Food Chemistry 405 (2023) 134826

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Single pot *in situ* aqueous derivatization and subsequent determination of streptomycin and dihydrostreptomycin residues in honey by means of mass spectrometry

İsmail Emir Akyıldız [a,b,*], Sezer Acar [a,b,1], Ece Kök Yetimoğlu [a,2], Sinem Raday [b,3], Özge Erdem [b,4], Dilek Uzunöner [b,5], Emel Damarlı [b,6]

[a] Chemistry Department, Marmara University, Istanbul, Turkey
[b] Balparmak R&D Center, Altıparmak Food Industry and Trade Inc., Istanbul, Turkey

ARTICLE INFO

Keywords:
Honey
Aminoglycosides
Residue
In situ derivatization
UHPLC-MS/MS

ABSTRACT

Streptomycin (STR) and dihydrostreptomycin (DSTR) are the typically encountered aminoglycoside (AMG) residues in honey. For AMG analysis, studies in literature involve impractical and expensive applications such as ion-pairing chromatography, immunoassays, pre and post column derivatizations, or SPE approaches. Pre-treatments of these methods are toilsome and costly. Herein, one-pot, aqueous *in-situ* derivatization method was presented as a superior protocol. Time and cost-efficient UHPLC-MS/MS method has been developed, and practical sample preparation was introduced. Satisfactory results were reported in method verification studies. The mean recovery values were 102.6% for STR and 101.3% for DSTR. Average values between 1.5% and 9.9% RSDs were found at intra and inter-day precisions. CCα (5.7 and 5.8 μg/kg) and CCβ (6.2 and 6.4 μg/kg) values were calculated for STR and DSTR respectively. AMG residues were found in 29 out of 110 analyzed samples using validated method. Described novelty enabled comprehensive analysis in an inexpensive and straightforward manner.

1. Introduction

In beekeeping, there is a struggle against plenty of pests, mites, and noteworthy insect-borne diseases. The most common types of bee diseases are American foulbrood (AFB), European foulbrood (EFB), European septicemia, Nosemosis, and Paratyphoid fever (Paratifo) (Shimanuki & Knox, 2000). The use of antibiotics for the control of bacterial diseases in beekeeping may arise antibiotic residues in honey. It may lose its quality and be harmful to human health if it involves deleterious xenobiotics (Aksoy, 2019). The well-known primary reasons for the presence of these residues in honey are the misusage of the drugs and applying improper drug retraction timeframes.

Aminoglycosides (AMGs) are used to treat a wide variety of infections and mainly focus on the eradication of gram-negative bacteria (Khondker et al., 2021). Streptomycin (STR) and dihydrostreptomycin (DSTR) are belonged to the AMG class and are commonly used pharmaceuticals in beekeeping to protect against brood diseases. These AMG drugs are typically encountered residues in honey and they devalue the product. In China, streptomycin one of the known AMG was the preferred antibiotic for the first time to eradicate a large AFB outbreak in 1997 (Ortelli et al., 2004). The European Union does not allow the use of veterinary drugs involving antibiotics in beekeeping. Thereby, there is no Maximum Residue Limit (MRL) for any antibiotics in honey (European Commission, 2010). It is emphasized that according to the Turkish

* Corresponding author at: Marmara University, Chemistry Department, Eğitim Street, Fahrettin Kerim Gökay Avenue, 34722 Kadıköy, Istanbul, Turkey. Altıparmak Food Industry and Trade Inc., Department of R&D, Çavuşbaşı Avenue, 34782, Çekmeköy, Istanbul, Turkey.
E-mail address: akyildizemir@hotmail.com (İ. Emir Akyıldız).
[1] ORCID: Sezer Acar: 0000-0001-9883-4385.
[2] ORCID: Ece Kök Yetimoğlu: 0000-0002-8551-5411.
[3] ORCID: Sinem Raday: 0000-0002-4683-070X.
[4] ORCID: Özge Erdem: 0000-0001-7883-9250.
[5] ORCID: Dilek Uzunöner: 0000-0001-6189-2020.
[6] ORCID: Emel Damarlı: 0000-0003-1082-2430.

https://doi.org/10.1016/j.foodchem.2022.134826
Received 13 June 2022; Received in revised form 23 October 2022; Accepted 30 October 2022
Available online 3 November 2022

Food Codex, Codex Alimentarius, and European Commission criteria, no drugs are allowed for beekeeping other than several anti-varroa preparations approved by the European Union. This means that zero tolerance is applied for antibiotic residues in honey. According to the 2003/181/EC legislation, it has been reported that without an MRL value, laboratory methods must be capable to quantify the lowest possible limits for residues (Commission, 2003). According to these performance criteria, the developed methods should be able to quantify the residues in line with maximum Residue Performance Limit (MRPL) concentrations.

