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Simultaneous screening and quantification of aminoglycoside antibiotics in honey using mixed-mode liquid chromatography with quadrupole time-of-flight mass spectroscopy with heated electrospray ionization

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An analytical method based on liquid chromatography with quadrupole time-of-flight mass spectrometry has been developed for the simultaneous determination of six aminoglycoside antibiotics in honey. The sample pretreatment included extraction with aqueous trichloroacetic acid followed by solid-phase extraction on Strata-X polymeric reversed phase cartridges. Liquid chromatography separation was performed on an Obelisc R zwitterionic type mixed-mode column. An ionBooster^{TM} heated electrospray source was used and showed enhanced ionization efficiency in comparison to a conventional electrospray source. The observed signal enhancement ranged from 3- (neomycin) to 16-fold (gentamicin C1). A data-dependent mass spectrometry acquisition approach was employed, in which the full mass spectrometry dataset provided quantification and a scheduled precursor list was used to trigger an alternating data-dependent acquisition of MS^2 spectra for confirmation purposes. The described method was validated in accordance to CD 2002/657/EC. Decision limit values were in the range 11.2–33.6 ng/g, and satisfactory performance characteristics were obtained for recovery (65–76%), repeatability (3.8–7.3%), and linearity (≥0.995). The method was applied to the analysis of 49 real honey samples from the country of Georgia. Streptomycin was detected in two samples at 117 and 35 ng/g, and gentamicin C1 was detected in one sample at 32 ng/g.

KEYWORDS

aminoglycoside antibiotics, heated electrospray ionization, honey, mixed-mode liquid chromatography, time-of-flight mass spectroscopy

1 | INTRODUCTION

Aminoglycosides (AGs) are a group of antibiotics either derived from *Streptomyces spp.* (streptomycin, neomycin, and spectinomycin) or *Micromonospora spp.* (gentamicin). This class of compounds is structurally defined by the presence of amino sugars bound by glycosidic linkage to a central hexose nucleus. AGs exhibit a broad spectrum of antimicrobial activity against various microorganisms, including gram-positive and gram-negative bacteria, mycobacteria, and protozoa [1,2], and are thus commonly used in veterinary medicine,

Article Related Abbreviations: AG, Aminoglycoside; ddMS/MS^2, data-dependent multiple reaction monitoring; DSTP, dihydrostreptomycin; DT, drying temperature; GEN, gentamicin C1; IB, IonBooster^{TM} high-temperature electrospray source; KAN, kanamycin; ME, matrix effect; MMSCC, matrix-matched standard calibration curve; MRLs, maximum residue limits; NEO, neomycin; SPC, spectinomycin; STP, streptomycin; TCA, trichloroacetic acid; VT, vaporizer temperature

but can also be applied for the treatment of bacterial infections in apiculture and agriculture, particularly for American and European foulbrood disease and fire blight of pome fruits. Therefore, traces of AGs could be present in honey and honey-based products [3–5]. The European Union has established a legal framework to ensure consumer protection by the means of setting Maximum Residue Limits (MRLs) for authorized substances. In the case of AGs, MRLs have been established in different matrixes, e.g., muscle tissue, kidneys, and milk, however the use of AGs is not authorized for the treatment of honey bees. Thus, there are no MRLs set by the EU in Commission Regulation No 37/2010 for the presence of AGs in honey and, the so-called "zero tolerance" level applies. Yet, there is no worldwide harmonization between the international laws regulating the use of antibiotics in apiculture, and honey imported from non-European Union countries might pose risks to public health in terms of unauthorized antibiotic residues [6]. The Rapid Alert System for Food and Feed (RASFF) in EU reported several cases in 2015 where honey samples originating from non-EU countries contained residues of streptomycin and dihydrostreptomycin [7].

