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Optimization and Comparisons for Separation, Detection and Quantification of 12 Aminoglycosides Using 2 Chromatographic Conditions by LC-MS/MS

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Abstract

Aminoglycosides are a family of antibiotics with important applications in veterinary medicine. Their ionic character, the similarity structures and the high polarity due to the presence of two or more amino and hydroxyl groups cause a difficulty in separation and make these compounds poorly retained on the reversed phase column. An analytical method for the separation and detection of 12 aminoglycosides has been optimized using two kinds of chromatographic conditions (*HILIC*, *Ion pairing*). In Hydrophilic Interaction, *ZIC_HILIC* column was used, by which the following parameters for the mobile phase were evaluated: concentration of ammonium acetate buffer, percentage of formic acid and effect of acid type. The maximum and adequate concentration of ammonium acetate for the majority of analytes was set to 30 mM. The percentage 0.1% of formic acid increases the response for the majority of analytes. On the other side, the use of 0.1% of trifluoroacetic acid improves the response when compared with the response obtained with 0.1% of formic acid except for Spectinomycin Dihydrostreptomycin and Streptomycin. For ion pairing chromatography, the concentration of pentafluoropropionic acid was tested and the greatest value appeared to be 9.2 mM. Therefore, the comparison between the two separation methods shows that the response area of the majority of analytes tested increases when using the ion pair mode. Also, the high value of S/N and the lower detection limit (5 - 15 μg·mL^{-1}) for most aminoglycosides studied make the ion pairing method more preferable than *HILIC* interaction.

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Keywords

Aminoglycosides, Ion Pairing, ZIC-HILIC, LC-MS/MS

1. Introduction

Aminoglycosides (AGs) are a large class of antibiotics that are characterized by two or more amino sugars linked by *glycosidic* bonds to an *aminocyclitol* component (**Figure 1**) [1].

They have been widely used in veterinary medicine and animal husbandry and they are distributed in the body after injection where little amount is absorbed from the gastro-intestinal tract. Thus, they are excreted unchanged in the urine [2]. Most of AGs are used against bacteria and parasites in the production of pork, chicken, beef, milk and eggs around the world, which could arouse side reactions and antimicrobial resistance to consumers [3]. These AGs interfere with bacterial protein synthesis by binding irreversibly to ribosome's, in order to interrupt the protein synthesis, which causes damage to the cell membranes [4].

Due to their toxicity and possible antibiotic resistance, considerable attention has been paid to the potential human health risk. Hence, the European Union (EU), the USA, China, Japan, and other countries have issued strict maximum residue levels (MRLs) for nine AGs in various animal origin foods [5]. To monitor these residues in live animals and animal products, two legislations settings are given in Council Directive 96/23/EC5, S.I. 507/98 and EU Commission Decision 2002/657/EC6. Even though, they are banned as growth promoters in the EU [6].

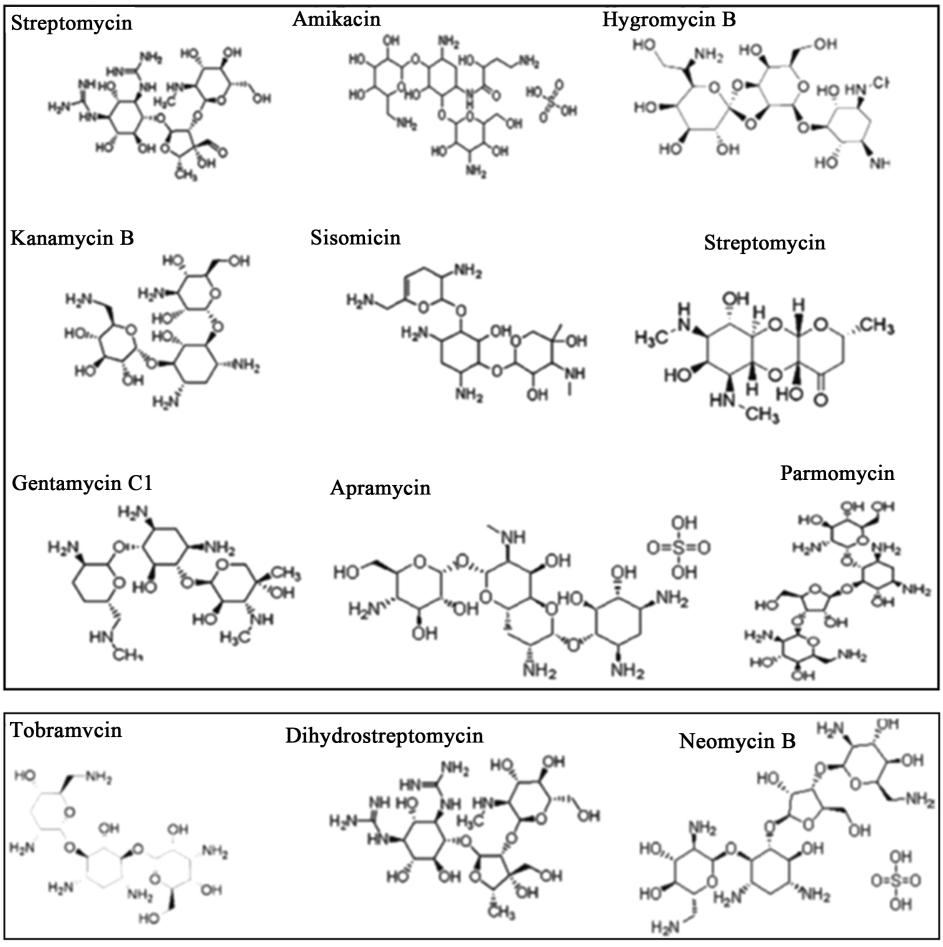


Figure 1. Chemical structures of the 12 AGs.