In the literature review, for AMG analysis, there are many applications that use molecularly imprinted polymers (MIPs) (Moreno-Gonzalez et al., 2015; Yang et al., 2017), immunoaffinity based (Liu et al., 2021) or Van der Waals interactions based conventional solid-phase extraction (SPE) sorbents in cartridge format (Contin et al., 2019; Kim & Kang, 2021; Young et al., 2014). Enzyme-Linked Immuno Sorbent Assay (ELISA) kits which are produced with specially synthesized antibodies are also quite common (Sachetelli et al., 1998; Xu et al., 2011). The SPE cleanup is known as costly and time-consuming for laboratories in the routine analysis. Besides, these techniques generally present low-yielded and irreproducible results. Since, binding, stripping, and back-eluting mechanisms mainstay of these techniques, the applications may generate losses of the target compound. In another respect, ELISA kits report total AMGs results instead of individual concentrations of each compound, which impede acquiring information about the subtype of AMGs. The biggest bottlenecks associated with ELISA kits at AMGs determination are the cross-reactivity phenomenon and their costs. To avoid this, highly specific antibodies are required, which makes the implementation of ELISA more expensive and unpropitious (Jiang et al., 2018; Jin et al., 2006). As instrumental analysis, the Liquid Chromatography (LC) technique is used commonly to separate the AMGs. These LC-based methods are applied for the determination of AMGs by combining ultraviolet (UV), fluorescent (FL), or mainly mass spectrometric (MS) detections (Glinka et al., 2020). Numerous other screening methods are also covered in the literature such as thin-layer chromatography (TLC) (Hubicka et al., 2009) and capillary electrophoresis (CE) (Huidobro et al., 2009). Several drawbacks can be stated as prevalent for the abovementioned methods including irreproducibility, insensitive detection, need for costly consumables, and non-user-friendly sample preparations. LC methods equipped with MS are the most discussed ones in literature when sensitivity is a constraint for AMGs detection for various matrices (Farouk et al., 2015; McGlinchey et al., 2008). Unfortunately, suggested LC-MS methods in the literature incorporate labor-intensive sample preparation steps. Highly polar molecular features of the AMGs have directed the researchers to develop LC methods by using ion-pairing reagents combined with octadecylsilane (C18) columns (Tölgyesi et al., 2018) or by using hydrophilic chromatography (HILIC) columns (Asakawa et al., 2018; Chen et al., 2017; Saluti et al., 2018). The use of ion-pairing additives is mostly preferred to combat the retention problem of AMGs (El Hawari et al., 2017; Megoulas & Koupparis, 2004). It is a well-known issue that the addition of such agents causes resistant contamination on the analytical column and remarkably high ion suppression is experienced. This hampers the ability of low levels of residue quantification and presents the necessity of aggressive cleaning procedures. The need for lengthy equilibration times prior to analysis can be expressed as yet another drawback of ion-pairing. Recently, the HILIC application is considered more favorable in AMG analysis. Researchers have reviewed the advantages of electrospray ionization (ESI) in combination with HILIC mode separations (Glinka et al., 2020). Even under isocratic conditions, HILIC provides excellent retention and separation from matrix components while providing baseline resolution (Peru et al., 2006). Like ion-pair chromatography, HILIC applications have also some challenges in honey analysis such as extended conditioning times, interferences of abundant polar substances such as carbohydrates and organic acids, and loss of sensitivity due to the matrix-dependent ion suppression effect. Alternatively, in order to avoid the use of ion-pair reagents and HILIC, researchers proposed derivatization-centric processes to retain and separate AMGs on the reversed-phase column. Pre-column and post-column derivatization approaches were applied to this extent (Chang & Yu, 2011; Driver et al., 2009; Pang et al., 2004). Nevertheless, it has seemed that the addition of a derivatization step into the sample pretreatment workflow leads to elongated total analysis time. Post-column derivatization procedures also need pre-extraction followed by clean-up at sample preparation steps to fulfill the sensitivity expectations. They are also not fully compatible to implement mass spectrometry (MS) based analysis methods due to the introduction of the non-volatile derivatization agents directly to the MS ionization source.

