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# A Comparative Review of Hydrophilic Interaction Chromatography (HILIC) with Traditional Chromatography in the Analysis of Aminoglycoside Antibiotics

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## ABSTRACT

Aminoglycoside antibiotics remain crucial for treating severe Gram-negative infections, yet their highly polar, polycationic nature presents ongoing analytical challenges. Traditional reversed-phase chromatography often proves inadequate, offering insufficient retention and poor peak symmetry due to limited interactions with hydrophobic stationary phases. Consequently, analytical techniques have increasingly shifted toward hydrophilic interaction liquid chromatography (HILIC), which offers improved retention, enhanced selectivity, and better compatibility with mass spectrometric detection. This review critically explores the development of chromatographic methods for aminoglycoside quantification, emphasizing methodological advancements, practical challenges, and the application of modern HILIC–MS/MS systems. Key structural elements of aminoglycosides, such as multiple amino and hydroxyl groups and the presence of 2-deoxystreptamine, significantly affect chromatographic behavior. Understanding these molecular features is essential for optimizing mobile-phase pH, buffer strength, and organic solvent composition to achieve effective separation. Recent research indicates that optimized HILIC methods can address previous issues related to ion suppression, low retention, and complex sample matrices, particularly in biological, pharmaceutical, and food safety analyses. Despite significant progress, challenges persist in method standardization, consistency across laboratories, and the development of universally applicable chromatographic conditions. This review compiles existing evidence, highlighting the strengths and limitations of current analytical methods, and suggests future directions for creating more sensitive, reproducible, and high-throughput methodologies. By integrating structural insights with modern chromatographic advancements, the review offers a comprehensive perspective aimed at enhancing aminoglycoside analysis across various research and regulatory settings.

## KEYWORDS

Aminoglycosides; hydrophilic interaction chromatography (HILIC); normal phase chromatography (NP); reversed phase chromatography (RP-HPLC); zwitterionic columns

## Introduction

HILIC (Hydrophilic Interaction Liquid Chromatography) provides an effective choice for analyzing polar substances. HILIC is a separation technique that integrates mobile phases characteristic of RP separations with stationary phases typically employed in normal-phase (NP) chromatography. In 1990, Alpert proposed the name “HILIC” to describe a separation technique where a moderately polar stationary phase interacts with polar analytes, eluting them using a relatively hydrophobic mobile phase. In 1975, Linden and Lawhead developed the technique of utilizing chemically modified silica with amine groups as the stationary phase (SP) to separate saccharides, with the mobile phase comprising acetonitrile (ACN) and water. Utilizing ACN and water as the eluent, Palmer investigated saccharides on amine-based columns in that same year and came to a similar conclusion. Other researchers, before Alpert’s work in 1990, had shown that HILIC as a separation method had been proven.<sup>[1]</sup>

Dr. Andrew Alpert, a pioneer in chromatographic science, first introduced the term Hydrophilic Interaction Liquid Chromatography (HILIC) in his seminal 1990 publication.

He described HILIC as a liquid chromatographic mode in which a polar stationary phase is used together with a mostly organic, water-restricted mobile phase. Under these conditions, a thin layer of water is retained on the surface of the stationary phase, creating a partitioning interface in which polar analytes interact strongly with the immobilized aqueous layer. This interaction increases the retention of hydrophilic compounds, making HILIC particularly suitable for polar and ionic analytes. Alpert originally positioned HILIC as an extension of normal-phase chromatography and developed several hydrophilic, neutral, silica-based stationary phases—such as polyhydroxyethyl and polysuccinimide phases—which significantly expanded the applicability of LC for highly polar molecules.<sup>[2]</sup>

Each with different molecular structure and charge, the polar metabolome consists of cationic, anionic, zwitterionic, and somewhat polar chemicals. Many physiological processes depend on these polar metabolites, which can also serve as health or disease markers. As a result, metabolomics, clinical chemistry, and biochemistry are now quite important areas of study as they examine polar metabolites. Because of its great sensitivity, accuracy, and unequalled flexibility, mass

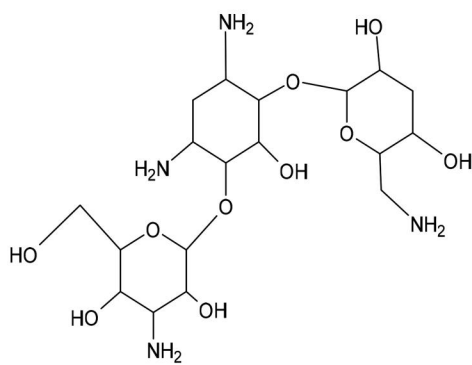
spectrometry (MS) is the most often used way to look at the polar metabolome. MS is commonly combined with a separation method such as HILIC in polar metabolome analysis, which mostly distinguishes chemicals depending on their hydrophilic properties.<sup>[3,4]</sup>

Using enriching methods to enhance difference and analysis of rather hydrophilic analytes has become increasingly important in recent years, especially in non-targeted analysis for thorough profiling of cell or tissue proteomes or glycomes.<sup>[5]</sup> One method to increase the resolution of polar analytes in RP columns is ion-pairing chromatography. This changes the retention of polar species by adding ion-pairing agents to mobile phase (MP), which act as a chemical link among stationary phase and polar species.<sup>[6]</sup> But MS analysis can show ionic suppression if an ion-pairing agent is present. HILIC is another commonly used method to separate these analytes by use of hydrophilic interactions among analytes and stationary phase.<sup>[7]</sup> This approach relies on hydrophilic interactions, like those with polysulfoethyl A or TSK gel Amide 80, to keep analytes on stationary phase. Highly polar or hydrophilic compounds are best kept in this way. Eluting these analytes based on raising mobile phase's polarity—for instance, using water. Many validations for this technique have come from separation of polar compounds, including carbohydrates and polar peptides, including glycopeptides and phosphopeptides. While HILIC can distinguish somewhat polar compounds, it is usually not appropriate for separating hydrophobic peptides. One might use either stand-alone RP or HILIC to administer two distinct injections of the same sample to solve this problem; however, twice the sample amount would be needed for examination.<sup>[8]</sup>

