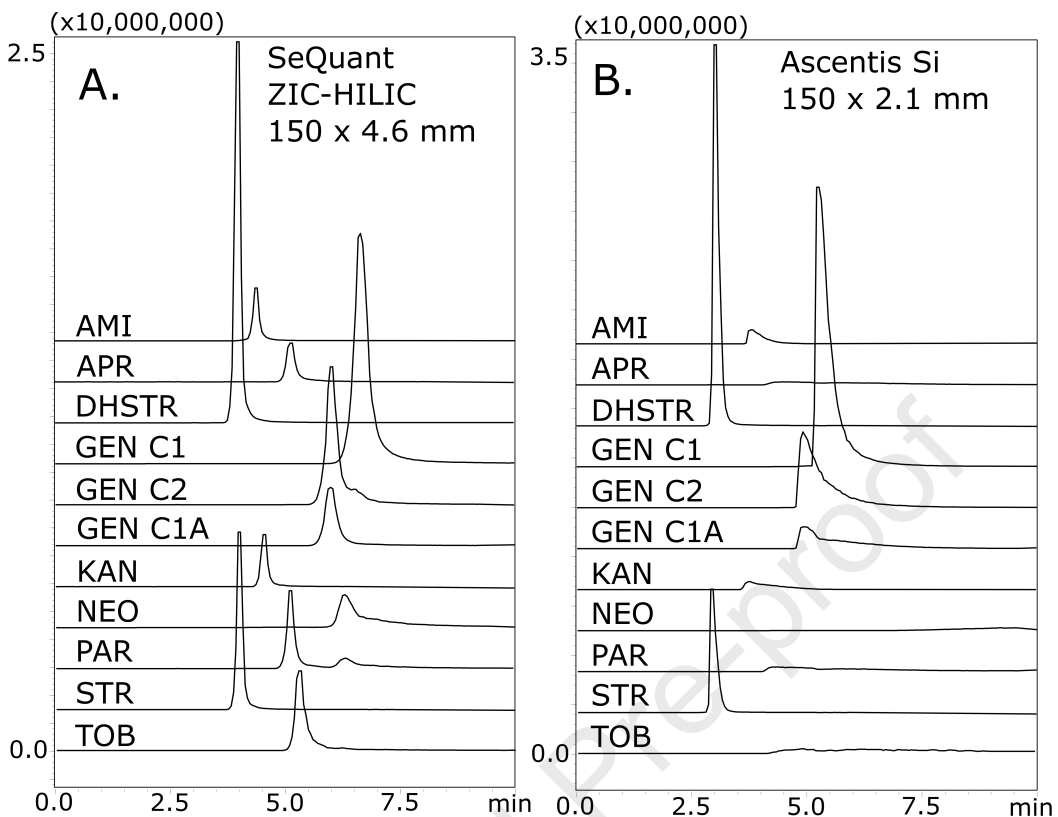
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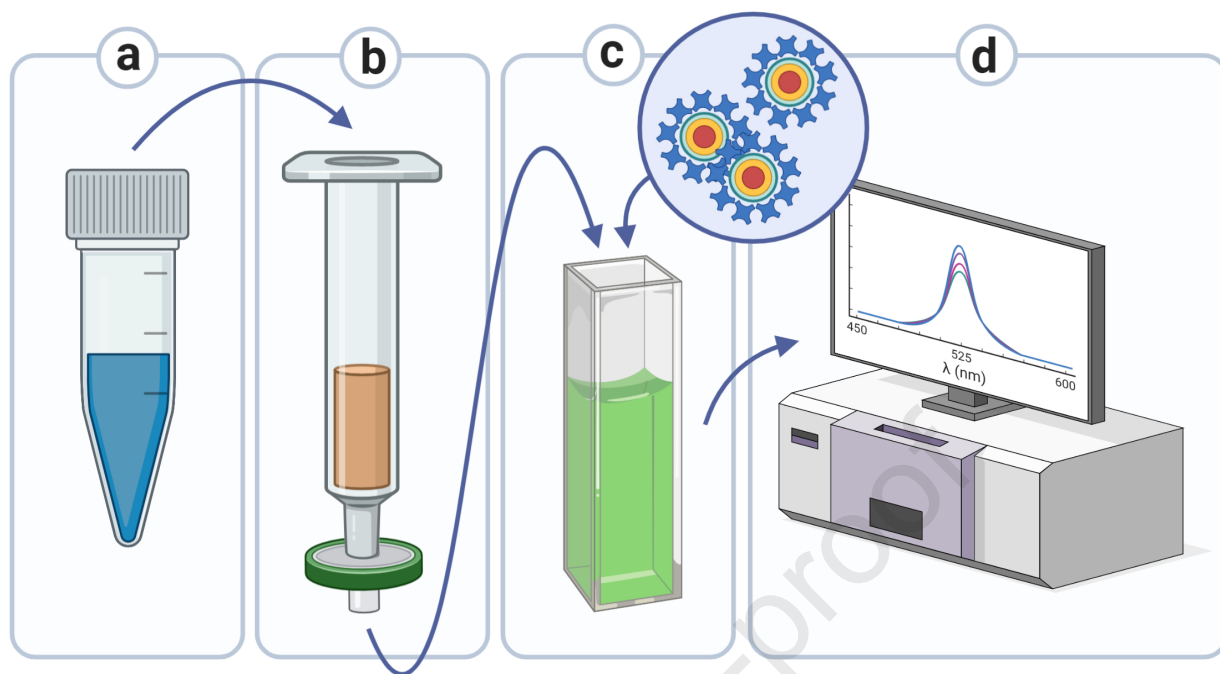




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- Reliable analytical methods are needed for determining aminoglycoside antibiotics.
- We review the state-of-the-art in sample preparation and detection techniques.
- Trends in the development of both LC-based and emerging methods are discussed.

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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