Therefore, the aim of the study was primarily to introduce a simplified, MS-compatible, and sensitive sample preparation process for analyzing AMGs in the honey matrix, which is arduous to analyze owing to their high polarity. In this study, single pot, aqueous *in situ* derivatization was designed as a superior sample preparation approach for AMG analysis in honey samples. By harnessing UHPLC-MS/MS system combined with this novel pretreatment protocol, a time and cost-efficient, versatile analysis method has been presented with high sensitivity and reproducibility.

2. Method and materials

2.1. Sample collection

Honey samples from different botanical origins were gathered from apiaries in Turkey from different regions including the Aegean, Central Anatolia, and the Mediterranean provinces. Blossom ($n = 57$), pine ($n = 24$), citrus ($n = 15$), and chestnut ($n = 14$) honey samples were collected in the season of 2021 and these samples were used for both method development study and for monitoring the frequency of the STR and DSTR residues. Researchers worked closely with beekeepers during harvesting season for being sure that collected honey samples were not adultered and sampling was done conveniently. We supplied and analyzed honeys of 4 different origins to verify the method is robust and selective against different matrix effects. Before analysis, for the confirmation of the botanical origins of honey samples, melissopalynological analysis was also performed and the samples were classified according to their botanical origins. All samples were taken in glass jars and hermetically sealed and they were kept at 4 °C until analysis.

2.2. Reagents and chemicals

Formic acid (reagent grade, ≥ 95%), 9-Fluorenylmethoxycarbonyl chloride (Fmoc-Cl, for HPLC derivatization, LiChropur™, ≥99.0%) were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany). Acetic acid, acetonitrile (ACN, LC grade), triethylamine (TEA), methanol (MeOH, LC grade), Sodium carbonate (Na_2CO_3, ACS grade, anhydrous, ≥ 99.5%), Sodium bicarbonate ($NaHCO_3$, ACS grade, anhydrous, ≥ 99.5%), boric acid (ACS grade, anhydrous, ≥ 99.9%) and sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$, ACS grade, ≥ 99.5%) were obtained from Merck (Merck KGaA, Darmstadt, Germany). Analytical standards of streptomycin sulfate, dihydrostreptomycin sulfate, and trimethoprim as internal standard (IS) were from Dr. Ehrenstorfer® GmbH (Augsburg, Germany). The purity of all the analytical neat standards was above 98.8%. Ultrapure water was obtained from Milli-Q Plus® from Millipore® (Bedford, MA, USA) and was used at all reagents and dilutions.

2.3. Preliminary survey using ultra high performance liquid chromatography - photo diode array (UHPLC-PDA)

In order to see which of the predicted derivatization agents can work most effectively, various derivatizing molecules were reacted with STR under constant reaction conditions using different buffers. Benzoyl chloride (BzCl, HPLC derivatization, LiChropur™, ≥ 99.0%) and phenyl

isocyanate (PIC, HPLC derivatization, LiChropur™, ≥ 99.0%) supplied from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany) and tested in parallel with Fmoc.Cl. Three different buffer systems were examined under a constant incubation time, derivatizing agent molarity, and temperature to obtain the most intense signal and single reaction product for the STR derivate. Carbonate buffer (pH; 9.2, 0.1 M), borate buffer (pH; 9.0, 0.1 M), and TEA additive (5% in ACN, v/v) as alkaline pH conditions were compared for labeling AMGs. In this preliminary investigation analysis, STR was spiked into 5 mL of 50:50 ACN:buffer (v/v, %) solutions at concentrations of 50, 100, and 150 mg/mL respectively. After the addition of each purposed derivatizing agent at 0.1 M concentration for each of 3 different buffered conditions (TEA, carbonate, borate buffers), reaction pots were incubated for a maximum of 1 h at room temperature using continuous stirring. Next, the solutions were filtered and injected into the UHPLC system without quenching the reactions. The reaction products were first scanned in the range of 200 to 750 nm using the Thermo Scientific® Accela UHPLC system (Waltham, Massachusetts, USA) equipped with an Accela 600 pump, an Accela autosampler, a column oven, an Accela PDA detector. The UV spectra were analyzed, then the sample was re-injected by choosing the fix-wavelength option. We chromatographically resolved the derivates by using a C18 column (Hypersil 150 length × 4.6 mm i.d., 5 μm) from Thermo Scientific® (Waltham, Massachusetts, USA) and utilizing a linear gradient elution via water and ACN containing 0.1% formic acid as mobile phases. The column temperature was maintained at 40°C and the injection volume was 20 μL. The flow rate was 1 mL/min and a 10 min runtime was established.