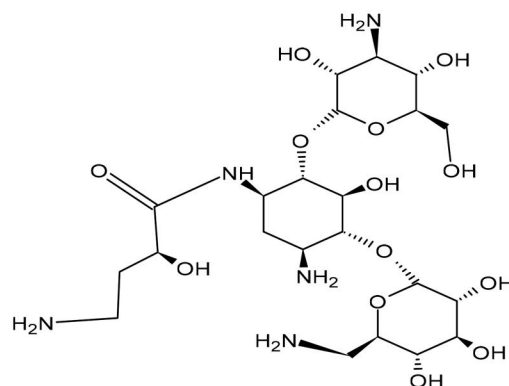
In ion exchange chromatography (IEC), analyte ions or highly polar compounds generate interactions among ions or electrostatic bonds with a stationary phase that has the opposite

charge. Cation-exchange and anion-exchange are 2 main kinds. Negatively charged SP and positively charged analytes may interact during cation exchange. However, in anion exchange, a positively charged SP interacts with negatively charged analytes. The analyte's overall electrostatic charge, charge density, as well as surface charge distribution, all have a significant influence on retention mechanism. The ionic strength of mobile phase eluent is typically increased to facilitate movement of charged analytes that interact ionically with SP during chromatographic separation.<sup>[9]</sup> For polar compound separation, HILIC is an alternative high-performance liquid chromatography (HPLC) technique. HILIC has been seen as a version of NP liquid chromatography in past, although separation process is more sophisticated than that of NP-LC.<sup>[10]</sup>

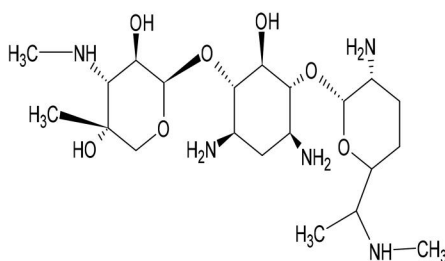
This review article emphasizes the recent developments in HILIC, a strong analytical method commonly used to separate polar aminoglycoside antibiotics. The article will look at the newest changes in HILIC column technology, mobile phase strategies, and ways to make analytical performance better. After this, we will go through the many ways that HILIC can be used, especially in pharmaceutical analysis, metabolomics, and bioanalytical areas. The advantages and constraints of HILIC will be underlined by means of a comparative study with other chromatographic methods, including RP, IEX, and NP Chromatography. Additionally, this review summarizes the major findings from recent studies and highlights the ongoing advancements in HILIC for the analysis of highly polar compounds such as aminoglycosides. The review concludes by outlining potential future developments and methodological improvements that could further enhance the reliability and applicability of HILIC in contemporary analytical science.



Kanamycin



Amikacin



Gentamycin

- The three structures illustrate key differences in functional groups and molecular frameworks, which influence their physicochemical properties and analytical behavior.
- These structural variations directly impact chromatographic retention, ionization efficiency in MS/MS, and overall detectability under HILIC conditions.
- The presence and position of amino and hydroxyl groups contribute to the compounds' high polarity, making them suitable candidates for HILIC-MS/MS analysis.
- The structural similarity among the three molecules highlights the inherent difficulty in achieving adequate chromatographic separation without optimized methods.
- Comparing these structures supports the rationale for developing tailored HILIC-MS/MS parameters to enhance selectivity and sensitivity.

### Scope and literature search strategy

Aim of this review focuses on comparing Hydrophilic Interaction Chromatography (HILIC) with traditional chromatographic techniques—including reversed-phase (RP), normal-phase (NP), and ion-exchange chromatography (IEX)—for the analysis of aminoglycoside antibiotics across pharmaceutical, clinical, food, and environmental matrices.

A structured literature search was conducted using the following scientific databases: PubMed, ScienceDirect, Scopus, Web of Science, and Google Scholar. Publications from 2004 to 2024 were considered to capture recent analytical advancements. The following keywords and combinations were used:

- “Aminoglycosides chromatography”
- “HILIC separation aminoglycosides”
- “Hydrophilic interaction chromatography + antibiotics”
- “Ion-exchange chromatography aminoglycosides”
- “Reversed-phase chromatography polar antibiotics”
- “LC-MS/MS aminoglycoside analysis”
- “Zwitterionic columns HILIC”
- “Chromatographic challenges of polar pharmaceuticals”

Additional references were identified from manufacturers' application notes (e.g., Shimadzu, Waters, Thermo Fisher Scientific) and cross-referencing relevant review articles. Only peer-reviewed studies and validated applications describing separation, sample preparation, or MS-based detection of aminoglycosides were included.

