

Application of Supported Liquid Membranes in the Multi-Residue Extraction of Aminoglycoside Antibiotics in Milk and Urine

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A supported liquid membrane has been used as a sample clean-up and/or enrichment technique in the recovery of a mixture of aminoglycoside compounds from cow's milk and urine. The aminoglycoside compounds studied include, neomycin, gentamycin (mainly gentamycin C_{1a}), and streptomycin. The supported liquid membrane (SLM) used to trap these compounds consisted of (1:1) 1-decanol:*n*-undecane. Several factors affecting the extraction efficiencies during SLM enrichment, such as donor and acceptor phases pHs, were studied. High performance liquid chromatography coupled to a positive ion electrospray-mass spectrometer was used as a detection method for these antibiotic compounds with their minor structural components. The extraction efficiencies obtained after SLM enrichment ranged between 60–90% for milk samples and 80–~100% for urine matrices. The detection limits obtained from urine was 0.23 $\mu g\,L^{-1}$ for neomycin, 0.31 $\mu g\,L^{-1}$ for gentamycin, and 0.16 $\mu g\,L^{-1}$ for streptomycin and from cow's milk were 0.34 $\mu g\,L^{-1}$ for neomycin, 0.42 $\mu g\,L^{-1}$ for gentamycin, and 0.20 $\mu g\,L^{-1}$ for streptomycin.

Extracts from matrices, whether of biological or environmental origin, normally contain many diverse interfering compounds included with the possible analytes of interest.[1,2] To minimize interferences due to these compounds a variety of sample pre-treatment techniques are usually employed. For example, protein precipitation has been reported as a sample preparation technique in the recovery of aminoglycosides from plasma matrices.[3–5] During protein precipitation, deproteinization is performed by a variety of solvents including, trichloroacetic acid, perchloric acid, methanol, ethanol, and acetonitrile. However, acidic precipitation has some drawbacks due to dilution, which results in decreased sensitivity, incomplete protein precipitation, and drug coprecipitation. Solid-phase extraction (SPE) has also been used as a sample clean-up for aminoglycosides. Normal phase SPE extraction cartridges, such as silica gel and cyanopropyl, have been reported to give much cleaner extracts.[5] However, the majority of SPE cartridges are for single use and this increases the cost of analysis due to the constant replacement of cartridges. This technique involves a number of sample handling steps, which may be time consuming and also may introduce errors due to the possibility of contamination. To minimize most of these problems, sample preparation methods involving flow systems are becoming popular because they are in most cases closed systems.[6] Sample clean-up and/or enrichment using a supported liquid membrane (SLM), connected to a flow system is such an example. The use of liquid membranes for extraction, clean-up and/or enrichment of samples from the environment was first reported by Audunsson.[6,7] In SLM, the sample comes in contact with the organic liquid solvent immobilized in a membrane (commonly a porous PTFE® membrane) where the analytes of interest are selectively extracted and enriched. On the other side of the membrane a stagnant acceptor solution traps the inactive (charged/ionized) sample analytes at their optimum pH values. Trapping analytes with stagnant acceptor solutions, results in an increase of the total concentration of the analytes and this led to efficient enrichment.[8,9] Supported liquid membranes have been used extensively on metal extractions,[10–14] and in the environmental and biomedical disciplines.[15,16] Recently, we reported the application of liquid membranes in the extraction and enrichment of several classes of antibiotics including benzimidazole anthelmintics,[17] sulfonamides,[18] and macrolide antibiotics.[19] Due to the advantages and simplicity of the SLM technique, there is a need to extend the enrichment of a wider range of veterinary drug residues found in biological matrices. Aminoglycoside antibiotics are among the veterinary drugs that have been reported to accumulate and contaminate the environment.[20,21] In veterinary medicine, aminoglycosides are used to treat animal diseases such as bacterial enteritis (scours) and mastitis and they also serve as prophylactic and growth promoters.[22,23] Due to the frequent use of these antibiotics in animal husbandry, residues have been reported in raw cow's milk and other edible tissues.[24] The occurrence of drug residues in food samples particularly those of animal origin is a serious health hazard. This has necessitated the development of rapid and sensitive methods to police these drugs to ensure the safety of consumers.