2.4. Preparation of reagents and standards

Streptomycin sulfate, dihydrostreptomycin sulfate, and trimethoprim standards (10.0 mg ± 0.05 mg) were weighed meticulously taking into consideration of their purity levels and they were prepared by dissolving them in 10 mL ultrapure water individually to get the concentration of 1.0 mg/mL. Each stock standard solution was further diluted to concentrations of 1.0 mg/L and 0.5 mg/L to get working mix solutions. Fmoc-Cl solution was prepared at a concentration of 0.2 M as derivatization reagent by dissolving in ACN. Sodium carbonate buffer pH 9.2 was prepared by mixing 80 mL of 0.2 M Na_2CO_3 and 920 mL of 0.2 M $NaHCO_3$ solution. ACN was added to this buffer with a ratio of 4:1 and this final reagent was used for both homogenization and extraction purposes.

2.5. Sample preparation procedure

For the sample preparation protocol of the final method, we determined; 0.2 ± 0.005 g of homogenized honey sample was accurately weighed into a 15 mL falcon tube and 50 μL of IS (100 μg/L) was added. IS solution was spiked into this solution to perform a normalization approach and to identify any sample pretreatment and/or system-dependent bias during analysis. 4.0 mL of homogenization/extraction buffer (sodium-carbonate buffer (pH 9.2)-ACN mixture (4:1, v/v, %) was added to the sample and homogenized by agitation. Afterward, 1 mL of 0.2 M Fmoc-Cl solution was added and vortexed for 20 *sec*. The sample was incubated at room temperature for 30 min by continuous shaking using a rotating shaker. 300 μL formic acid was added and mixed thoroughly for terminating the derivatization reaction. The resulting solution was subjected to a syringe filter (0.45-μm PVDF membrane filters, Interlab®, Arnavutköy, Istanbul, Turkey) to get rid of any particles and transferred into the amber glass vials for injection. Quantitation of the unknown samples was carried out with the aid of matrix-matched calibration curves on 6 concentration levels. For this purpose, appropriate amounts of working mix solutions of each STR and DSTR were spiked into the blank honey to get 5.0, 10.0, 15.0, 20.0, 50.0, and 100 μg/kg calibration levels.

Table 1
Selected Reaction Monitoring (SRM) transitions and related mass spectrometry parameters selected for STR/DSTR residues determination in honey by UHPLC-ESI-MS/MS.

Analyte	Retention Time, (min)	Precursor ion, $[M + H]^+$, (*m/z*)	Product ions (*m/z*), (Q/q1/q2)	Collision Energies, (eV), (Q/q1/q2)
STR-FMOC[a]	3.4	804	154/221/245/263	50/50/50/40
DSTR-FMOC[b]	3.4	806	154/245/263	50/50/40
Trimethoprim-FMOC[c] (IS*)	3.5	514	318/292/180	25/25/25

Cone voltages for AMGs were 70 V and cone voltage for IS was 30 V. Dwell times were 0.005 *sec*., *m/z* ions used for quantification (Q) and for confirmation (q1, q2). *IS; Internal Standard. [a]STR-FMOC; 9-Fluorenylmethoxycarbonyl labelled Streptomycin, [b]DSTR; 9-Fluorenylmethoxycarbonyl labelled Dihydrostreptomycin, [c]Trimethoprim-FMOC; 9-Fluorenylmethoxycarbonyl labelled Trimethoprim.