### Recent advancement in HILIC for analysis of polar pharmaceuticals

Improving the peak shape of basic, neutral, and acidic medicinal and chemical solutes in HILIC has been under focus in recent methodological research. Peak shape in HILIC is affected by several factors, including the variety of MPs across a broad pH range, composition and volume of injection solvent, and utilizing salt buffers in MP<sup>10</sup>. Different pH and ionic strengths of buffers influence balance between hydrophilic and ionic retention processes, so influencing

peak shape. For instance, ionic strength of formic acid in a high percentage of ACN is fairly low, which can reduce production of water layer on SP. On the other hand, ammonium ions tend to encourage water layer formation, potentially obscuring ionic interaction effects. Consequently, in formic acid, the peak form for ionizable solutes—especially basic substances—was much worse than in ammonium formate.<sup>[11]</sup> At same water pH levels, ammonium formate exhibited markedly different selectivity on a silica column in contrast to strong acids comprising TFA (trifluoroacetic acid) and HFBA (heptafluorobutyric acid) ammonium salt buffer. TFA and HFBA exhibited constrained pH range in water solutions (w/w pH 1.9–2.8), yet demonstrated an expanded range of true thermodynamic pH in 90% ACN (ss pH 2.4–5.2), which might provide marginally enhanced ionic strength; in contrast, ammonium salt buffers keep significantly greater ionic strength and yield superior peak shape. Although, when analyzing basic substances on a hybrid bare silica phase, a mismatch among injection solvent (95-0% ACN, v/v) and MP (kept at 95% ACN, v/v) led to a greater degradation in peak shape; this effect has stronger than that of increasing injection volume.<sup>[12,13]</sup>

### Development of new HILIC stationary phase

HILIC SP are typically divided in two main categories: silica-based and polymer-based. Silica-based phases are mainly employed and categorized through their chemical properties, including neutral (e.g., amide or diol), positively charged (e.g., amine or triazole), negatively charged (e.g., polysuccinimide or bare silica), as well as zwitterionic phases, or through their structural configurations, including fully porous, superficially porous (commonly referred to as core-shell), and monolithic silica. Numerous earlier analyses have extensively discussed most HILIC SPs, emphasizing the significance of creating new SPs to enhance and implement the HILIC method.<sup>[14]</sup> Majority of new columns have been derived from silica SPs, with click chemistry being a favored approach for modification. “Click chemistry” involves reactions that are quick, highly selective, adaptable, or produce high yields when involving 2 molecular elements.<sup>[15]</sup>

A variation of Huisgen 1,3-dipolar cycloaddition, the copper-catalyzed azide-alkyne cycloaddition (CuAAC) creates 1,4-disubstituted<sup>[1–3]</sup>-triazoles from terminal alkyne and an aliphatic azide when copper is present. Utilized in many different disciplines, this reaction is essential to click chemistry. A new zwitterionic HILIC SP known as click lysine SP (CLSP) has been created through covalently bonding L-azido lysine to silica using CuAAC process. Later, this new SP had been applied to separation of hydrophilic and polar substances, including carbapenems, organic acids, and cephalosporins. The analysis of the retention mechanism indicated that CLSP may encompass partitioning, hydrogen bonding interactions, and electrostatic interactions, among other elements.<sup>[16,17]</sup>

### **Development of new HILIC mobile phases**

The composition of MP in HILIC is fundamentally equivalent to that in standard RP HPLC, often comprising a blend of water and organic solvents like methanol, ACN, tetrahydrofuran, isopropanol, or ethanol. Nevertheless, influence of the MP composition on retention in HILIC differs markedly from its effect in RP HPLC. SP is less hydrophilic than MP in HILIC, while MP is more hydrophilic in RP HPLC. HILIC exhibits significantly more hydrophilic interactions than RP HPLC, such as IEX or hydrogen bonding among analytes and the SP. This is evident when homologues with similar or identical polar functional groups—like sucrose, raffinose, and stachyose—are separated on an HILIC column.<sup>[18]</sup>

A significant advantage of HILIC separation phase has been enhanced signal in MS, which is ascribed to the highly organic mobile phases' more efficient desolvation. This happens because numerous prevalent organic solvents, such as ACN, have a lower surface tension and density than water, which improves the effectiveness of desolvation. In ESI, the vapor pressure of the solvent also affects the rate of solvent evaporation from droplet surface, favoring organic solvents that are more volatile than water. However, irrespective of the column chemistry, the majority of strong bases are better ionized at pH3. On amide column, XICs for 2 compounds with positive ESI-MS and 2 mixtures with negative ESI-MS has been illustrated. These results, in combination with retention data, indicate that the majority of compounds examined by HILIC will ionize (low pH for bases, high pH for acids) to obtain maximal retention and MS signal response.<sup>[19,20]</sup>

### **Analytical challenges of aminoglycosides**

#### **Lack of chromophoric groups and detection challenges**

Aminoglycoside antibiotics, including gentamicin, amikacin, and tobramycin, inherently lack strong chromophores, which makes them difficult to detect using standard UV detectors without first undergoing chemical derivatization.<sup>[20]</sup> Their primary and secondary amine groups do not absorb significantly in the UV-visible spectrum, necessitating the use of derivatizing agents like FMOC-Cl, OPA, or dansyl chloride to enhance detection. This process adds complexity, time, and potential variability to the analytical procedure. While derivatization improves UV response, it also poses the risk of incomplete reactions or degradation, which can lead to inaccurate quantification.<sup>[3]</sup>

#### **High polarity and ionizability**

Aminoglycosides are highly polar and possess multiple ionizable amino groups, which pose difficulties in traditional reversed-phase chromatographic systems. These compounds interact strongly with water and weakly with hydrophobic stationary phases, resulting in minimal retention and poor resolution in RP-HPLC unless ion-pairing agents are used. This polarity also leads to peak tailing and poor peak shape

because of strong interaction with residual silanol groups on column. HILIC and ion exchange chromatography have shown improved performance in retaining and separating these compounds due to their ability to handle charged, polar analyte.<sup>[3,20]</sup>