We report here the use of SLM as a sample clean-up and enrichment technique before the separation and detection by LC-ESI-MS. SLM in comparison with disposable SPE cartridges, is reusable and utilizes a minimal amount of organics, hence an environmental friendly technique. Moreover, the majority of Public Health Authorities worldwide have certified the use of mass spectrometry for confirmation of the presence of antibiotic residues in foodstuffs.[25]

Experimental

Reagents and Chemicals. The aminoglycoside antibiotics (gentamycin, neomycin, and streptomycin) and 1-decanol were from Fluka Chemika (Buchs, Switzerland). Sodium hydroxide

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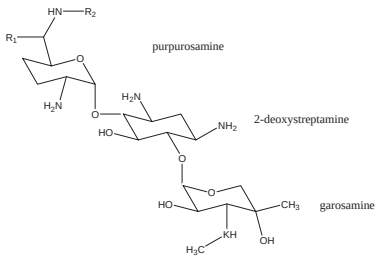
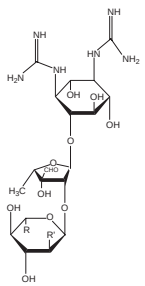
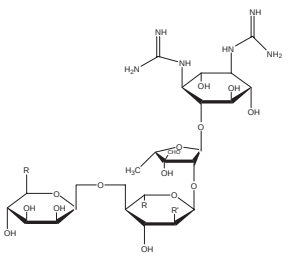
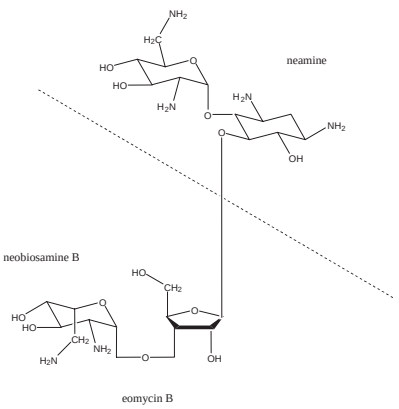
Table 1. Structures of Aminoglycosides

Name	Structure of the compound	pK_a value
Gentamycin C_1; $R_1 = R_2 = CH_3$ Gentamycin C_2; $R_1 = CH_3$, $R_2 = H$ Gentamycin C_{1a}; $R_1 = R_2 = H$		8.20
Streptomycin A R = CH_2OH R′ = $NHCH_3$		7.17
Streptomycin B R = CH_2OH R′ = $NHCH_3$		7.17
Neomycin B		9.03

pellets (98%), *n*-undecane, formic acid, disodium hydrogen phosphate, potassium hydrogen phthalate, and hydrochloric acid were from Saarchem (Krugersdorp, South Africa). HPLC grade acetonitrile, methanol, and trichloroacetic acid were from BDH Laboratory (Poole, England). The chloroform used was from Rochelle Chemicals (Johannesburg, South Africa). A Millipore Alpha-Q Ultra Pure Ionex gradient A10 system was used for water purification (Molsheim, France). All mass measurements were carried out on a Sartorius Supermicro S4, electronic ultramicro balance (Goettingen, German). The structures for the aminoglycoside compounds studied are shown in Table 1.