2.6. UHPLC-ESI-MS/MS analysis conditions

Chromatographic separation was achieved using a Waters® (Milford, Massachusetts, USA) ACQUITY UPLC system equipped with a binary solvent delivery system, a column oven, and an autosampler. Reversed-phase interaction-based separation was accomplished on a Waters® ACQUITY BEH (Bridge Ethylene Hybride) C18 column (2.1 mm i.d., x 50 mm length, 1.7 μm). Chromatographic analysis was carried out with a gradient mobile phase consisting of solution A (water, 0.05% acetic acid) and consisting of solution B (MeOH-ACN (1:1, v/v, %), 0.05% acetic acid) at a flow rate of 0.35 mL/min. The run time was 5 min and the linear gradient conditions were as follows: 0–1.30 min., 5% B; 1.30–2.5 min., 5–95% B; 2.5–3.5 min., 95–5% B; 3.50–3.51 min., 5% B; 3.51–5 min. The column temperature was maintained at 40 °C and the injection volume was 50 μL. For eliminating the matrix components at the initial steps of the injection, column effluent was directed to the waste position by operating the embedded valve on a mass spectrometer until 2 min. Mass spectrometry acquisition was achieved using Waters® Xevo-TQ triple quadrupole mass spectrometer (Milford, Massachusetts, USA). All analytes were ionized using electrospray ionization (ESI) in positive mode. Parameters for the ESI source were set as follows: capillary voltage 1.0 kV, source temperature 150 °C, desolvation temperature 450 °C, cone gas flow 50 L/h, and desolvation gas flow 850 L/h. Data were collected in selected reaction monitoring (SRM) mode. Dwell times for each compound were optimized for getting the best peak shape and sufficient data points. All ESI and MS parameters were optimized individually for each target compound and listed in Table 1. Data acquisition was performed using Mass-Lynx software with the TargetLynx program (Waters®, Milford, Massachusetts, USA). **(**Table 1 **near here)**.

2.7. Method validation

The entire validation study was conducted in accordance with the European Commission 2002/657/EC standards (Commission, 2002) and by following the validation workflow from our previous work **(**İsmail Emir et al., 2021**)** to test the applicability of the developed method. In this context, for the linearity study, 6 concentrations of 2 analytes were analyzed in triplicate. Matrix-matched calibration curves were generated by plotting the concentrations of each analyte versus the peak areas. Inter-day precision (reproducibility, RSD_R) and intra-day precision (repeatability, RSD_r) studies were accomplished by calculating the relative standard deviations (RSD%). For the intra-day precision test, working mix solution (500 μg/L) was spiked into the blank honey samples at 3 concentrations (5.0 μg/kg, 20.0 μg/kg, 100.0 μg/kg) and they were analyzed as seven replicates ($n = 7$) within one day. For the

inter-day variability test, the same procedure was performed by 3 different analysts for consecutive 3 days ($n = 7$) using freshly prepared reagents. Decision limit (CCα) and detection capability (CCβ) were determined for each substance at signal-to-noise ratios (S/N) of about 3 and 10, respectively. Recovery/accuracy test was carried out by analyzing the blank samples which were contaminated with the known amount of STR/DSTR at three different concentrations (5.0 μg/kg, 20.0 μg/kg, 100.0 μg/kg). The calculated areas were compared with post-extraction spiked samples at equal concentrations. Each sample was analyzed in triplicate and the percentages of recoveries were reported on average. For the method robustness, spike standards were analyzed under deliberate changes in method parameters. Observed variations were expressed as relative standard deviations (RSD). At ruggedness tests, 2 different analysts worked on 2 different instruments using the same samples and methods. Results were reported as average recovery. Testing the specificity of the methods was carried out by comparing the signal/noise (S/N) ratios of the blank sample injections ($n = 10$) in comparison to the same samples spiked with known amount of standards.

3. Results & discussion

As general analytical approaches in the literature for AMGs analysis, the use of SPE cartridges in sample pretreatment steps and application of post-column derivatization, ion-pairing chromatography, or HILIC as chromatographic considerations may cause many shortcomings and difficulties in terms of economic, labor, and detection efficacy. It was anticipated within the scope of the study, implementing a pre-column derivatization step for the AMGs before their separation and detection would be the most efficient way regarding the rapid determination of molecules. From our point of view, if the correct derivatization agent is selected and pertinent analytical conditions are met, monitoring of AMGs in honey matrix can be performed in a single pot format without applying post-extraction or post-derivatization clean-up techniques. Due to the ability of the honey matrix to form a homogeneous solution with any aqueous buffer, it allowed us to work as an aqueous *in situ* condition without the need for any additional extraction process. We hypothesized that this situation also facilitates the development of a dilute and shoot sample pretreatment procedure that enables single-pot derivatization and can be completed without prolonged and expensive sample preparation steps. Within this study, the dilute and shoot preparation strategy employed in a single-pot format was combined with *in situ* aqueous derivatization and a method was developed to simultaneously quantitate the STR and DSTR residues in honey.