Consequently, there is a great need to develop sensitive and reliable analytical methods for monitoring the residues of trace level AGs in complex matrices. Several analytical methods have been described in literature for determination of AGs in biological samples (plasma, urine, milk…) [7]-[10], edible tissues (kidney, muscle…) or varieties of water samples (Hospital water, wastewater….) [11].

AGs are characterized by thermal stability and non-volatility, requiring long derivatization time using *GC* or *GC-MS* [12]. Many authors have overcome the problem of the lack of *UV* chromophore or fluorophore for the AGs by using derivatizing agents for the detection by ultraviolet absorbance (*UV*) or by fluorescence (*FLD*) [13]. In this field, the analysis by liquid chromatography (*LC*) often entails pre- or postcolumn derivatization, followed by the detection with *UV* or *FLD* [12] [14]-[16]. However, the derivatization steps render the analytical process moretime consuming and may even introduce impurities. Another problem associated with derivatization is the possibility of self degradation of these derivatives few hours after their formation. This leads to a poor reproducibility caused by their instability and a low yield of derivatives [17]. Therefore, high-performance liquid chromatographic (*HPLC*) methods in combination with tandem mass spectrometry may have the greatest potential for accomplishing multi-residue identifications in complex matrices [18]. For AGs ionization in MS, the analysis depends on two main types of atmospheric pressure ion sources: the electrospray ionization (*ESI*) and the atmospheric pressure chemical ionization (*APCI*). In fact, the *ESI* is the most commonly used and few publications relied on the *APCI* for AGs ionization by *LC-MS* [19] [20].

Sensitivity and selectivity related problems have prompted several researchers to use liquid chromatography coupled to tandem mass spectrometry (*LC-MS/MS*) to analyze AGs [5] [15] [18]. AGs are well known for their high polarity, thus, they are not retained on the reversed-phase columns [5] [21]. This fact presents a big analytical challenge. So, there were numerous methods to produce some retention of the chromatographically unretained AGs using either ion pairing agents or hydrophilic interaction chromatography which is recently cited by researchers but no explanation was found for their choice [5] [21]-[23]. Our paper contributes to a better understanding of both, the advantages and disadvantages of these two methods *HILIC* and *Ion pairing*.

The main objective of our paper is to develop and optimize a reliable and generic *LC-MS/MS* method in order to quantify 12 AGs in water. The originality of our work is related to the presentation and discussion of the potential factors affecting response and sensitivity using *ion pairing* or *HILIC* for separation.

2. Experimental

2.1. Materials, Chemicals and Reagents

Apramycin sulphate (APR) (purity 98.5%), Gentamycin-2.5-sulphate hydrate (GEN) (purity 96.5%), Tobramycin (TOB) (purity 93%), Streptomycin sulphate (STR) (purity 98%), Dihydrostreptomycin sesquisulfate hydrate (DIH) (purity 99%), Spectinomycin dihydrochloride hydrate (SPE)(purity 99%), Paromomycin sulphate (PAR) (purity 90%) and Neomycin sulphate (NEO) (purity 90%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg-Germany). Sisomycin sulphate salt (SIS) (purity 87%), Kanamycin sulphate (KAN) (purity 77%), Amikacin Hydrate (AMK) (purity 98%) and Hygromycin B (HYG) (purity 66%) were purchased from Sigma-Aldrich (St. Louis, MO).

Water, acetonitrile (ACN) for LC-MS CHROMASOLV and pentafluoropropionic acid (PFPA) (purity 97%) were obtained from Sigma-Aldrich (GmbH Riedstr steinheim, Germany). Ammonium acetate (AmAc) and trifluoroacetic acid (TFA) were from MERCK (Darmstadt, Germany) and formic acid (FA) from BDH laboratory (England).

2.2. Preparations of Standard Solutions

Due to the high sorption affinity of the AGs to polar surfaces, only laboratory equipments made of polypropylene were used during sample preparation and storage. The use of organic acid, such as 1% FA in stock solution allows the adsorption to plastic tubes.

Stock standard solutions preparations (~1000 $\mu g \cdot mL^{-1}$): 0.02 g of each AG were dissolved in 25 mL volumetric flask and then reconstituted with water at 1% FA. The solutions were stored at 4˚C and were stable for at least 8 months. For the optimization of MS/MS parameters (tuning), an individual standard solution was prepared at 5 $\mu g \cdot mL^{-1}$ in ACN–Water (1:1, v/v). For the standard calibration curves, a mixture of standard solution at highest concentration (35 $\mu g \cdot mL^{-1}$) was prepared in water at 1% FA. This solution was then appropriately diluted to lower concentrations.

2.3. Mass Spectrometer Conditions

Analysis was applied using HPLC from Agilent (Agilent technologies, USA) series 1200 coupled with MS/MS triple quadruple Agilent 6410. The Agilent HPLC was equipped with an automatic degasser, a quaternary pump, a cooled autosampler and an ESI ion source. The optimization was carried out by infusing 5 $\mu g \cdot mL^{-1}$ of each standard in the HPLC mobile phase into the MS/MS system for automatic tuning to achieve the maximum response. Then, MS/MS data acquisition was performed in Multiple Reaction Monitoring (MRM) mode where two precursor-product ions were chosen for each AGs and listed in **Table 1**. The precursor ions of all analytes were $[M + H]^+$, except SPE, which forms intensive water adducts. SPE has an unusual structural feature in which the carbonyl group is hydrated in an aqueous solution [24]. Therefore, $[M + H_2O + H]^+$, *m/z* 351 was selected as the precursor ion for MS/MS analysis.