Preparation of Standards. Each of the aminoglycoside antibiotics was dissolved in 50:50 (v/v) water/methanol to make a stock solution of 1000 mg L^{-1}. The solution was stored at 4 °C but warmed-up to room temperature before use. The corresponding weights for each aminoglycoside compound were; gentamycin (1.3130 mg) and streptomycin (1.5080 mg). Neomycin was obtained in a solution form and its concentration was 1000 mg L^{-1}. The working standards were prepared by dilution of the stock solutions with water/methanol (50:50 v/v) to the required concentration. Use of glass containers during the sample preparation was mostly avoided due to the high sorption affinity of these compounds to the polar surfaces of glass containers. Polypropylene containers were therefore used throughout this work, except during quantitative spiking where glass containers were utilized for a very short period of time.

Extraction from Milk Matrix. The milk samples were filtered through GF/B Whatman glass microfibre filters to remove solid particles. Portions of 10 mL of filtered milk were transferred into 100 mL volumetric flasks and quantitatively spiked with a

mixture of aminoglycosides to give the desired concentrations, ranging from 0.1 μg L^{-1} to 1 mg L^{-1}. Then 10 mL of 0.05 M trichloroacetic acid was added and the mixture was transferred into plastic centrifuge tubes containing 10 mL of chloroform and centrifuged at 3000 rpm for 10 min to remove proteins. The process was repeated at least three times and the aqueous layer was pooled and subjected to further processing using SLM.

Extraction from Urine Matrix. A 10 mL aliquot of urine collected from Gaborone City Council Abattoirs was transferred to a 100 mL volumetric flask. Known amounts of aminoglycoside mixtures were quantitatively added to make concentrations ranging from 0.1 μg L^{-1} to 1 mg L^{-1}. Then 10 mL of acetonitrile was added to the 10 mL spiked urine samples and the mixture was centrifuged at 3000 rpm for 15 min in plastic centrifuge tubes and the supernatant was collected. The process was repeated and the supernatant was pooled and transferred to another tube containing 10 mL of chloroform. The mixture was vortexed for 10 min and then centrifuged for 5 min at 3000 rpm. The extract was then concentrated with a stream of nitrogen and then subjected for further processing with SLM.

SLM Set-Up for the Extraction of Aminoglycoside Compounds. The SLM device was fabricated at the Department of Analytical Chemistry, Lund University, Lund, Sweden. The SLM set-up used was similar to the one described by Jönsson and Mathiasson.[9] The liquid membrane used was prepared by impregnating a Millipore filter, made of PTFE®, FG type with a pore size of 0.2 μm with (1:1) 1-decanol/*n*-undecane. The liquid membrane was made by soaking the PTFE® filter for 30 min, turning it over every 15 min. After assembling the SLM unit, both the donor and acceptor channels were flushed with water to remove the excess organic solvent. Minipuls 3 peristaltic pumps, Gilson (Villiers-Le-Bel, France), were used to pump and control the flow rates of the solutions for both donor and acceptor phases. The flow system was made up of acid resistant tubes, Elkay Products, (Shrewsbury, MA, USA), with internal diameters of 1.2 and 0.60 mm for the donor and acceptor, respectively, connected by 0.5 mm internal diameter PTFE® tubing and Alex screw fittings. The sample and buffer merged in a PTFE® tee connection and then mixed in a coil before entering the donor channel.

Membrane Enrichment. Different concentrations of aminoglycoside samples spiked in urine and milk matrices and the donor buffer (0.05 M disodium hydrogen phosphate/0.1 M sodium hydroxide at pH 12) were delivered with a peristaltic pump to the extraction system. The sample to buffer ratio was 2:1, flow rate of 0.3 mL min^{-1} and an extraction time of 30 min were used in this work. The aminoglycoside compounds were enriched with a stagnant acceptor solution (0.1 M potassium hydrogen phthalate/0.1 M hydrochloric acid pH 3). After processing the sample for 30 min the system was washed by flushing the donor phase for 5 min, while the acceptor phase was kept stagnant. The system was left to stand for 10 min to allow for the diffusion of analytes from the membrane to the acceptor phase and also to minimize memory effects. The acceptor solution containing the aminoglycosides was then transferred quantitatively into 2 mL volumetric flasks using a peristaltic pump. The acceptor channel was then flushed with the acceptor solution for 5 min to clear the system of any traces of analyte before the commencement of the next extraction. A 20 μL aliquot of the enriched sample was then introduced into the HPLC system coupled to an electrospray-mass spectrometer.