There is a wide range of analytical methodologies described in the literature for the determination of AGs in products of animal origin, for example, a commonly used method for analysis of streptomycin residues in food involves LC combined with post-column derivatization and fluorometric detection [8]. However, the majority of methods used for this purpose over the past two decades have relied on one particular instrumental technique: HPLC–MS, because AGs are highly hydrophilic non-volatile compounds lacking any chromophore or fluorophore groups, therefore satisfactory method performance is difficult to achieve by other separation and detection techniques [9,10]. Due to the aforementioned properties, AGs are not adequately retained on conventional reversed-phase HPLC columns, unless derivatized. Several authors have reported solutions to this issue by using ion-pairing agents, such as heptafluorobutyric acid (HFBA) [11,12]. However, it has been noted that ion-pairing agents induce ion suppression and long-term usage of HFBA or other fluorinated acids negatively affect the performance of mass spectrometer, hence increasing the overall maintenance costs.

A more favorable approach is the use of HILIC, which is specifically designed to separate highly polar compounds. This technique has been successfully applied for the determination of AGs by Zhu et al. (2008) and has shown superior performance compared to a previously described reversed-phase methodology, as it did not require additional mobile phase additives or laborious derivatization procedures and provided an additional dimension in terms of chromatographic interactions by harnessing the ionic properties of AGs to control the analyte distribution between mobile and stationary phases [13].

As mentioned above, the detection of AGs is usually performed by MS, especially by MS/MS [14]. In addition, recent technological advances have opened new possibilities for applications involving high-resolution mass spectrometry (HRMS) and hybrid HRMS [15,16], combining high resolving power with the well-known advantages of MS/MS. Although highly sophisticated instrumental techniques have been applied by several authors, the reported sensitivity still remains relatively low, largely due to the poor ionization efficiencies of AGs by conventional ESI sources, therefore alternative ionization approaches must be investigated to increase the overall sensitivity, particularly in this case when "zero tolerance" criteria are applied for the determination of unauthorized antibiotics.

The most significant challenge in such analyses is achieving efficient extraction of the target AGs and providing good recovery in successive clean-up steps. Various sample pretreatment techniques are described in the literature but the most commonly used is deproteinization with a protein precipitation agent, such as trichloroacetic acid (TCA), followed by SPE. The sorbent selection in most cases is limited to three types of SPE packings: cation exchange [17–20], hydrophilic–lipophilic interaction sorbents (especially Waters Oasis HLB) [13,21], and cartridges containing molecularly imprinted polymers (MIPs) [22].

The aim of this study was to develop a highly selective and sensitive SPE based HILIC–Q-TOF-ddMS/MS^2 method for the determination of six regulated AGs in honey and to evaluate the advantages of a high-temperature ESI source over conventional ESI in terms of the ionization efficiency of AGs. To the best of our knowledge, this is the first time a Q-TOF-based method is presented that is capable of simultaneous screening and quantification of AGs at low ppb level.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Streptomycin sulfate (STP, 98.0%), neomycin sulfate (NEO, 90.0%), spectinomycin dihydrochloride hydrate (SPC, 98.0%), and gentamicin-2,5-sulfate hydrate (GEN, 96.5%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Kanamycin sulfate (KAN, 99.0%) was obtained from Sigma–Aldrich (St. Louis, MO, USA), and dihydrostreptomycin sesquisulfate (DSTP, 98.0%) was supplied by Fluka (Buchs, Switzerland). Deionized water (18 MΩ cm) was produced by a Milli-Q water-purification system (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile, formic acid (≥98%), trichloroacetic acid (≥98%), sodium hydroxide (≥98%), EDTA disodium salt dihydrate (≥98.5%), and ammonium formate (≥99%) were purchased from Sigma–Aldrich. SPE was performed on Strata-X PRP cartridges (200 mg/3 mL)

obtained from Phenomenex (Torrance, CA, USA). Stock standard solutions were individually prepared by dissolving the appropriate amount of the analytical standard in water (10 mL) to give a final concentration of 1 mg/mL (calculated for base). Working solutions were prepared weekly by diluting each stock standard solution with water to a final concentration of 0.1 and 0.01 mg/mL. For the reason that AGs can be easily adsorbed on such polar surfaces as amber glass, all standard solutions were stored at −18°C in polypropylene tubes instead of glass containers.