The MS/MS instrument was operated in positive ion mode using nitrogen gas as a collision gas and also as nebulization gas at 30 and 40 psi respectively. The source temperature was maintained at 350˚C and capillary voltage was set at 4 kV. The fragment ions, the optimized cone voltage and the collision energy are shown in **Table 1**. Fragment ions with the highest intensity were chosen for quantification and the dwell time was fixed at 25.

2.4. Chromatographic Separation

Two types of LC conditions were applied for the analysis of the 12 AGs: *Ion pairing* chromatography and *HILIC*. To optimize chromatographic separation, preliminary experiments were performed using both methods.

2.5. ZIC_HILIC Conditions

Separation of AGs was performed on a SeQuant *ZIC_HILIC* PEEK column 50 mm * 2.1 mm, 3.5 µm particle sizes. The column was contained in a thermostated column oven maintained at 30˚C. Mobile phases were: A-Ultrapure water with 30 mM AmAc + ACN+ TFA (95/5/0.1) and B: Ultrapure water with 2 mM AmAc + ACN + TFA (5/95/0.2). The elution gradient program (A: B) was applied: (0:100) to (70:30) in 2 min at 0.3 $mL \cdot min^{-1}$; (70:30) to (95:5) in 1 min at 0.3 $mL \cdot min^{-1}$: (95:5) to (5:95) in 1 min at 0.5 $mL \cdot min^{-1}$; (5:95) to (0:100) in 1 min at 0.3 $mL \cdot min^{-1}$. Then, the column was equilibrated for 5 min until the next injection with the flow rates 0.3 $mL \cdot min^{-1}$; the total run time of the method was 19 min. An injection volume of 10 µL was employed for all samples tested.

2.6. Ion Pairing Chromatography

A suitable reversed phase separation of a mixture of AGs was achieved with a 150 mm * 4.6 mm 5 µm Zorbax Eclipse C18 column. Mobile phases were: A-Ultrapure water + 0.1% PFPA and B: ACN. The elution gradient

Table 1. The optimal parameters for multiple reaction monitoring (*MRM*) for 12 AGs.

Aminoglycosides	Precursor ion (*m/z*)	Cone voltage (V)	Quantification ion (*m/z*)	Collision energy (V)	Confirmation ion (*m/z*)	Collision energy (V)
Apramycin	540.3	90	217	25	378	15
Gentamycin	478.3 464.3 450.1	120	322.2 322.1 322	10	156.9	20
Dihydrostreptomycin	584.3	120	262.9	30	245.9	35
Streptomycin	582.2	120	263.1	35	245.9	35
Néomycin	615.3	120	160.9	35	292.9	25
Spectinomycin	351.1	120	333.1	15	207.1	20
Paromomycin	616.3	100	162.9	40	324.1	20
Tobramycin	468.2	120	162.9	20	324.2	10
Amikacin	586.3	110	162.9	35	425.3	15
Sisomycin	448.3	100	254.1	15	271.1	15
Kanamycin	485	60	324.2	15	163.2	5
Hygromycin	528.2	110	177	30	352.1	25

program (A:B) was applied: (95:5) to (50:50) in 10.6 min; (50:50) to (0:100) in 0.4 min; (0:100) to (95:5) in 3 min. Then, the column was equilibrated for 3 min until the next injection; the total run time of the method was 16 min using 0.8 mL·min^{-1}. A 10 µL injection volume was also used in this method.

3. Results and Discussion

3.1. HILIC-ESI-MS/MS Optimization

Recently, a *HILIC* phase prepared by graft polymerization to incorporate 3-sulfopropyl dimethylalkyl ammonium inner salts, *i.e.* sulfoalkylbetaine functional groups onto silica and polymer particles has released. Sometimes, this phase is called a zwitterionic phase. These types of columns are available from SeQuant (Ume, Sweden), as *ZIC_HILIC* columns. Originally, this phase was prepared for cation exchange chromatography, and earlier reports described the separations of inorganic salts and proteins in fully aqueous mobile phases [25]. This phase can retain not only polar and charged compounds, but it also interacts with neutrally charged analytes (containing either positive or negative charges), increasing selectivity in the analysis of a wide range of compounds. This phase is widely accepted by many researchers, and used often in more recent publications [26].

As mentioned in Section Introduction, highly polar compounds are not retained and are eluted in the void volume of column when using reversed-phase separation. Polar analytes can be more strongly retained in the *HILIC* mode and are eluted by increasing the percentage of aqueous portion in the mobile phase. The stationary *HILIC* phase described in literature is a zwitterionic silica gel. Therefore, the *HILIC* technique is suitable for the analysis of polar compounds (e.g. folates [27], carbohydrates [28], peptides [29] or natural products [30]) with MS/MS detection. The separation mechanism of *HILIC* is opposite to that of reversed phase liquid chromatography (*RPLC*). Using a solvent with 95% ACN, a complete retention of the hydrophilic analytes was observed for more than 20 min. Otherwise, when using a solvent with 90% water, the hydrophilic analytes were eluted with the front [31].

A wide variety of *HILIC* columns are commercially available with different functionalities of stationary phase and other features (*ZIC_HILIC*, *Acquity UPLC BEH HILIC*…) [32]. Mobile phase composition is an additional important consideration in LC–ESI-MS/MS, especially for trace analysis, in order to achieve high ionization and sufficient separation with minimum matrix interference [32].

Ishii. R *et al*. found that *ZIC_HILIC* column was better than other columns in separating the AGs and resolving the corresponding peak form. According to their work, ACN alone was better as a mobile phase than methanol (MeOH) or a mixture of ACN /MeOH (1:1, v/v).

In *HILIC*, the presence of water in the mobile phase is fundamental for the establishment of a stagnant enriched aqueous layer on the surface of the stationary phase into which analytes may selectively be partitioned. The use of a buffered mobile phase is crucial in order to achieve acceptable repeatability for the LC separation of charged compounds, since electrostatic interactions between the solute and the stationary phase are controlled by the buffer. The concentration of the buffer should be low to avoid ionization suppression in the ESI.