High Performance Liquid Chromatography-Mass Spectrometry. A Hewlett Packard HP 1100 HPLC system consisting of a DAD detector, binary pump system, and thermostatted column compartment, coupled to Thermo Electron LCQ^{DECA} ion trap mass spectrometry (Thermo Electron, San Jose, California, USA) was used for all separation and detection of the antibiotics. Aminoglycosides were separated on a RP18 XTerra, 250 mm × 3.0 mm × 3.5 μm column at a flow rate of 150 μL min^{-1} and a mobile phase consisting of A = 25 mM formic acid in water and B = 25 mM formic acid in acetonitrile, using gradient elution as shown in Table 2.

Table 2. The Gradient Elution Parameters for Separation of Aminoglycosides

Time /min	Flow rate /μL min^{-1}	A	B
0.00	150	100	0
5.00	150	100	0
10.00	150	85	15
15.00	150	65	35
30.00	150	55	45
40.00	150	50	50
60	150	50	50

Results and Discussion

Optimization of the SLM System for the Extraction of Aminoglycosides Residues. For efficient enrichment of analytes, the conditions that govern the mass transfer of analytes from the donor to the acceptor across the membrane and the entrapment of the analytes in the acceptor phase must be optimized. The analytes in the sample solution need to be in an uncharged form before their diffusion across the hydrophobic membrane. The partition coefficient of the analyte molecules between the organic liquid membrane and the aqueous donor phase has to be as large as possible for the target molecules and small for the interfering compounds.[26,27] However, it should not be too large as to cause difficulties in stripping into the acceptor buffer phase. Also, an efficient trapping (conversion of the analytes into the inactive form that prevent back-diffusion into the donor channel) must take place on the acceptor side. Therefore, the following parameters, which include the selection of appropriate liquid membranes, donor pH, acceptor pH, and enrichment time, were optimized for each of the veterinary drugs studied.

Choice of the Liquid Membrane. The composition of an organic liquid membrane plays an important role in governing the extraction efficiency and selectivity of the analyte.[26,27] Several liquid membrane solvents were tried to enrich aminoglycoside antibiotics spiked in urine and milk samples and the extraction efficiencies were monitored under the same conditions. The liquid membrane consisting of 1-decanol was relatively successful in extracting a mixture of aminoglycoside compounds in comparison with other liquid membranes used. However, the extraction efficiencies obtained with 1-decanol were low due to the low partition coefficient of the analytes. This was probably due to the high viscosity of 1-decanol, which reduced the diffusibility of the analyte. In order to achieve a high mass-transfer coefficient, the use of less viscous organic liquid membranes is a requirement. This requirement was achieved by mixing 1-decanol with *n*-undecane at various ratios and optimization experiments were carried out. From the

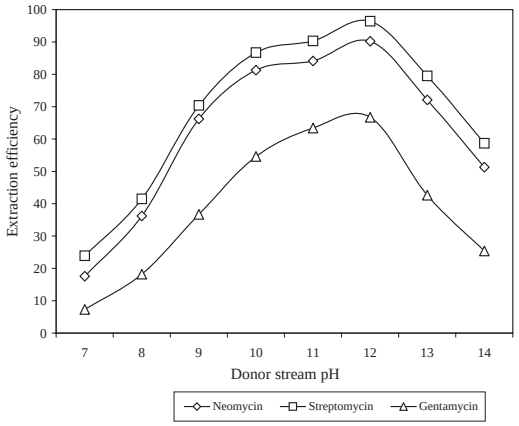


Fig. 1. Donor pH optimization for aminoglycosides: membrane, 1-decanol:*n*-undecane (1:1), acceptor pH = 3, extraction time = 30 min, and flow rate = 0.3 mL min^{-1}.

results, it was found that the mixture of 1-decanol with *n*-undecane (1:1) led to a reduction of the viscosity of the liquid membrane which resulted in the highest relative recoveries. This composition of liquid membrane resulted in a significant increase in the permeability of the analytes into the membrane and therefore was used in all subsequent enrichments of aminoglycosides in different matrices. The 1:1 ratio of 1-decanol/*n*-undecane liquid membrane was found to be optimum (results not shown).