### **Sample preparation complexity**

Due to their high aqueous solubility and lack of volatility, aminoglycosides often require a sample-preparation procedure before chromatographic analysis. In biological matrices, techniques such as protein precipitation, solid-phase extraction (SPE), and liquid-liquid extraction (LLE) are commonly employed to reduce matrix effects in plasma, serum, or urine. These procedures must be carefully optimized to prevent analyte loss, as aminoglycosides readily bind to matrix proteins or may co-elute with other highly polar metabolites. In pharmaceutical formulations, excipients and co-formulated active ingredients can further interfere with detection if insufficient sample cleanup is performed.<sup>[21-23]</sup>

### **Stability issues in biological matrices**

Aminoglycosides may undergo degradation or bind to components in biological matrices, affecting their stability during storage and analysis. Their tendency to interact with proteins and nucleic acids can alter recovery and reproducibility unless handled under controlled conditions (e.g., low temperature, appropriate pH). Freeze-thaw cycles, prolonged storage, and enzymatic activity in biological samples can contribute to degradation or chemical transformation of the analytes. Therefore, stability studies and proper storage conditions are essential parts of method validation for accurate quantification in pharmacokinetic or therapeutic drug monitoring studies.<sup>[24,25]</sup>

### **Limitations of conventional detection methods**

Standard detection techniques like UV and fluorescence are often inadequate for aminoglycoside analysis due to poor sensitivity and specificity. While derivatization can address UV limitations, it is not ideal for high-throughput or MS-based workflows. More advanced detection methods, including evaporative light scattering detection (ELSD), pulsed amperometric detection (PAD), or tandem mass spectrometry (MS/MS), have become preferred choices.<sup>[26]</sup> These detectors can quantify aminoglycosides without derivatization and offer higher sensitivity and selectivity, especially when coupled with HILIC or ion exchange separation methods. However, the adoption of such techniques requires specialized equipment and expertise.<sup>[26]</sup>

### **Comparative analysis of chromatographic techniques for aminoglycoside analysis**

Because of their strong polarity and polycationic properties, aminoglycoside antibiotics require particular chromatographic

methods for proper separation and measurement. Among the several approaches now accessible, Normal Phase (NP) Chromatography, HILIC, RP-HPLC, and IEX are usually under review. Regarding retention capacity, HILIC is excellent for aminoglycosides.<sup>[27]</sup> This is ascribed to the polar interactions and hydrogen bonding among analyte and SP, which renders it especially successful for compounds missing UV chromophores. In contrast, RP-HPLC usually shows inadequate retention for aminoglycosides unless derivatization or ion-pairing additives are used, therefore complicating method development and interfering with detection. Through electrostatic interactions among positively charged amines of antibiotics and negatively charged stationary phase, IEX—especially cation-exchange chromatography—also effectively keeps aminoglycosides. Normal Phase Chromatography (NPC) usually lacks the selectivity required for the complicated matrices in which aminoglycosides are tested, and offers just limited retention.<sup>[4,27,28]</sup>

Another key consideration in choosing a chromatographic approach for aminoglycoside analysis is mass spectrometry (MS) compatibility. HILIC scores the best in this regard since it uses volatile mobile phases, including acetonitrile with ammonium formate or acetate buffers, which are totally MS-compatible. RP-HPLC, while also utilized with MS, exhibits modest to great compatibility depending on the particular technique circumstances, as it experiences signal suppression when ion-pairing agents are used. IEX mostly uses nonvolatile buffers like phosphate or sulfate, which aren't great for MS detection unless a lot of desalting or buffer changing is done. Normal Phase Chromatography's dependence on nonpolar solvents like hexane or isopropanol, which are challenging to evaporate and can harm MS sources, makes it naturally incompatible with MS.<sup>[29–31]</sup>

Regarding derivatization, HILIC has a significant advantage since it enables direct injection and detection of underivatized aminoglycosides, therefore, saving time and lowering possible mistakes in sample handling. On the other hand, RP-HPLC sometimes calls for derivatization with chemicals like *o*-phthalaldehyde (OPA) or FMOc to allow UV or fluorescence detection, that are time-consuming or could compromise quantification accuracy. While generally not requiring derivatization, IEX techniques depend more on post-column derivatization or indirect detection methods, especially for non-chromophoric molecules. NPC occasionally calls for derivatization to increase solubility or detection; however, owing to other intrinsic limits, it is less often used for aminoglycoside analysis.<sup>[28,32,33]</sup>

These approaches likewise differ greatly in composition of MP. Usually utilizing MP high in acetonitrile (up to 90%) with a lower amount of aqueous buffer, HILIC improves polar analyte retention while still being MS-compatible. When analyzing aminoglycosides, RP-HPLC techniques make use of aqueous buffers containing organic modifiers such as methanol or acetonitrile, sometimes with extra ion-pairing agents. IEX chromatography uses buffered aqueous mobile phases with carefully regulated pH and ionic strength to maximize ion exchange interactions. Often nonvolatile, these buffers need a lot of method tweaking. Normal phase

chromatography uses nonpolar mobile phases like hexane or chloroform, which makes it less environmentally friendly and not very good for biological samples.<sup>[28]</sup>

Another consideration separating these approaches is reproducibility. While older generations of columns were more likely to drift, HILIC offers great reproducibility when using current column techniques and appropriate equilibration. With decades of method validation and extensive use in pharmacopeial operations, RP-HPLC still holds the gold standard for repeatability. Although they are fairly reproducible, IEX techniques are extremely sensitive to changes in pH and buffer concentration, which might influence resolution and retention timings. Normal Phase Chromatography exhibits the least repeatability, frequently affected by variations in stationary phases and environmental moisture across batches that might arbitrarily change retention behavior.<sup>[27,34,35]</sup>

Sample preparation also varies across techniques. HILIC allows for relatively straightforward sample introduction, with many methods accommodating direct injection of diluted biological matrices such as plasma or urine. RP-HPLC typically requires extensive sample cleanup and derivatization to overcome matrix interferences and improve retention. IEX methods often need pretreatment steps such as solid phase extraction (SPE) to remove interfering ions or proteins. NPC involves challenging sample preparation due to solubility limitations of polar analytes in nonpolar solvents and the risk of overloading the column, making it the least preferred method for routine aminoglycoside analysis.<sup>[36,37]</sup> The analysis results are summarized in Table 1, providing a comprehensive overview of the key findings and insights.