Using zwitterionic *ZIC_HILIC* columns, many problems were observed with the constancy of the retention times and backpressure after 250 samples. Analytes injected on new columns have up to 100% longer retention times than the same analytes after 20 injections on this column. To remove remaining organic solvent and polar impurities, 20 µL of 0.5M sodium chloride solution were injected after 15 samples. If the backpressure increases or a shift in the selectivity is observed, an initial washing with deionised water is required to remove organic solvent and polar impurities, followed by a flush with 0.5 M sodium chloride solution. Removing salt solution with sufficient water is recommended and finally the column is filled with 80% ACN [31].

The effect of acid and salts added to the mobile phase for accelerating and stabilizing the ionization, was examined. AGs are eluted from *HILIC* columns starting with a high concentration of AmAc. It seems necessary to use concentrated AmAc in the mobile phase for eluting all the tested compounds from the analytical column, especially NEO, APR and GEN. In order to investigate the efficiency of *ZIC_HILIC*, we also designed experiments to compare the chromatographic responses of 12 AGs. Furthermore, mobile phase gradient conditions were also optimized in order to obtain the best chromatographic results with minimum analysis time. Therefore, we presented and discussed the effect of the percentage of organic solvent (ACN), the concentration of AmAc at the starting time, the percentage and the acid type used in the mobile phase, on the separation response area and the corresponding peak form of each compound. All experiment was performed in triplicate and the RSD were in the range 1% - 20%.

3.2. Effect of Acetonitrile on Aminoglycoside Retention

The effect of ACN on analyte retention was investigated. The experiment demonstrated that, as the percentage of ACN in the starting gradient increased, the retention also increased. Above 60% ACN, most of the analytes appear to be retained. The organic modifier/aqueous portion ratio is the predominant factor in providing sufficient retention in *HILIC*. In this issue, the peak of GEN, PAR, NEO, SIS, TOB and APR has a broad and poor form when the percentage of ACN decreases. Thus, this result is related to the decreases of the AmAc concentration (Phase B) and the strong elution with the high percentage of water.

Hence, our optimized gradient started with 95% ACN then after 2 min the percentage decreases. In addition, the flow (0.3 $mL \cdot min^{-1}$) in the starting gradient with 95% ACN ensured a good retention and separation for all AGs (**Figure 2**). Furthermore, the effect of ACN on the ending gradient was much less influential as the one in the starting gradient.

3.3. Effect of Ammonium Acetate Concentration on AGs Response

The mobile phase in *HILIC* separation plays important roles and its pH conditions are generally controlled by buffer solutions with a 10 - 65 mM salt concentration. With respect to the MS sensitivity, the AmAc was superior to the ammonium formate. Therefore, the AmAc was used in different concentrations to obtain sharp peaks and to achieve good responses for all AGs.

Figure 3 shows that the optimum concentration of AmAc was found at 30 mM except that of HYG and AMK. Once the concentration of AmAc increased, the area of majority of AGs was decreased. That's because increasing the concentration of AmAc induced an increase of the ionic strength in the mobile phase. As a consequence, the response of AGs was highly influenced and decreased. This is related to the compound properties. The high ionic strength in the mobile phase lead to discharges and poor spray performance of ionization by *ESI*.

The response of DIH, STR and GEN did not show a considerable change upon decreasing the concentration of AmAc.

Another fact assumed that the form of peaks of most AGs was improved by increasing AmAc concentration. The late eluted AGs (APR, PAR, SIS, TOB) showed broad peaks when the concentration of AmAc was decreased. This led to a poor limit of detection for this compound. On the other hand, the retention times were increased by raising the concentration of the buffer.

Neomycin has a bad response and a broad peak in *ZIC_HILIC* column. Hence, many researchers found a great need to add 120 - 150 mM AmAc and a 1% FA in the mobile phase [1]. However, this was impossible to be done in our work as it negatively influenced the response of other AGs.

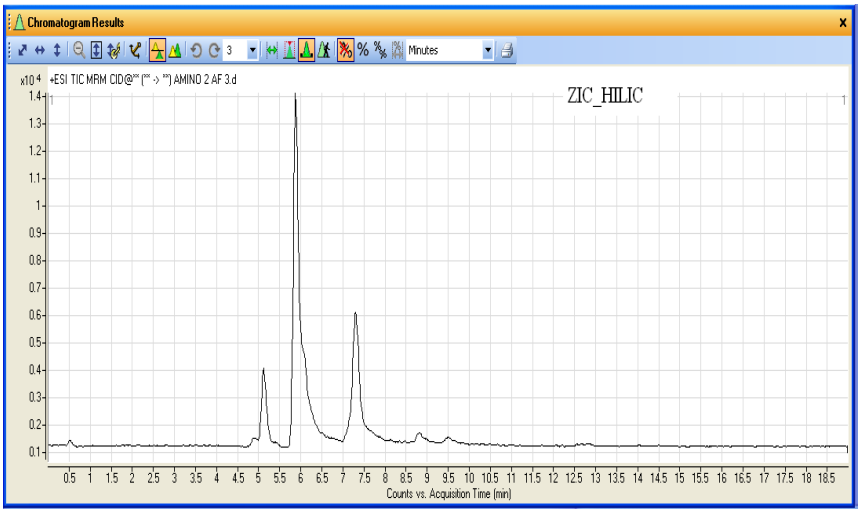
3.4. Effect of Percentage and Type of Acid in the Mobile Phase on the Ionization for AGs