2.2 | Samples

A total of forty nine samples of honey were collected from different areas of the country of Georgia during the summer of 2016. An aliquot of approximately 25 mL was taken from each sample, transferred to a 50 mL polypropylene tube and stored at −18°C before the analysis.

2.3 | Sample preparation

Each sample of honey (5.00 ± 0.01 g) was weighed into a 50 mL polypropylene tube. The extraction solution (20 mL of 1% TCA in deionized water) was added, the mixture was vortexed for 1 min and shaken for 10 min using a mechanical shaker. The pH of the mixture was then adjusted to 5.0 ± 1.0 with 1.0 M NaOH solution. After that, the sample was centrifuged at 3500 rpm for 10 min and the supernatant was treated by SPE clean-up procedure using a Strata-X polymeric RP cartridge (200 mg/3 mL). The cartridge was preconditioned sequentially with 3 mL of methanol and 3 mL of deionized water. After loading a 5 mL sample aliquot, the column was washed with 6 mL of water, dried for 5 min under vacuum, and eluted with 3 mL of aqueous 10% formic acid v/v into a 15 mL polypropylene tube. Then 50 µL of 1.0 M ammonium formate solution was added to the final extract, the container was vortexed for approximately 10 s and 250 µL of the final extract was transferred to an HPLC vial.

2.4 | Chromatographic separation

Chromatographic separation was achieved using a Dionex UltiMate 3000 rapid separation LC system (Thermo Scientific, Sunnyvale, CA, USA) comprising a binary high-pressure gradient pump, an autosampler, and a thermostatted column compartment on an Obelisc R (2.1 × 150 mm, 5 µm particle size) analytical column obtained from SIELC Technologies (Prospect Heights, IL, USA). The mobile phase consisted of (A) aqueous 1% formic acid solution v/v, (B) acetonitrile, and (C) water. The flow rate was 0.5 mL/min and the injection volume was 15 µL. The oven and sample compartment temperatures were set to 30 and 14°C, respectively. The following gradient conditions were used: 0–0.5 min, isocratic 0% A, 90% B, 10% C; 0.5–4.5 min, linear increase from 0 to 95% A, linear decrease from 90 to 5% B, linear decrease from 10 to 0% C; 4.5–9.0 min, isocratic 95% A, 5% B, 0% C; 9.0–9.1 min, return to the initial conditions and 9.1–15.0 min post-run equilibration at the initial conditions.

2.5 | Acquisition of mass spectra and data analysis

Mass spectra were acquired using a Compact Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a IonBooster^{TM} (IB) high-temperature ESI source, which also was purchased from Bruker Daltonics. The instrument was equipped with a conventional ESI source for comparison during the method optimization stage. The mass calibration of the Q-TOF instrument was performed before each sequence by direct infusion of aqueous 1 mM sodium formate solution at the flow rate of 2 µL/min. The mass accuracy (≤5 ppm) during the sequence was maintained using the external calibration approach by injecting the aforementioned calibrant once every five samples using a 2 min long isocratic LC method with the initial HPLC conditions (0% A, 90% B, 10% C). The instrument was operated in positive ionization mode by alternating the acquisition between full MS at the *m/z* range of 50–1000 and data-dependent MS^2 spectra of the scheduled precursor list (see Supporting Information Table S1 in supplementary material). All spectra were scanned at the rate of 2 spectra/s, and 3 MS^2 spectra were acquired for each compound with a cycle time of 2 s. The operating parameters of mass spectrometer were optimized as follows: the end plate offset was 500 V, dry gas temperature and flow rate were set at 200°C and 3.0 L/min, respectively. In the case of the conventional ESI source, the capillary voltage was 4500 V and the dry gas flow rate was set at 10 L/min, while for IB, the capillary voltage was reduced to 1000 V and the dry gas flow rate was 3 L/min. The use of IB introduced two extra parameters that were absent in the case of the standard ESI source: the charging voltage and vaporizer temperature that were set at 300 V and 350°C, respectively. For instrument control and data acquisition, otofControl 4.0, HyStar 3.2 (Bruker Daltonics), and Chromeleon Xpress software (Thermo Scientific) were used. DataAnalysis 4.3 software (Bruker Daltonics) was used for post-run mass calibration and processing of the data.