### Summary of chromatographic methods for aminoglycoside analysis

The analysis results are summarized in Table 2.

The summarized methods demonstrate a clear trend toward the increased use of HILIC-MS/MS for aminoglycoside analysis across biological, food, and pharmaceutical matrices. Unlike reversed-phase chromatography, which typically requires ion-pairing reagents or derivatization to retain highly polar aminoglycosides, HILIC provides strong and reproducible retention without compromising MS compatibility. Normal-phase and ion-exchange techniques remain useful but often exhibit longer run times, require extensive sample cleanup, or lack selectivity. Zwitterionic and diol-type HILIC phases show particularly good performance, supporting their growing adoption in regulatory and high-throughput workflows.<sup>[28,38]</sup>

### Applications of HILIC in analysis of aminoglycoside antibiotics

HILIC offers exceptional utility in analyzing aminoglycoside antibiotics, particularly due to its strong retention of highly polar molecules without requiring ion-pairing agents.

**Table 1.** Comparative analysis of chromatographic techniques for aminoglycoside analysis.

Aminoglycoside	Technique	HILIC Column/mode	Matrix	Detection	Key advantages	Recent reference (year)
Gentamicin (C1/C1a/C2)	HILIC	ZIC-HILIC (zwitterionic)	Milk, animal muscle	LC-MS/MS	Strong retention; excellent MS response; no derivatization	Diez et al., <i>Anal. Chim. Acta</i> (2015)
Gentamicin (non-derivatized)	UPLC-HILIC	Porous graphitic carbon (PGC-HILIC)	Pharmaceutical	LC-MS	Fast, high-resolution separation of C-components	Rodriguez et al., <i>Anal Bioanal Chem</i> (2015)
Amikacin	HILIC	Diol-HILIC	Human plasma	UPLC-QTOF-MS	High sensitivity; minimal matrix effect	Minohara et al., 2021
Amikacin	UHPLC-HILIC	Zwitterionic mixed-mode	Porcine tissue	LC-MS/MS	Multi-residue capability (15 aminoglycosides)	Sun et al., 2023
Kanamycin A	HILIC	Click-TE-Cys zwitterionic	Food	LC-MS/MS	Highly selective separation; stable retention	Wei et al., <i>J. Sep. Sci.</i> (2014)
Kanamycin & Neomycin	HILIC	ZIC-HILIC	Honey & milk	LC-MS/MS	High recoveries; good ionization	Nowacka-Kozak et al., <i>Molecules</i> (2023)
Tobramycin	HILIC	Diol-HILIC	Plasma	UPLC-QToF-MS	Better than ion-pair RP-LC; no derivatization	Minohara et al., 2021
Neomycin B	HILIC + MISPE	ZIC-HILIC	Milk-based foods	LC-MS/MS	Molecularly imprinted polymer extraction improves selectivity	Moreno-González et al., <i>Talanta</i> (2017)
Streptomycin	HILIC	Silica-HILIC	Animal tissues	LC-MS/MS	Good separation; MS-friendly	Kahsay et al., <i>J Pharm Biomed Anal</i> (2014)
Apramycin	HILIC	Silica-amide HILIC	Poultry meat	LC-MS/MS	High retention; stable MS response	European Food Safety Authority Method, 2021
All major aminoglycosides (13 analytes)	HILIC (Regulatory method)	Zwitterionic HILIC	Meat, milk, eggs	LC-MS/MS	Rapid (<5 min) multi-residue screening	Shimadzu EU Method Package, 2023
15 aminoglycosides (multi-residue)	HILIC	ZIC-HILIC	Porcine tissues	LC-MS/MS	Matrix-robust, highly selective	Wang et al., 2023

**Table 2.** Summary of chromatographic methods for aminoglycoside analysis.

Feature	HILIC	RP-HPLC	IEX	Normal Phase Chromatography (NPC)
Retention capacity	Excellent (polar interactions, H-bonding)	Inadequate (unless derivatization/ion-pairing used)	Effective (electrostatic interactions with positively charged amines)	Limited (lacks selectivity for complex matrices)
MS Compatibility	Best (volatile mobile phases: acetonitrile with ammonium formate/acetate buffers)	Modest to good (depends on technique, signal suppression with ion-pairing agents)	Poor (nonvolatile buffers like phosphate/sulfate, requires desalting)	Naturally incompatible (nonpolar solvents challenging to evaporate, harm MS sources)
Derivatization	No derivatization required (direct injection and detection)	Often required (e.g., OPA, FMOC for UV/fluorescence detection), time-consuming, potential for errors	Generally not required, but often relies on post-column derivatization or indirect detection	Occasionally required (to increase solubility/detection), less common for aminoglycosides
Mobile Phase (MP)	High acetonitrile (up to 90%) with aqueous buffer	Aqueous buffers with organic modifiers (methanol/acetonitrile), sometimes with ion-pairing agents	Buffered aqueous mobile phases with controlled pH and ionic strength	Nonpolar mobile phases (e.g., hexane, chloroform)
Reproducibility	Great (with current column technologies and proper equilibration)	Gold standard (decades of method validation, extensive pharmacopeial use)	Fairly reproducible (sensitive to pH/buffer concentration changes)	Least repeatable (affected by variations in stationary phases and environmental moisture)
Sample preparation	Relatively straightforward (direct injection of diluted biological matrices)	Extensive cleanup and derivatization often required (overcome matrix interferences, improve retention)	Often needs pretreatment (e.g., SPE to remove interfering ions or proteins)	Challenging (solubility limitations, risk of overloading column), least preferred for routine analysis