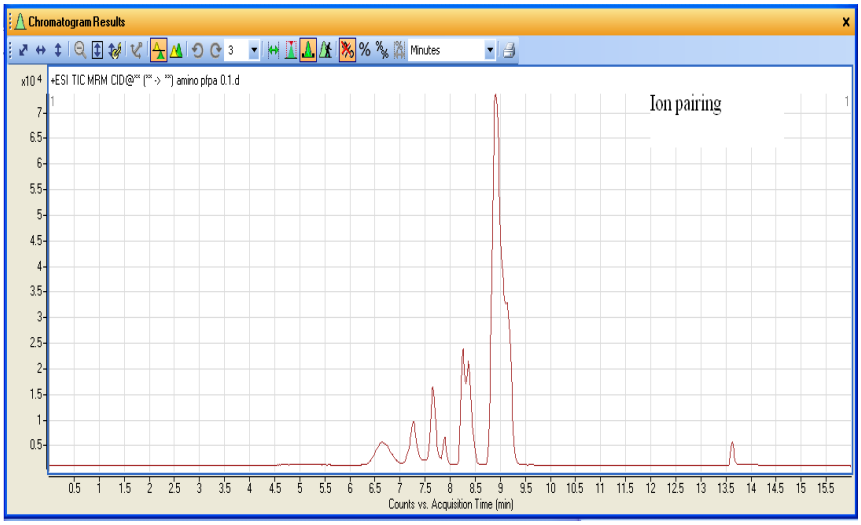
The effect of three different percentages of FA (0.1, 0.2 and 0.5) added to the mobile phase A was studied. We found that the retention times were not affected when varying the pH of the mobile phase. This indicates the high selectivity and the pH independence of all compounds. Most of AGs showed an increase in response when changing the percentage of FA from 0.5 to 0.1. So the percentage 0.1% (pH 4.30) was shown to be better than 0.2% (pH 3.51) and 0.5% (pH 3.22) (**Figure 4**). However, GEN, DIH, STR presented an exception as their response increase when 0.5% FA was used (**Figure 4**).

Another experiment studied the effect of response for all AGs substituting FA by TFA. When using 0.1% TFA (pH 4.88), the results presented in the **Figure 5** showed an improvement in the peak area for majority of AGs (AMK-HYG-KAN-NEO-APR-GEN-PAR-SIS-TOB) to the detriment of 0.1% FA (pH 4.30). In contrast, the remaining AGs (SPE-DIH-STR) revealed an improvement in the response when using 0.1% FA.

3.5. Ion Pairing Optimization

Most of previously reviewed methods are mainly based on reversed phase separation due to its applicability for a wide range of neutral compounds of different polarities. However, the main drawbacks encountered when using reversed phase for the most highly polar compounds, that they are poorly retained and produce poor peak shapes. Hammel *et al.* [33] proposed ion pair chromatography for multiresidue antibiotics analysis in honey, including AGs. However, ion pair separation requires ion-pair reagent (mainly TFA, heptafluorobutyric acid, phosphoric acid, etc.) in the mobile phase composition to regulate the retention of polar compounds on the stationary phase [32].


Chromatogram Results
+ESI TIC MRM CID@"" ("" -> "") AMINO 2 AF 3.d
ZIC_HILIC
Counts vs. Acquisition Time (min)


Chromatogram Results
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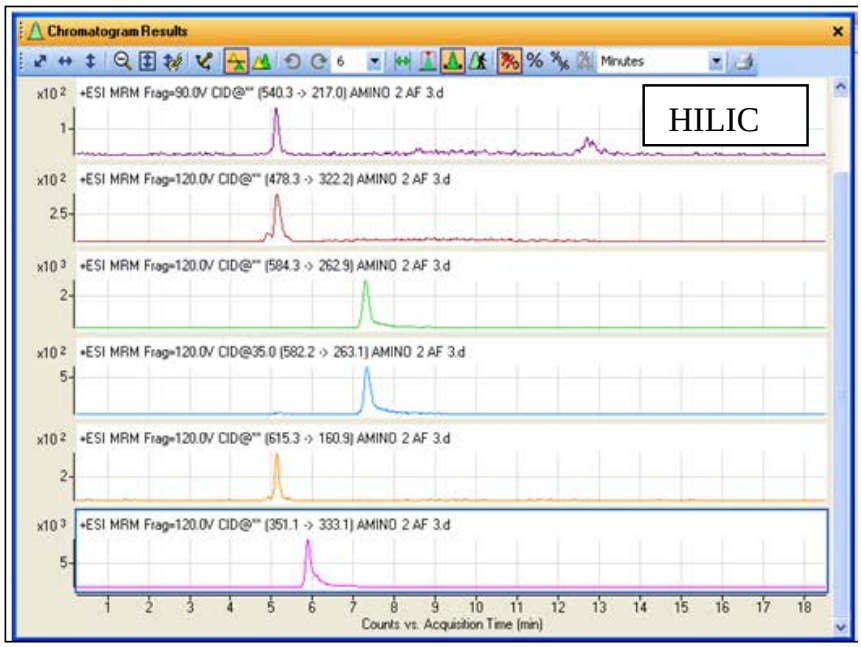
Ion pairing
Counts vs. Acquisition Time (min)


Chromatogram Results
+ESI MRM Frag=90.0V CID@"" (540.3 -> 217.0) AMINO 2 AF 3.d
HILIC
+ESI MRM Frag=120.0V CID@"" (478.3 -> 322.2) AMINO 2 AF 3.d
+ESI MRM Frag=120.0V CID@"" (584.3 -> 262.9) AMINO 2 AF 3.d
+ESI MRM Frag=120.0V CID@35.0 (582.2 -> 263.1) AMINO 2 AF 3.d
+ESI MRM Frag=120.0V CID@"" (615.3 -> 160.9) AMINO 2 AF 3.d
+ESI MRM Frag=120.0V CID@"" (351.1 -> 333.1) AMINO 2 AF 3.d
Counts vs. Acquisition Time (min)