Albeit the preferred sample preparation technique is greatly significant, it is also crucial to adjust the best conditions for each parameter at the selected workflow in analytical method development. For this reason, the extraction of AMGs from honey and the derivatization procedure was optimized carefully. In this context, the sweet spots of each sample preparation step were determined experimentally together with ideal LC conditions.

3.1. Combining the dilute & shoot pretreatment with LC-MS monitoring as a novel AMGs analysis platform

At LC-MS/MS analysis of AMGs in honey samples, abundant and hydrophilic honey matrix components such as sugars, minerals, amino acids, phenolics, and organic acids can introduce strong matrix effects and can cause poor chromatographic resolution and low sensitivity (Machado De-Melo et al., 2018). This complex content may affect the ionization efficiency of the target analytes. It is necessary to separate AMGs from the abovementioned hydrophilic environment to be able to monitor trace amounts of residue in honey. Owing to highly polar guanidinium moieties and glycosidic bonds of AMGs, targeted pharmaceuticals show similar retention and extraction properties with most of the indicated hydrophilic and abundant molecules. Therefore, we envisaged that rapid derivatization of AMGs by targeting their primary amines can distinguish the compounds from non-amine-carrying molecules (e.g. organic acids, minerals, phenolics, sugars, etc.). By selecting the conceivable derivatizing agent, labeling methodology could also allow us to retain AMGs at reversed-phase chemistry. Applying reversed-phase conditions can enable the depletion of the abundant hydrophilic ingredient of honey before MS detection by using an embedded valve on the mass spectrometer. We designed the sample preparation principle to perform an on-line clean-up by sending the non-derivatized hydrophilic substances to waste while retaining the AMGs on a C18 column. By switching the position of the MS-embedded six-port divert valve just before the elution time of AMGs, we managed to get responses with an attenuated matrix effect. The derivatizing strategy was indispensable for us to specifically extraction of STR and DSTR in a bulk, hydrophilic matrix environment, and subsequent screening. This analytical approach also paved the way for implementing the dilute and shoot technique.

3.2. UHPLC-PDA preliminary survey results

Since most of the derivatization agents have UV chromophores, we thought that it would be reasonable to track the performance of the yield by UHPLC-PDA monitoring before the optimization study using a certain amount of derivatization agents, appropriate buffers, and analytical standard of STR without the honey matrix. We first aimed to test several derivatizing agents as pre-column derivatization options for tagging the AMGs. For this, BzCl, PIC, ethyl chloroformate, 2,4-dinitrofluorobenzene, N, N disuccinimidyl carbonate, ninhydrin, o-phthalaldehyde (OPA), sodium 1,2 naphthoquinone-4-sulfonate and Fmoc-Cl molecules were investigated. It was observed that 2,4-dinitrofluorobenzene, N, N disuccinimidyl carbonate, ninhydrin, and sodium 1,2 naphthoquinone-4-sulfonate were unsuccessful to produce apparent STR derivates. Therefore these agents were abrogated from the experimental design. A method that can be used for the derivatization of both the amino and carboxylic acid groups of amino acids using alkyl chloroformates for LC-MS analysis was assessed as a powerful tool for the simultaneous measurement of AMGs. For this purpose, we have intended to use ethyl chloroformate. Besides, the formation of fluorescent isoindoles from the OPA reagent requires also a free amino group on the molecule to be derivatized (Tsikas et al., 1999). Thereby, these agents were estimated as versatile derivatization reactants for STR and DSTR targets. The designed reactions should be triggered in the organic phases which are catalyzed by an organic base such as pyridine or picoline (Husek & Simek, 2006). Volatile natures of the final products and potential prerequisites as organic solvent extraction of the resulting apolar derivates by employing liquid–liquid extraction followed by solvent exchange application were the major obstructs to the implementation of the dilute and shoot methodology. The application also extended the duration of the sample preparation workflow and the high cost of the analysis was foreseen. Furthermore, honey is a very complex foodstuff and derivatizing reagents could be attacked by the nucleophilic parts of many matrix-dependent compounds. Hence, derivatization reagent for multiple functional groups (e.g. amine, carboxyl, aldehyde, hydroxyl) was not proper to get an ideal response for AMGs specifically. Further research we conducted by evaluating the labeling efficiencies of the BzCl, PIC, and Fmoc.Cl agents in comparison which can work in the aqueous environment, it was observed that the experiments using BzCl and PIC agents produced multiple AMG derivates at linear STR concentrations. The binding of STR molecules from more than one primary amine functional moiety of the pointed out agents greatly increased the molecular size and did not highlight a homogeneous distribution. While some of the STRs made a single point linkage with the specified derivatization reactants, other parts of STRs made a conjugation from multiple points. This caused variation in the final product and thus the splitting of the STR signal occurred. Since miniaturized and practical sample preparation was aimed, the requirement for a long incubation