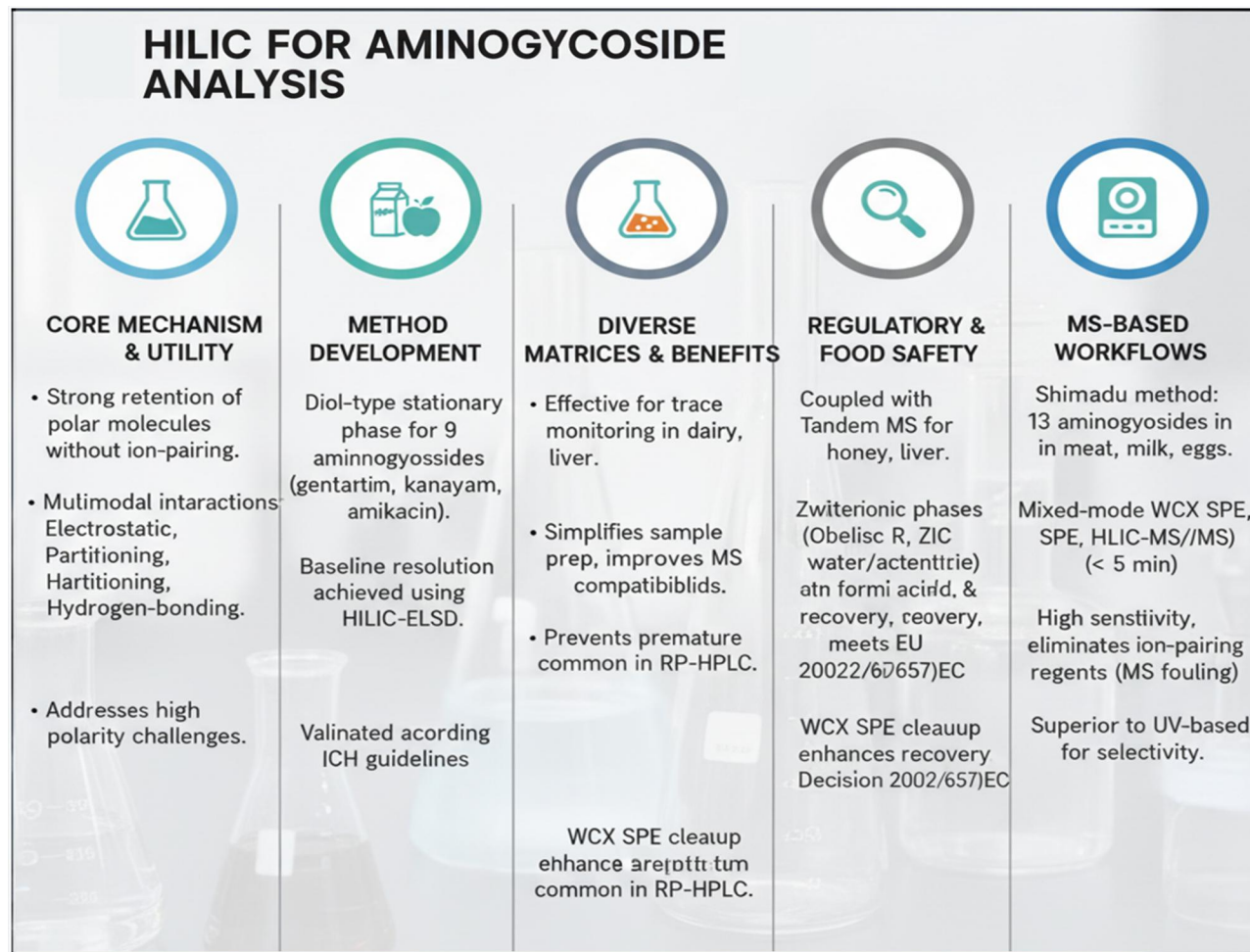
A diol-type stationary phase was effectively used in an HILIC-ELSD method to separate nine aminoglycosides, including gentamicin, kanamycin, and amikacin, with complete baseline resolution and validation according to ICH guidelines. Retention was driven through multimodal mechanisms comprising electrostatic, partitioning, as well as

hydrogen-bonding interactions, highlighting HILIC's ability to address separation challenges posed by aminoglycosides' high polarity.<sup>[38,39]</sup>

The effectiveness of HILIC extends across diverse sample matrices such as dairy, food, and biological fluids, where aminoglycoside residues must be monitored at trace levels.

**Table 3.** Applications of HILIC in analysis of aminoglycoside antibiotics.

Application	Sample/matrix	Analytical purpose	Detection	References
Pharmaceutical quality control	Bulk drugs and formulations	Separation and quantification of aminoglycosides without ion-pairing reagents	ELSD	Alpert et al.
Multi-residue food safety analysis	Milk, honey, liver	Determination of trace-level aminoglycoside residues for regulatory compliance	LC-MS/MS	Kaufmann et al.
Veterinary drug residue monitoring	Meat, milk, eggs	Simultaneous determination of multiple aminoglycosides	LC-MS/MS (MRM)	Shimadzu et al.
Dairy and food product testing	Dairy products and processed foods	Sensitive analysis of highly polar aminoglycosides	LC-MS/MS	Mol et al.
Bioanalytical comparative studies	Standard mixtures	Comparison of HILIC and ion-pair RP chromatography	UV / MS	Kato et al.