Influence of the Donor Channel pH on the Extraction Efficiencies. To study the influence of the donor pH on the extraction efficiency of aminoglycosides, the pH was varied between 7–14. The results showed that the extraction efficiency increased with increasing pH to a maximum of about 12 (Fig. 1). This demonstrated that the donor pH had an effect over the extraction efficiency. A drop in the extraction efficiency might have been due to the fact that the conditions for neutrality were no longer maintained at pH values higher than optimal. The observed results were in agreement with the theoretical predictions, i.e. the donor pH should be at least 3.3 pH units above the pK_a value of the basic analyte.[28] The pK_a values for neomycin, streptomycin, and gentamycin are 9.03, 7.17, and 8.20, respectively.[29]

Effect of the Stagnant Acceptor Solution pH. The pH of the stagnant acceptor phase also has an essential role in determining the degree of extraction of the target analytes. Aminoglycosides display a hydrophilic and basic character and thus should be trapped in acidic acceptor solutions. A pH of 3 was applied to the acceptor for this experiment (after optimization, Fig. 2). Again this was in agreement with the theoretical predictions which suggest that the acceptor solution must be at least 2 pH units below the pK_a value of the basic analytes for complete trapping.[28,30,31]

SLM Enrichment of Aminoglycosides in Milk and Urine Samples. Aminoglycoside antibiotics were enriched from milk and urine, a metabolic by-product matrix. Figure 3 shows the results of the aminoglycoside antibiotics recovered from the milk matrix. For a mixture with similar concentration levels studied, the extraction efficiencies of SLM used were between 70–90, 65–~80, and 60–~75% for gentamycin, neomycin, and streptomycin, respectively. The order of recoveries

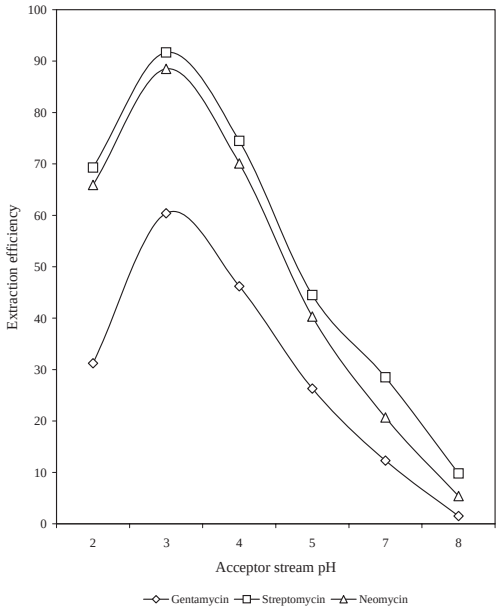


Fig. 2. Acceptor pH optimization for aminoglycosides: membrane, 1-decanol:*n*-undecane (1:1), donor pH = 12, extraction time = 30 min, and flow rate = 0.3 mL min^{-1}.