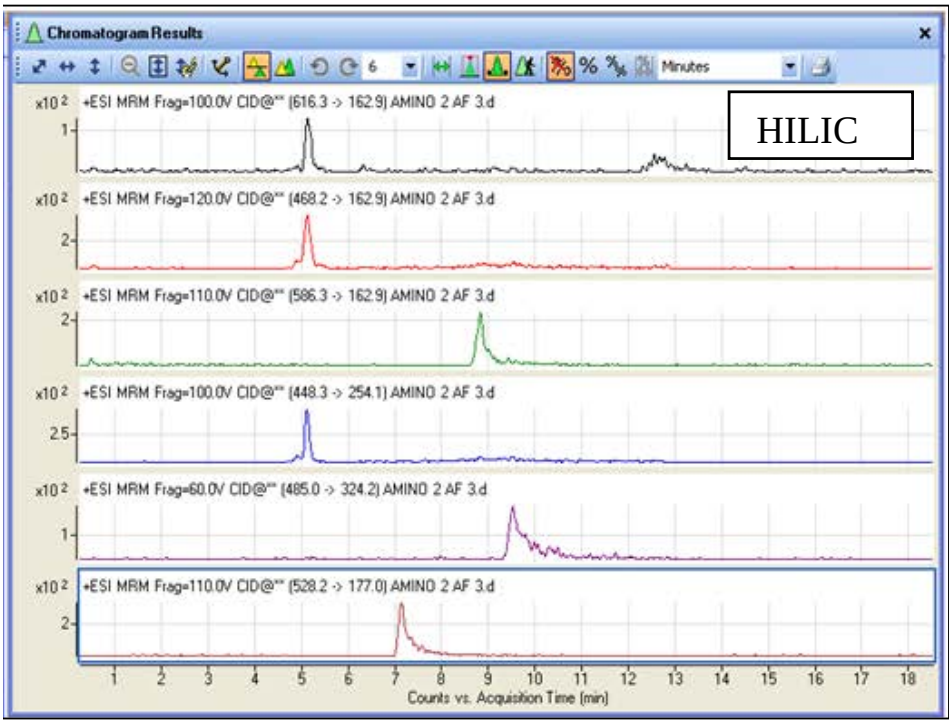


Figure 2. TIC obtained for the 12 AGs by the two methods of separation (HILIC and Ion Pairing) at a 100 (μg·mL^{-1}) and chromatogram obtained at 95% ACN (starting gradient) in ZIC-HILIC column.

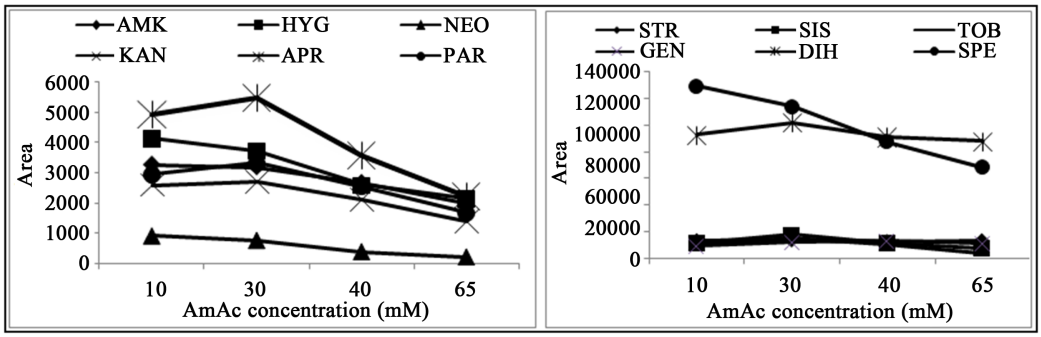


Figure 3. Effect of the buffer (ammonium acetate AmAc) concentration in the mobile phase A on the response of the 12 AGs.

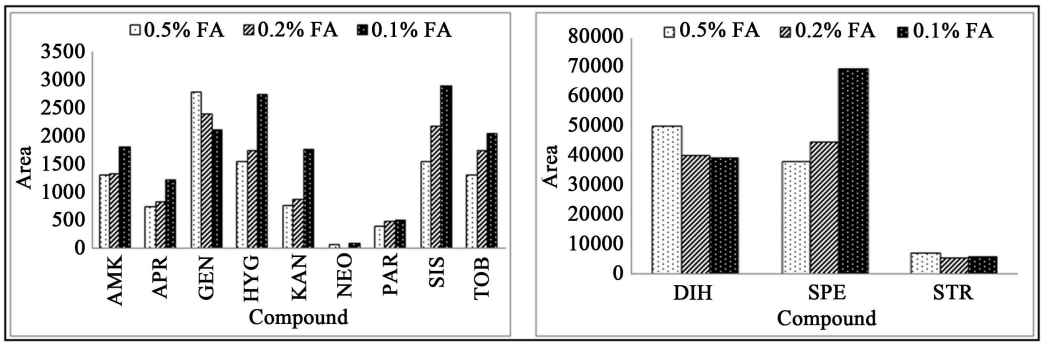


Figure 4. Effect of the percentage of Formic acid (0.5%, 0.2% and 0.1%) in the mobile phase A on the response of AGs.