**Figure 1.** Applications of HILIC in analysis of aminoglycoside antibiotics.

A comprehensive review noted that HILIC is advantageous for high-polarity antibiotics because it eliminates the need for ion-pairing reagents, simplifies sample preparation, and improves compatibility with mass spectrometry detection. This makes HILIC particularly suitable for samples where reversed-phase typically fails due to premature elution in the void volume.<sup>[13]</sup>

In food safety and regulatory monitoring, HILIC attached with tandem mass spectrometry has been extensively utilized to detect aminoglycosides in milk, honey, and liver. For example, zwitterionic stationary phases (Obelisc R, ZIC-HILIC) allowed efficient multi-analyte separation using

water/acetonitrile mobile phases with formic acid. A sample cleanup protocol using polymeric weak cation-exchange (WCX) SPE cartridges enhanced recovery and reduced matrix effects, enabling compliance with EU regulatory requirements such as Decision 2002/657/EC.<sup>[40]</sup>

Shimadzu's method package for simultaneous quantification of thirteen aminoglycosides in meat, milk, and eggs demonstrates HILIC's versatility in residue analysis. Utilizing mixed-mode weak cation-exchange SPE for sample cleanup and fast (< 5 min) HILIC-MS/MS with two MRM transitions per compound, the method achieved high sensitivity and eliminated need for ion-pairing reagents that often

foul MS instruments. Second confirmation mode with expanded MRMs further enhances identification confidence in regulatory applications.<sup>[30]</sup>

A study comparing HILIC with ion-pair reversed-phase chromatography (IP-LC- Ion-Pair Liquid Chromatography) for aminoglycosides under UV detection revealed that while HILIC exhibited limited selectivity for five tested antibiotics—particularly amikacin and kanamycin—IP-LC with borate complexation and octanesulfonate pairing offered resolution for some compounds. However, HILIC's utility improves dramatically when coupled with MS detection. This illustrates that HILIC's true strength lies in MS-based workflows rather than UV-based systems, reinforcing its growing adoption in bioanalytical contexts.<sup>[13,40]</sup> Table 3 and Figure 1 indicate the applications of HILIC.

### **Comprehensive summary of aminoglycoside evaluation via HILIC and alternative chromatographic methods**

#### **(A) Pharmaceutical contexts**

Analysis of gentamicin components in raw drug materials stability-indicating techniques for amikacin and tobramycin Impurity profiling without derivatization using HILIC-MS/MS.

#### **(B) Clinical and biomedical contexts**

Therapeutic drug monitoring in plasma/serum UPLC-QToF analysis for swift identification of aminoglycosides Quantification in micro dialysis and urine samples.

#### **(C) Food and veterinary contexts**

Screening and confirmatory LC-MS/MS methods for detecting residues in milk, meat, egg, and fish EU regulatory compliance (Decision 2002/657/EC)—Zwitterionic HILIC phases for multi-residue analysis.

#### **(D) Environmental and industrial contexts**

Detection of aminoglycoside contaminants in water environments and Monitoring fermentation media during antibiotic production.

### **Future outlook of HILIC**

#### **HILIC-MS in complex matrix analysis**

In the quantitation of aminoglycosides in complex biological or fermentation media, HILIC-MS methods have shown remarkable sensitivity and robustness while avoiding derivatization. Optimized a HILIC-LC-MS assay for spectinomycin and kanamycin in fermentation broth, achieving calibration down to 0.01 µg/mL for spectinomycin and 1 µg/mL for kanamycin using SPE cleanup with strong cation-exchange sorbents. This method, validated per ICH standards, demonstrated excellent linearity, precision, recovery, and minimal matrix effects, highlighting HILIC-MS's

suitability for trace-level detection in complex aqueous matrices.<sup>[30]</sup>

#### **HILIC at elevated pH for therapeutic drug monitoring**

High-pH mobile phases in HILIC workflows have proven effective for quantifying aminoglycosides without derivatization, making them suitable for therapeutic drug monitoring (TDM). A robust LC-MS/MS approach developed for amikacin in cellular uptake and pharmacokinetic research employed ammonium hydroxide to increase mobile-phase pH, thereby improving retention by reducing ionization and promoting stronger interaction with the stationary phase. This streamlined approach eliminated derivatization and avoided ion-pair reagents, yielding high specificity and sensitivity across cell lysate and serum matrices.<sup>[41]</sup>

#### **Zwitterionic HILIC columns for multi-residue screening**

Zwitterionic HILIC stationary phases have emerged as the preferred choice for high-throughput multi-residue screening of aminoglycosides across food and animal matrices. Waters' Atlantis Premier BEH Z-HILIC column, combined with HILIC-MS/MS and mixed-mode weak cation-exchange SPE cleanup, enables simultaneous detection of up to 13 aminoglycosides (e.g., gentamicin C1-C2a, kanamycin, neomycin) in under five minutes per sample, without using ion-pairing agents. This method improves sensitivity, identification confidence (with MRM spectrum mode), and workflow efficiency in regulatory residue testing.<sup>[42]</sup>

### **Summary and conclusion**

Analysis of aminoglycoside antibiotics remains significant analytical challenge because of their structural characteristics, which include high polarity, lack of chromophores, and variable ionization properties. This has led to the development and comparison of several chromatographic techniques over the years. Among these, HILIC has emerged as highly effective alternate, especially when comparing traditional RP, IEX, and NP chromatography methods.