2.6 | Validation study

Validation was performed according to the guidelines laid down by Commission Decision 2002/657/EC. The final version of the method was validated for decision limit (CCα), detection capability (CCβ), linearity, repeatability (RSD_r), within-laboratory reproducibility (RSD_{wR}), and recovery using the in-house validation concept. The experimental

design of the validation and the necessary calculations were performed with InterVAL 3.3.2.4 software (QuoData, Dresden, Germany). Two leading factors were chosen for the validation design: the operator and the distinctive characteristics of sample matrix, as it was possible to divide the targeted sample batch into two separate groups based on their specific traits: low viscosity dark-toned honey and high viscosity light-toned honey. LOQs were established empirically by analyzing a set of spiked honey samples with decreasing concentrations of AGs. The lowest observable concentration with S/N ratio of ≥10 was established as the LOQ. All intra- and interday validation parameters were evaluated by the following experimental procedure: two distinctive honey samples (in random order) were fortified with five different known concentrations of AGs mixture (five replicates per level) by two operators within a period of two days. The sample concentrations were calculated using a method matrix-matched standard calibration curve (MMSCC). To simplify these experiments, the initial study design was slightly modified so that the assignment of operators would not be randomized between the days. Overall, 50 samples including the method matrix-matched calibration standards were analyzed during the validation study. The results of the validation study are summarized in supplementary material (Supporting Information Table S2) and discussed in Section 3.53.5.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of LC

Considering the highly polar nature of AGs and, thus, their poor retention on conventional RP columns, it was decided to use a zwitterionic-type mixed-mode Obelisc R LC column from SIELC Technologies, which has been successfully applied for the analysis of AGs by other authors and has shown superior separation performance due to the unique stationary phase containing cationic groups attached close to the silica surface and anionic groups that are separated by hydrophobic chains [17,23]. The initial optimization experiments were carried out using a binary mobile phase consisting of 1% v/v formic acid in water (A) and 1% v/v formic acid in acetonitrile (B), using gradient conditions previously described by Diez et al. [17]. The elution was performed according to the following program: 0–4 min, 10–95% A; 4–5 min, 95% A; 5–8 min, 10% A. The obtained chromatogram showed satisfactory peak symmetry for all target analytes, but the relative separation between AGs was insufficient and all compounds were eluted in a narrow time interval. To maximize the differences of relative retention times, various gradient conditions were examined. A higher initial portion of acetonitrile and lower gradient slope were shown to produce a slightly better separation, at the same time increasing the peak width and enhanced peak tailing, especially for strongly retained compounds such as NEO and GEN, thus, impaired peak symmetry. As noted before by Alechaga et al. [23], the predominant separation mechanism in this case involved hydrophilic interactions between the stationary phase and AGs, namely, cation exchange occurring at the carboxyl sites of the column. Furthermore, the elution order of AGs correlated with the number of protonation sites for each compound (including STP and DSTP where each guanidine group can bind only one proton), suggesting controlled protonation of the stationary phase as the key for selective separation. For that reason, three mobile phase systems consisting of aqueous 1% v/v formic acid (A), acetonitrile (B), and water (C) were tested to gradually change the mobile phase from neutral to acidic. Thus, at the beginning of the gradient program, interactions between the basic functional groups of AGs and the stationary phase would be favored, while low pH of mobile phase at the end of the gradient program would fully protonate the carboxyl sites and diminish the ion exchange interactions. However, we encountered peak symmetry problems during the optimization procedure (peak tailing, peak splitting and floating retention times) for early eluting compounds, especially for SPC. Based on the findings of Diez et al. [17] where the author noted that ammonium counterions may disrupt the interactions between the charges in the Obelisc R stationary phase, further addition of ammonium formate was examined and showed that concentrations above 25 mM in the injection phase were sufficient to eliminate most of the disturbances. As shown in Figure 1, this approach led to a considerably better relative separation of AGs in comparison to the previously described binary gradient program, while generally preserving peak symmetry, although slight peak tailing for later eluting compounds could still be observed.