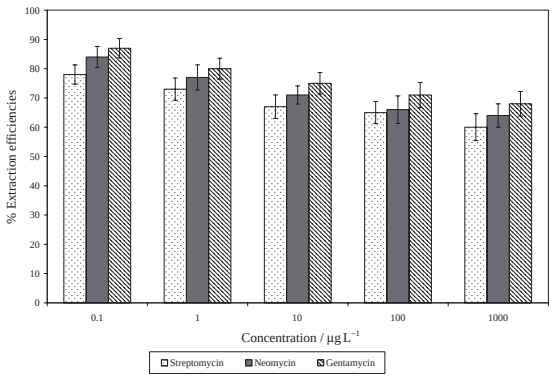


Fig. 3. Percentage of aminoglycoside antibiotics recovered from spiked cow's milk as extracted with SLM. Donor pH = 12, acceptor pH = 3, and enrichment time = 30 min. Error bars represents standard deviation values ($n = 5$). Error bars were calculated using Microsoft Excel software.

from the milk matrix was gentamycin > neomycin > streptomycin. The presence of only hexose sugar moieties in the gentamycin structures was probably responsible for higher efficiencies in comparison with the other two compounds. The six-membered ring sugars (hexoses) displayed more stability than the five-membered ring sugars (pentoses). Neomycin and streptomycin possessed pentose sugar moieties in addition to hexoses. In general there was about a 15% decrease in the % E extracted from milk as the concentration increased in the concentration levels studied. Figure 4 shows the results of the SLM enrichment for a mixture of aminoglycosides in the urine matrix for the concentration levels studied. The recovery of these drugs from urine ranged between ~85–~100, ~75–~90, and 80–~95% for gentamycin, neomycin, and streptomycin, respectively. In comparison with milk, urine does not

contain proteins or lipids and hence less interferences present, which were probably responsible for the higher extraction efficiencies. Enrichment at low concentrations gave the highest extraction efficiencies. It was also observed that the order of extraction efficiencies in urine had changed in comparison with milk. The observed order was gentamycin > streptomycin > neomycin. The extraction efficiency for streptomycin from urine increased to almost as high as that of gentamycin. Milk for instance contains a lot of fat, and is always considered to be a complex emulsion of fat droplets in aqueous milk plasma. It consists of water, proteins, carbohydrates (mainly in the form of lactose sugar), lipids, enzymes, minerals, phosphatides, and other compounds.[32,35] Moreover, milk plasma is not homogeneous but contains a colloidal solution of globular proteins and a dispersion of serum proteins called casein micelles.[32] Due to the physicochemically different phases occurring in milk, drugs may sometimes be unevenly distributed and may also be predominantly found in one phase.[32] This may result in the non uniform recoveries of the components in the mixtures. Urine, on the other hand, is composed mainly of nitrogenous compounds such as urea, uric acid, and creatine.[35] Aminoglycosides, which are themselves basic and polar, have slight differences in their polarities and thereby interact differently with molecules of different biological matrices. Gentamycin which is less polar than the other two and which is made up of only hexoses, had the highest recoveries in both urine and milk matrices. The presence of only hexoses might be responsible for its high relative stability in the liquid membrane compared with the other two compounds possessing both hexoses and pentoses. However, neomycin extracted better than streptomycin did in milk, but the reverse was observed in the urine matrix. The polarities of the compounds in relation to the matrices might have accounted for this trend. Streptomycin being more polar than neomycin, might have attained more stability in the presence of polar functionalities in the urine matrix as was observed in neomycin in milk matrix.

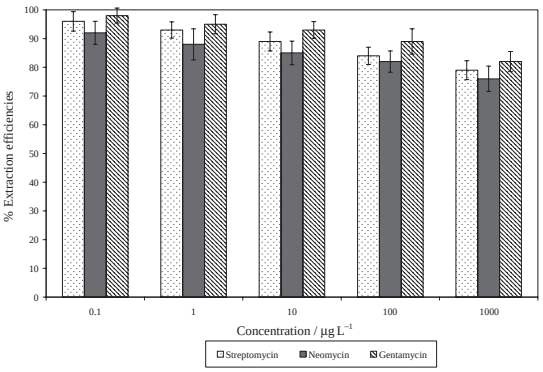


Fig. 4. Percentage of aminoglycoside antibiotics recovered from spiked urine samples as extracted with SLM. Donor pH = 12, acceptor pH = 3, and enrichment time = 30 min. Error bars represents standard deviation values ($n = 5$). Error bars were calculated using Microsoft Excel software.