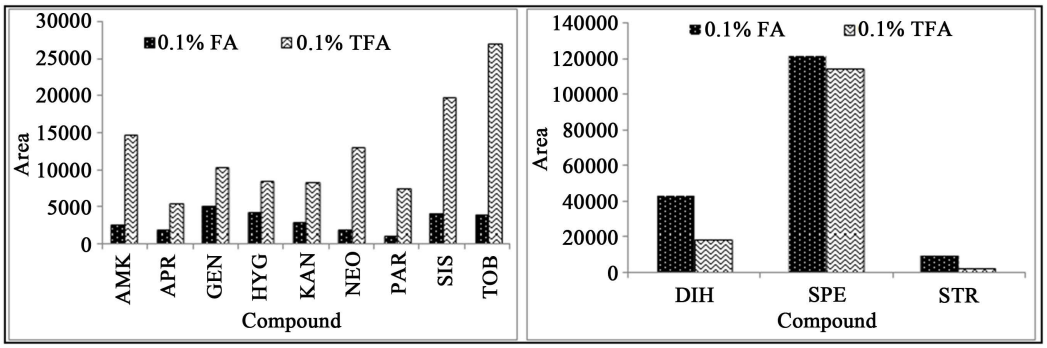


Figure 5. Effect of the acid type, TFA and FA, in the mobile phase A at 0.1% on the response of all AGs.

Therefore, some volatile and fluorinated ion-pair reagents including TFA, PFPA and heptafluorobutyric acid (HFBA) are commonly used in the previously published methods for the analysis of AGs combined with mass spectrometry [17] [23] [34].

When HFBA and PFPA are compared, PFPA can be considered as a better reagent, because it does not adsorb to the stationary phase as HFBA strongly does and the retention is clearly obtained through an ion-pair mechanism [35]. On the other hand, with TFA as an ion pair agent, dramatic ion suppression in the ESI occurs. Whereas with HFBA the ion suppression was limited [9] [22].

In our work, the PFPA was used as an ion pairing reagent. Their concentration was optimized to get less signal suppression in the ESI. Therefore, the maximum concentration of additives that can be used depends on the design and technical solutions of the ion-source of the mass spectrometric instrument, e.g. orthogonal electrospray instruments can often tolerate higher concentration of additives [36]. **Figure 6** presents the comparison of response by using modified concentrations of PFPA [0.92 mmol·L^{-1} (0.01%), 9.20 mmol·L^{-1} (0.1%), 46 mmol·L^{-1} (0.5%)] in the mobile phase. This parameter was found in triplicate and the RSD was between 1% - 12%.

All compound's areas present an optimum result at 0.1% PFPA. The variation of the results of the peak areas for AGs was designed between 18% - 80% when comparing 0.1% and 0.5% PFPA. This result became logic if one takes into account the suppression of signal in the ion source (ESI) with the use of high concentration of ion pair reagent. On the other hand, the area response of AGs was better when 0.1% PFPA was used at the expense of 0.01% PFPA. The deviation of response was 5% to 77%. Consequently, 0.01% PFPA is not sufficient to ensure the optimum retention of analyte in the column.

3.6. Comparison between HILIC and Ion Pairing

AGs are very hydrophilic compounds and are traditionally difficult to retain on conventional *HPLC* columns. Hence, ion-pair reversed phase LC is preferred to chromatograph aminoglycosides on a C18 column. The use of an ion-pairing reagent in the mobile phase has been reported earlier [5] [22] [23] [37]. On the other hand, the application of *HILIC* is known to be as an alternative for the determination of extremely polar compounds. Recently, literature and research on hydrophilic interaction chromatography (*HILIC*) have been increasing drastically, along with various stationary phases developed. *HILIC* is a kind of normal-phase liquid chromatography (*NPLC*), and has attracted the attention of researchers that study the separation of polar compounds in a wide variety of scientific fields. In *HILIC* mode, a mixture of water and organic modifiers (mostly ACN) is employed with a polar stationary phase. Structural variations in *HILIC* type stationary phases are wider than those found in reversed-phase applications. This technique is suitable for *ESI-MS*, because of the compatibility of the aqueous organic mobile phase to *ESI-MS*, which is a very powerful tool in detecting and identifying a wide range of polar compounds [26].

The confirmation and quantification of AGs by *LC-MS/MS* was evaluated by *ZIC_HILIC* and *ion pairing* separation. As seen in **Figure 2**, the ion pairing separation was found better for most of AGs. The response by ion pair reagent showed an increase of the obtained value with *ZIC_HILIC* conditions (**Figure 7**). An exception of DIH, STR and SPE, the area was improved in *HILIC* than in ion pair mode. This led to higher S/N ratios and low limits of detection for the method using *ion pairing* separation.

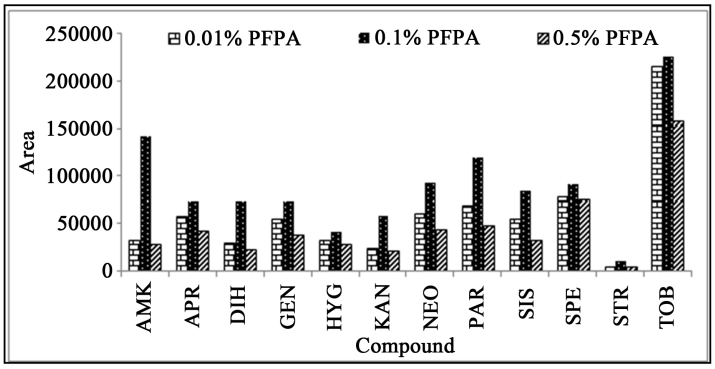


Figure 6. The area response for AGs using 3 different percentages (0.01%, 0.1% and 0.5%) of PFPA.