3.2 | Optimization of ionization

One of the main goals of this study was to investigate possibilities for enhancing the overall method sensitivity by applying the IonBooster^{TM} (IB) high-temperature ESI source, which contains an additional heated spray zone and works under atmospheric pressure conditions. In previous works by Kempf et al. [24] and Chepyala et al. [25] IB was successfully used for the determination of psychotropic drugs, showing an enhanced ionization for most analytes, including polar compounds with high pK_a values, therefore, indicating its potential for this particular application. Preliminary optimization revealed that only two parameters (drying temperature (DT) and vaporizer temperature (VT)) significantly affected the ionization efficiency, which was in agreement with the findings of other authors [24,25]. To evaluate the impact of IB temperature parameters, a series of optimization experiments were carried out by analyzing a fortified honey sample with a concentration of 100 ng/g. An increase of DT from 200 to

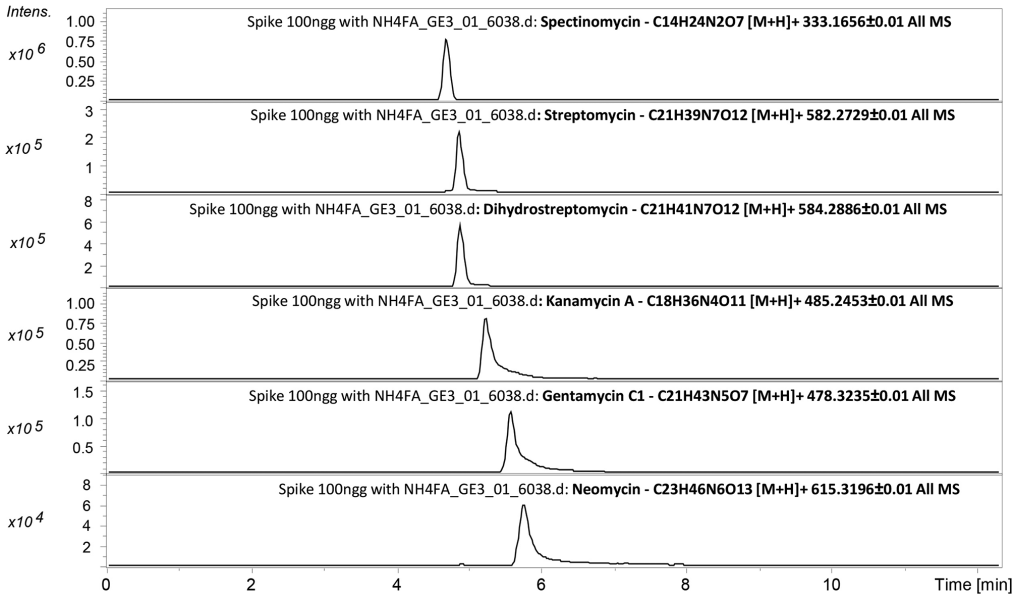

FIGURE 1 Extracted ion chromatograms of $[M+H]^+$ species of AGs in a spiked honey sample (100 ng/g) using the fully optimized method

400°C led to signal enhancement for all target analytes (temperatures above 400°C were not tested as they exceeded the manufacturer's recommendations), however, increasing the VT resulted in deterioration of the signal, indicating thermal decomposition of analytes within the ion source. The largest signal enhancement in comparison to ESI was observed for GEN (16-fold increase), followed by SPC, STP, and DSTP (at least eightfold increase for each), yet the sensitivity for KAN (threefold increase) and NEO (fivefold increase) remained almost constant during the optimization experiments as DT and VT did not have any notable impact on the ionization efficiency (Figure 2.).