Separation and Detection of a Mixture of Aminoglycoside Compounds with LC-ESI-MS. Reverse-phase separation with an acidified organic mobile phase was used for all separations. The effect of column temperature was also studied by varying the column temperature from 25 to 45 °C. The retention time decreased with increase in column temperature. The temperature of 40 °C was selected on the bases of peak

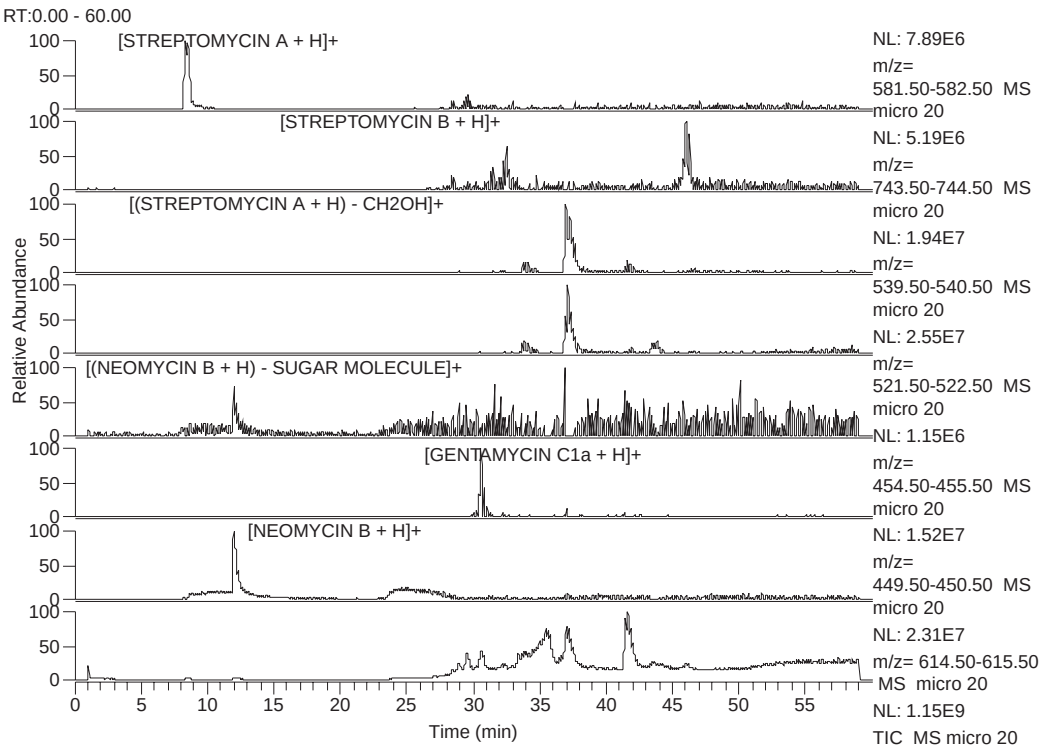


Fig. 5. SLM/LC-ESI-SIM-MS of a mixture of aminoglycosides extracted from spiked urine matrix; concn = 0.1 μg L^{-1}. Donor pH = 12, acceptor pH = 3, and enrichment time = 30 min.