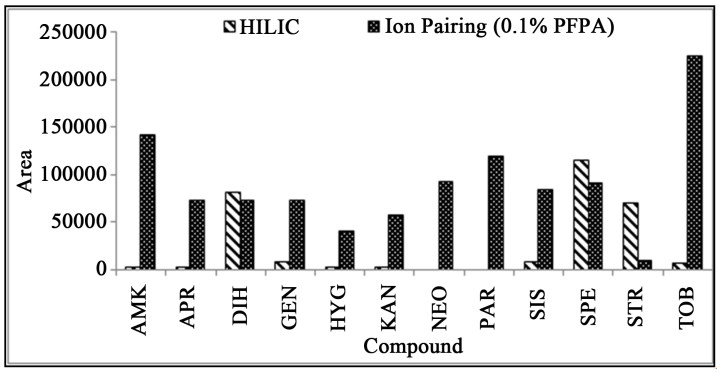


Figure 7. Comparison of area response of AGs by the two methods of separation (*HILIC and Ion pairing*).

Repeatability of the two separation methods were described as the value of relative standard deviation (RSDs) of the areas obtained for each analyte after the replicate (n = 3) analyses of spiked water blank samples which ranged between 0.5% - 20%. LODs were experimentally determined at a signal to noise ratio (S/N) of 3 and presented for each AGs. Then, **Table 2** present the calculated values (at the same concentration 700 µg·mL^{-1}) of S/N and the limit of detection of the two methods of separation used in our work and certified results was cited above. Linearity of the two methods (HILIC, Ion pairing) compared was determined by injection of blank water samples spiked at different concentration levels ranged between 5 to 500 µg·L^{-1}. Each compound showed good linearity in studied working range, with coefficient of determination (R^2) greater than 0.98 (**Table 2**). The best coefficient of determination (0.982 - 0.998) was related to *Ion pairing* method for the majority of AGs.

ZIC_HILIC columns need mobile phases with high organic content. This type of column gives large peak areas when used without modifiers, but they required high concentrations of AmAc to achieve reasonable peak shapes. But, the high concentration of AmAc greatly suppressed the response from the mass spectrometer [22].

Another problems were found, the *ZIC_HILIC* column need more time for conditioning in comparison to the C18 column by using PFPA reagent. This fact was demonstrated by the injection of standards several times at the beginning of conditioning. The better result of separation by *ZIC_HILIC* was performed for each AG after 1 h conditioning.

Moreover, the column of *ZIC_HILIC* needs a wash for several hours by water and 0.5 mM *NaCl* solution in order to eliminate the back pressure after 250 injection samples. As a consequence, the use of ion pairing modifiers and reversed phase for *LC* was preferred over *HILIC*. The ion pair system was showed to be stable after two to three injections. It produced significantly higher sensitivities for late eluting AGs than *HILIC* and was very stable regarding the injection of high salt containing extracts.

The PFPA reagent was used to regulate the retention of AGs on the C18 column. But many researchers reported the serious problem of the affected MS performance by ionization suppression and the contamination of the ion source when using the ion pair reagent [32].

Table 2. Coefficients of determination (R^2) for the calibration curves signal to noise (*S/N*) ratio, Limit of detection ($\mu g \cdot min^{-1}$) and retention times (Tr) of the two methods (*HILIC* and *Ion Pairing*).

	HILIC				Ion pairing			
Aminoglycosides	R^2	LOD (ppb)	S/N	Tr (min)	R^2	LOD (ppb)	S/N	Tr (min)
Amikacin	0.991	10	3.12	9.7	0.998	5	148	8.2
Apramycin	0.988	90	3.96	5.3	0.991	5	138	8.8
Dihydrostreptomycin	0.996	5	11.4	5.4	0.994	5	57.4	7.2
Gentamicin	0.989	60	4.99	5.3	0.989	5	204	9.1
Hygromycin	0.994`	5	8.08	7.2	0.949	5	10	7.2
Kanamycin	0.995	90	3	10.2	0.982	5	138	8.3
Neomycin	0.420	200	4.05	5.3	0.994	5	161	9.3
Paromomycin	0.981	200	3.1	5.1	0.991	5	277	8.8
Sisomycin	0.979	200	2.10	5.2	0.990	5	168	9
Spectinomycin	0.942	5	52	6.7	0.979	15	12.6	6.7
Streptomycin	0.987	5	9.06	5.4	0.993	15	17.6	9.1
Tobramycin	0.968	200	3.4	5.3	0.987	5	169	8.9

Sometimes, it is reported that these reagents adhere to the line on *LC* and *MS* detectors, which reduces the *MS* sensitivity and requires maintenance of *LC* and *MS* equipment due to these contaminants [38].

Finally, regarding all the results and the problems cited above, the separation by reversed phase C18 using the PFPA is more suitable than the separation by *ZIC_HILIC*. Yet, cleaning the instrument was not found to be a relevant problem when using the ion pair reagent.

4. Conclusions

Two types of separation conditions (*HILIC*, *Ion pairing*) were examined and optimized to improve the chromatographic parameters for the analysis of 12 aminoglycosides by *LC-MS/MS*.

In *ZIC_HILIC* column, different concentrations of ammonium acetate and formic acid have been tested. As a result, 30 mM ammonium acetate and 0.1% FA appear to induce better conditions for most analytes. As regards to the pentafluoropropionic acid (PFPA), an optimum concentration was evaluated for ion pairing using Zorbax Eclipse C18 column (9.2 $mM \cdot L^{-1}$). The advantages and the disadvantages of the two methods were discussed and reported. Both ion pairing and *HILIC*, demonstrated good separation of the majority of compounds tested.

When comparing the response area of *HILIC* and ion pairing, most of the AGs showed an increase of around 99% by ion pairing. However, Dihydrostreptomycin, Streptomycin and Spectinomycin showed better area responses when separation was made with *HILIC*. Both limit of detection and S/N ratio were better when using ion pairing. On the other hand, the problems for the constancy of the retention times and the backpressure in the column make the ion pairing a reliable and powerful technique comparing to *HILIC*.

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