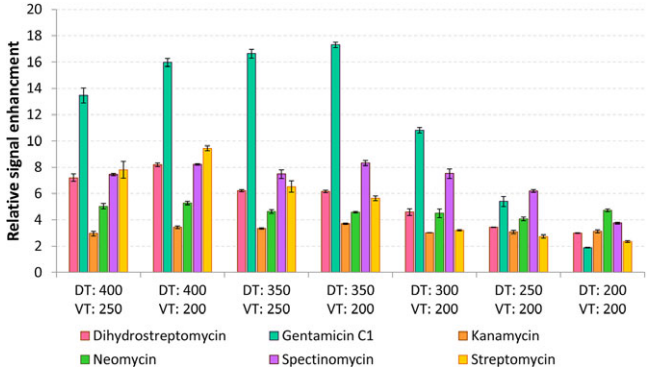

FIGURE 2 The impact of DT and VT on the signal enhancement when using IB source for spiked honey sample with analyte concentration of 100 ng/g ($n = 3$ for each experiment). The relative signal enhancement was calculated with respect to ESI source when analyzing the same sample extract

3.3 | Optimization of Q-TOF-ddMS/MS^2 procedure

To fulfill the identification point (IP) criterion specified in Commission Decision 2002/657/EC (three IPs for group B substances in foodstuffs), a data-dependent MS/MS^2 approach was selected. Infusion experiments were carried out for each compound in full scan and multiple reaction monitoring modes to examine the ionization and fragmentation patterns, respectively. Doubly charged ions were observed for all analytes with the exception of SPC. Nevertheless, reaction monitoring data revealed that singly charged species produced higher intensity fragmentation spectra and had more distinct fragmentation patterns. Thus, the Q-TOF instrument was optimized to acquire the highest abundance of $[M+H]^+$ species and the collected data were used to set up a scheduled precursor list for the data-dependent MS/MS^2 approach. The final optimized MS parameters can be found in section 2.5. During the optimization study, it was noticed that only one parameter, the ion energy, which is the energy difference between hexapole and quadrupole, was noticeably responsible for the reduction of doubly charged species. A series of experiments were performed to optimize the ion energy value by averaging 30 s intervals of full scan spectra with a step of 2 eV and comparing the obtained intensities of $[M+H]^+$ and $[M+2H]^{2+}$ species. The most suitable values were found at 12 eV (for GEN, KAN, and NEO) and 24 eV (for STP and DSTP), where the abundance of singly charged species was more than 95% and the absolute intensity of $[M+H]^+$ was close to the maximum for each compound (Supporting Information Figure S1). Each value was applied on a separate time segment, which correlated with the corresponding

retention time for each target analyte. To avoid faulty precursor selection in data dependent MS/MS^2 scan (3 MS^2 spectra per precursor), smart exclusion mode was enabled, meaning that the precursors were discriminated between steep and gradual (background noise). Additionally, a precursor reconsideration algorithm was applied, e.g., if a precursor was already observed and the MS^2 spectrum was measured, the particular precursor was marked as found, however, if another MS peak for the same precursor was detected in the scheduled time interval, additional MS^2 analysis was performed. This approach helped preventing an un-triggered ddMS/MS^2 scan in the case of closely eluting isobaric compounds. An example of ddMS/MS^2 acquisition for STP and DSTP precursors is illustrated in Supporting Information Figure S2.

3.4 | Optimization of the sample extraction and SPE procedures

Several studies have been conducted to find the most efficient extraction procedure and SPE conditions for the determination of AGs in various matrixes [18,20,26], indicating that the most promising sample preparation approach includes acidic sample treatment with TCA as protein precipitation agent, followed by SPE cleanup. Regardless of the common usage of 5% TCA solution for sample extraction, in this study, the samples were treated with 1% solution of TCA, as honey contains considerably lower amounts of protein than animal tissues or milk and excessive use of TCA could negatively affect the following SPE procedure according to a study by Wang et al. [26]. No other additives were selected for the proposed application, as the extraction yields during preliminary method development experiments were not substantially affected by such frequently used extraction medium enhancers as EDTA and sodium formate.