Table 3. Detection Limits for Aminoglycosides[a)]

Compound	Urine		Milk	
	This work	Literature	This work	Literature
Neomycin	$0.23\,\mu g\,L^{-1}$	$3.5\,\mu g\,kg^{-1}$ (39)[b)]	$0.34\,\mu g\,L^{-1}$	$10\,mg\,L^{-1}$ (37)
Gentamycin	$0.31\,\mu g\,L^{-1}$	$0.5–2.5\,\mu g\,kg^{-1}$ (40)[b)]	$0.42\,\mu g\,L^{-1}$	$10\,mg\,L^{-1}$ (37)
Streptomycin	$0.16\,\mu g\,L^{-1}$	$0.01\,mg\,kg^{-1}$ (limit of quantification) (41)[c)]	$0.20\,\mu g\,L^{-1}$	$30\,\mu g\,kg^{-1}$ (36); $10\,mg\,L^{-1}$ (37); $1\,\mu g\,L^{-1}$ (38); 8 $\mu g\,kg^{-1}$ (42)

a) NB: Values in parentheses refers to references cited. b) Matrix was animal tisuue. c) Matrix was honey.

Table 4. MRLs for Different Aminoglycosides Antibiotics as per EU[a)] and FDA[b)] Regulations[33]

	Maximum residual limits (MRL)/$\mu g\,L^{-1}$											
	Milk		Eggs		Muscle		Liver		Kidney		Fat	
	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA
Gentamycin (chickens, turkeys)						100		100		100		100
Gentamycin (cattle)	100				100		200		1000		100	
Gentamycin (swine)					100	100	200	300	1000	400	100	400
Neomycin (chickens)			500		500		500		5000		500	
Neomycin (turkeys)					500	1200	500	3600	5000		500	7200
Neomycin (cattle, swine, sheep, goats)	500	150			500	1200	500	3600	5000	7200	500	7200

a) EU = European Union. b) FDA = Food and Drug Administration.

symmetry, resolution, and retention time of compounds of interest. Figure 5 shows a typical separation of the aminoglycoside compounds extracted from a urine matrix. In electrospray-mass spectrometry, a protonated molecular ion and fragment ion, due to the loss of a CH_2OH group, were observed for streptomycin A. Similarly, a protonated molecular ion and fragment ions were observed from neomycin B. However, only the protonated molecular ion was observed from gentamycin C_{1a}. In monitoring these compounds protonated molecular ions were used for all three compounds. In addition, fragment ions due to the loss of CH_2OH were used for streptomycin A.

Detection Limits (DL). Detection limits (DLs), were calculated as the analyte concentrations giving a signal equal to the blank signal (yB) plus three standard deviations (for $n = 5$) of the blank (sB), that is, yB + 3sB.[34] The DL obtained from urine extraction was $0.16\,\mu g\,L^{-1}$ for streptomycin, $0.23\,\mu g\,L^{-1}$ for neomycin, and $0.31\,\mu g\,L^{-1}$ for gentamycin. For the milk matrix, the DL values were $0.20\,\mu g\,L^{-1}$ for streptomycin, $0.34\,\mu g\,L^{-1}$ for neomycin, and $0.42\,\mu g\,L^{-1}$ for gentamycin. The detection limits obtained in this work were compared and found to be better than data that has been reported using different methods and approaches[35–42] (Table 3).

Method Precision and Accuracy. In the determination of precision (proximity of experimental values to each other)[34] and accuracy (proximity to the true value),[34] a bovine milk matrix was spiked with a mixture of aminoglycosides of known concentrations ranging from $10\,\mu g\,L^{-1}$ to $1\,mg\,L^{-1}$. The results for accuracy were as high, above 90%, while the % relative standard deviation values ranged from 1.3 to 5.2 (for $n = 5$).

Conclusion

SLM demonstrated higher extraction efficiency at lower concentrations, when these sample preparation techniques were incorporated with LC-ESI-MS. These compounds were detected at levels that are far below the stipulated maximum residual limits (MRLs) by the European Union (EU) and Food and Drug Administration (FDA) (Table 4).[33] In addition, statistical data from the results obtained in this work also supports the potential of SLM as a clean-up and/or enrichment method for mixtures of aminoglycoside compounds in different biomatrices. SLM can therefore be suitable for the routine analyses of these compounds and/or similar antibiotic residues in food samples.

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