Reversed Phase Chromatography, though widely used, suffers from poor retention of aminoglycosides due to their polar nature. The common workaround involves derivation or utilization of ion-pairing agents—both of which increase sample preparation time and risk MS source contamination. Conversely, Ion Exchange Chromatography offers good retention and selectivity for charged aminoglycosides, but the requirement for nonvolatile buffers (e.g., phosphate or sulfate) reduces its MS compatibility. Furthermore, method development in IEX is often matrix-dependent and less flexible across applications. Normal Phase Chromatography, though theoretically applicable, has largely fallen out of favor due to reproducibility issues and limited compatibility with aqueous samples.

In contrast, HILIC addresses many of these limitations. Ability of HILIC to retain highly polar mixtures without derivatization, along with its compatibility with volatile

buffers and MS detection, makes it particularly valuable in modern pharmaceutical and food safety testing laboratories. Studies show that HILIC-MS/MS offers lower limits of detection (LODs), faster analysis time, and simpler sample preparation—especially when used with SPE (solid-phase extraction) cleanup protocols like WCX (weak cation exchange) cartridges.

One particularly promising area is application of zwitterionic stationary phases in HILIC, for instance BEH Z-HILIC and Click TE-Cys columns. These phases combine strong retention with high peak symmetry and low carryover for aminoglycosides like kanamycin, gentamicin, and spectinomycin. Moreover, newer developments, such as high-pH mobile phases and mixed-mode columns, allow selective tuning of retention and improve method flexibility without compromising MS performance.

Importantly, the comparative assessment in this review suggests that while RP and IEX methods still have niche roles in QC and regulatory environments, HILIC offers the best all-around performance for multi-residue screening, therapeutic drug monitoring, and pharmacokinetic profiling of aminoglycosides. However, challenges remain in terms of method transferability, retention mechanism complexity, and optimization of mobile phase composition. As column chemistries and MS detection technologies continue to evolve, HILIC will likely become even more dominant in aminoglycoside analysis in the years to come.

HILIC has been recognized as possessing strong and adaptable technique for analyzing aminoglycoside antibiotics, especially due to its high selectivity for polar compounds and excellent compatibility with mass spectrometry. Compared to RP, IEX, and NP Chromatography, HILIC offers superior retention and separation of aminoglycosides without requiring derivatization or ion-pairing agents, thereby simplifying the analytical workflow and enhancing detection sensitivity in complex biological or food matrices. Recent advancements, including the development of novel zwitterionic and click chemistry-based stationary phases, have further refined HILIC's performance, enabling high-throughput and multi-residue analysis within short run times and minimal sample preparation.

The challenges historically associated with aminoglycoside analysis—such as poor UV absorption, high polarity, and instability in biological matrices—are now effectively addressed through optimized HILIC-MS/MS Systems. Innovations such as elevated-pH mobile phases, polymer-based sorbents for cleanup, and the use of volatile buffers have significantly enhanced reproducibility, ionization efficiency, and quantitation accuracy. These improvements underscore the growing relevance of HILIC in pharmaceutical quality control, clinical therapeutic drug monitoring, and food safety testing.

Comparative Advantages of HILIC over RP, NP, and IEX for Aminoglycosides

- Reversed-phase(RP):
- Aminoglycosides exhibit minimal retention due to high polarity. Ion-pairing reagents (e.g., HFBA, TFA) improve

retention but reduce MS sensitivity and contaminate the system.

- Normal-phase(NP):
- Offers retention but uses non-aqueous mobile phases incompatible with biological samples. Poor reproducibility and limited robustness.
- Ion-exchange(IEX):
- Provides strong retention but requires high-salt buffers, which are problematic for MS. Salt removal steps increase sample-prep workload.
- HILIC (Advantage):
  - Excellent retention of hydrophilic analytes
  - High organic content promotes MS ionization
  - No derivatization or ion-pairing reagents required
  - Good peak shape with zwitterionic and diol stationary phases
  - Suited for complex matrices (milk, plasma, food, fermentation media)

HILIC allows direct injection of samples extracted into aqueous-organic mixtures and provides better method transferability across labs compared to ion-pair LC and NP modes. Recent research highlights that the choice of stationary phase strongly influences aminoglycoside separation behavior. Zwitterionic HILIC columns show enhanced selectivity for structurally similar aminoglycosides such as gentamicin C1, C1a, and C2. Meanwhile, mixed-mode HILIC-ion exchange materials offer additional retention mechanisms that help resolve closely eluting impurities. These developments indicate a shift toward hybrid stationary phases and MS-compatible workflows that minimize sample preparation requirements.

Looking ahead, integration of HILIC with advanced detection techniques like high-resolution MS and machine learning-assisted data interpretation is expected to revolutionize bioanalytical workflows. Continuous evolution in column chemistries and mobile phase compositions will further expand HILIC's applicability to a broader range of analytes, offering new possibilities for the high-sensitivity quantification of trace-level antibiotics in diverse matrices.

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Sumithra M provided conceptualization, supervision, managed project administration; Santhosh K contributed to literature review, data collection, and primary drafting, manuscript writing and final editing.

## Author contributions

CRedit: **Santhosh K**: Data curation, Formal analysis, Resources, Writing – original draft, Writing – review & editing; **Sumithra M**: Conceptualization, Investigation, Project administration, Supervision, Validation, Visualization.

## Ethical approval

No ethical approval was required for this study. This study did not involve the participation of human subjects, so no ethical approval or informed consent was needed.

## Research involving human and animal rights

The corresponding author affirms, on behalf of all authors, that human and animal rights were upheld in the study. Moreover, this study did not involve humans or animals.

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