The initial strategy for the proposed SPE procedure was using a weak cation mixed-mode stationary phase (Phenomenex Strata-X-CW), which is particularly designed for selective retention of strong bases. However, a generic elution approach with 1, 5, and 10% formic acid in three most common elution media (methanol, water, and acetonitrile) gave satisfactory results only with STP, DSTP, and SPC, while NEO, GEN, and KAN remained bound to the stationary phase and apparently required stronger elution conditions that would not be compatible with the developed HPLC method. Alternatively, some authors [27,28] report different approach involving reversed-phase functionalized polymeric sorbent cartridges (Waters Oasis HLB). For that reason, a structurally similar SPE column (Phenomenex Strata-X-PRP) was selected and evaluated for this study. The main difference between both SPE cartridges is that the Strata-X-PRP stationary phase contains piperidone moieties, whereas the Oasis HLB contains pyrrolidone groups. This slight divergence should not significantly affect the general retention mechanisms, suggesting that the SPE procedure can be adapted from applications that use Oasis HLB (and vice versa). Therefore, 1 mL of aqueous 10% formic acid (FA) v/v followed by 3 mL of acetonitrile was used for the initial elution procedure (adapted from Dasenaki et al. (2016)) [28]. As expected, the results from elution profile analysis suggested that the aqueous FA alone was directly responsible for the elution of AGs, while the subsequent addition of acetonitrile increased the co-elution of matrix components (Supporting Information Figure S3). Due to the sensitivity of IB source towards matrix effects, the decision was made to avoid using an additional organic solvent as elution medium. A series of experiments with different volumes (2, 3, 4, and 5 mL) of various FA solutions (1, 2.5, 5, and 10%, v/v) were conducted using a spiked honey sample (100 ng/g, adjusted to a pH of 7±1 with 1 M NaOH solution) to evaluate the SPE conditions in terms of analyte recovery. The highest recoveries were achieved with aqueous 10% FA v/v, although only a small difference was observed between 2.5, 5, and 10% in terms of analyte recovery. A volume of 3 mL proved to be sufficient for eluting the compounds of interest. Nevertheless, relatively low recoveries were observed for GEN and KAN (<50%). To address this issue, an additional series of experiments were carried out by extracting a honey sample fortified with AGs standard mixture (100 ng/g) with the previously selected SPE eluent at different pH values. Results showed (Figure 3.) that the target analytes can be separated in two distinct groups, where GEN and NEO exhibit the highest affinity towards the stationary phase at low pH values, while the rest of AGs adsorbed better at neutral or slightly basic conditions. Fairly acceptable recoveries (65 to 76%) were obtained at pH 5, and these were the conditions selected for the optimized method. The reason behind low extraction recoveries might be an oversaturation of SPE cartridge, because honey contains high levels of carbohydrates, which are also retained on the stationary phase during the clean-up procedure, thus

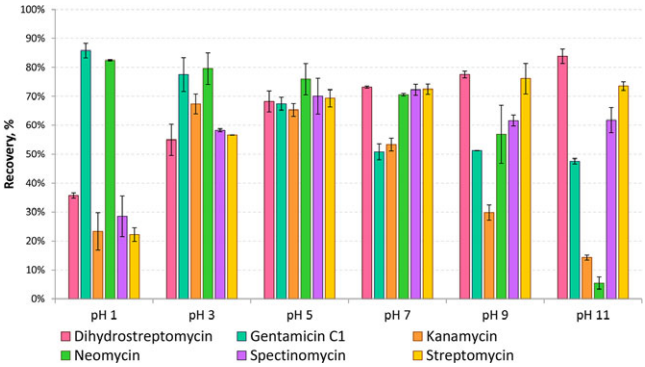

FIGURE 3 Recoveries for blank samples spiked at 100 ng/g and extracted at different pH values ($n = 3$)

occupying active sites and decreasing the absolute analyte recovery.

The matrix effect (ME) was evaluated by analyzing identically prepared blank honey samples ($n = 5$) that were fortified with known concentrations of AGs at the end of sample preparation procedure (Supporting Information Figure S3). The peak areas obtained for the target analytes were compared to the peak areas obtained for the calibration standard at the corresponding concentration levels. The ME values were calculated by the following formula (1):

$$ME(\%) = \frac{Peak\ area_{\text{fortified honey sample}} - Peak\ area_{\text{standard}}}{Peak\ area_{\text{standard}}} \times 100\% \quad (1)$$

Signal enhancement was observed for GEN (+10 ± 5%), KAN (+12 ± 4%), and NEO (+7 ± 2%), while signal suppression occurred in the case of STP (–18 ± 2%), DSTP (–16 ± 3%), and SPC (–13 ± 1%). Even though the sample extracts were already significantly diluted, ME still prevailed, pointing towards the necessity to apply the MMSCC approach to compensate for the loss of analytes and ME.

3.5 | The validation study

The validation was carried out in accordance with Decision 2002/657/EC, using an in-house validation concept (described in Section 2.6). Five concentration levels were investigated per compound. The limit of quantification was determined empirically and subsequently used as the lowest level for the validation. The following fortification levels were selected for the validation study: 10, 25, 50, 100, and 250 ng/g. Notably, in the case of NEO, where the sensitivity was lower due to the broadening of chromatographic peaks, values of 25, 50, 100, 250, and 500 ng/g were used instead. Quantification was performed by using the MMSCC technique. Decision limit (CCα) and detection capability (CCβ) was in the range of 11.2–33.6 and 13.4–47.9 ng/g, respectively. A complete summary of the method performance parameters is provided in Supporting Information Table S2. The repeatability remained almost constant for all target analytes throughout the validation ($3.8\% \leq RSD_r \leq 7.3\%$), however, the within-laboratory reproducibility was relatively poor, especially for NEO (19.7% for the lowest fortification level). Nevertheless, satisfactory values of accuracy were achieved, largely because of the MMSCC approach. The compound identification criteria (based on the MS^2 fragment ratios) were fulfilled for 48 out of 50 samples. Both of the remaining cases involved an outlying fragment ion ratios of NEO, which exceeded the permitted range of 0.17 ± 0.05 (Q1/Q2), suggesting that more distinctive fragmentation patterns might be required for future applications.

3.6 | Application of the method

The developed method was applied for the determination of AGs in honey samples. All samples were analyzed in accordance with the previously described procedure. Quantification was performed using the MMSCC approach. MMSCC were prepared on the same day as the unknown samples using the same conditions. A total of 49 samples from various regions of Georgia and having different composition were analyzed during the study. Residues of STP were detected in two samples (117 and 35 ng/g), and one sample contained GEN at the level of 32 ng/g. Our results indicate that presence of antimicrobial residues in honey is a relevant issue, which needs to be addressed to ensure the safety of consumers.

4 | CONCLUSIONS

In this study, we developed a simple sample preparation procedure followed by LC–Q-TOF-ddMS/MS^2 analysis with scheduled precursor list for simultaneous screening and quantification of AG residues in honey. The final optimized sample pretreatment procedure showed satisfactory recoveries on polymeric RP-SPE cartridges other than Oasis HLB.

Three mobile phase systems involving gradual increase of acidity proved to be the most suitable for a zwitterionic-type mixed-mode LC column (Obelisc R). An IB source was superior over a conventional ESI source in terms of ionization efficiency, indicating that it could be a promising choice for future applications involving AGs. In addition, the IB provided another advantage by ensuring the absence of $[M+Na]^+$, $[M+NH_4]^+$, and $[M+K]^+$ ionic species and reducing the amounts of doubly charged ions which were abundant in the MS spectra acquired with a conventional ESI source. Moreover, the sensitivity of the method is within the range necessary for the quantification of AGs in honey (on-column LOQs ranged from 50 to 125 pg), thus proving that this approach can keep up with conventional triple quadrupole MS/MS systems. A comparison in terms of method sensitivity between our study and the reports from other authors can be found in Supporting Information Table S3 highlighting that Q-TOF-MS can be used not only for screening purposes, but also for comprehensive residue analysis.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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