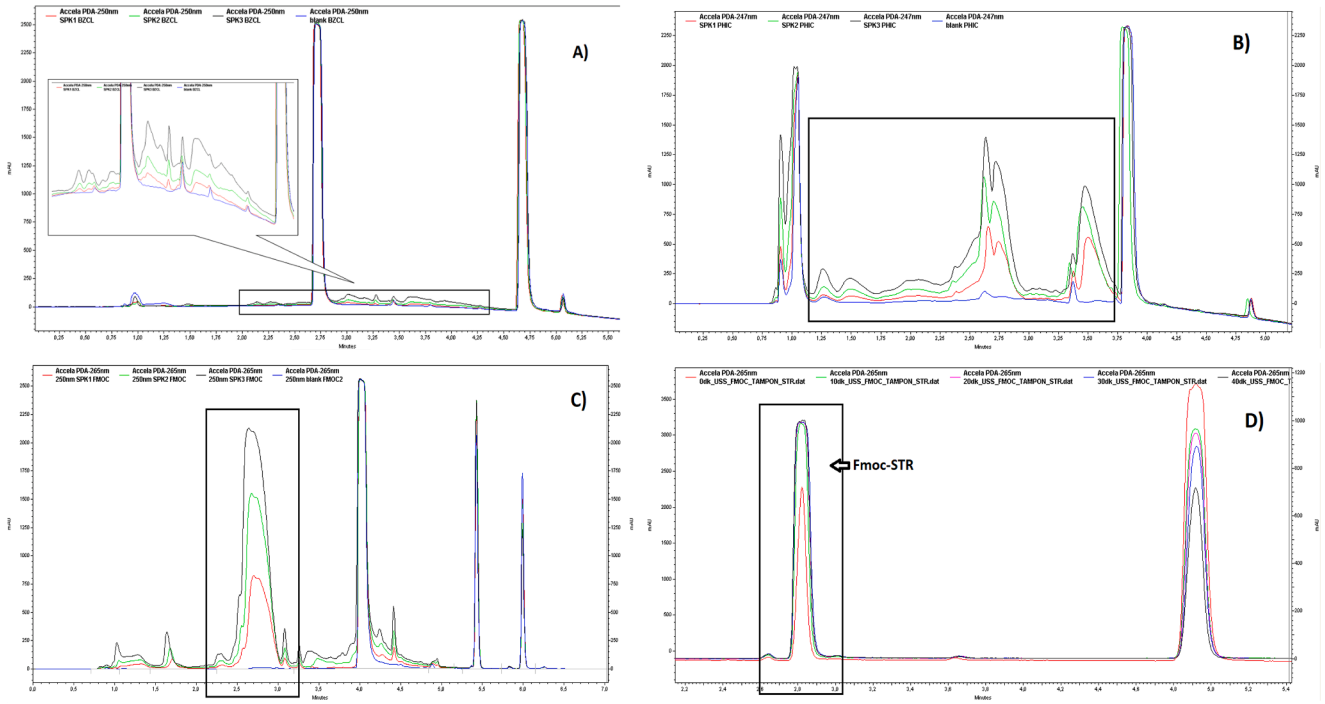


Fig. 1. Results of the preliminary investigations performed to determine the convenient derivatizing agent at the UHPLC-PDA system. A) Chromatogram of the formation of multi-derivative products at linear STR concentrations as a result of the derivatization reaction using BzCl; B) Chromatogram of the formation of multi-derivative products at linear STR concentrations as a result of the derivatization reaction using PIC; C) Chromatogram of the formation of uniform derivate at linear STR concentrations as a result of the derivatization reaction using Fmoc.Cl; D) Illustration of the studies using Fmoc.Cl to determine the appropriate incubation time as chromatogram comparisons.

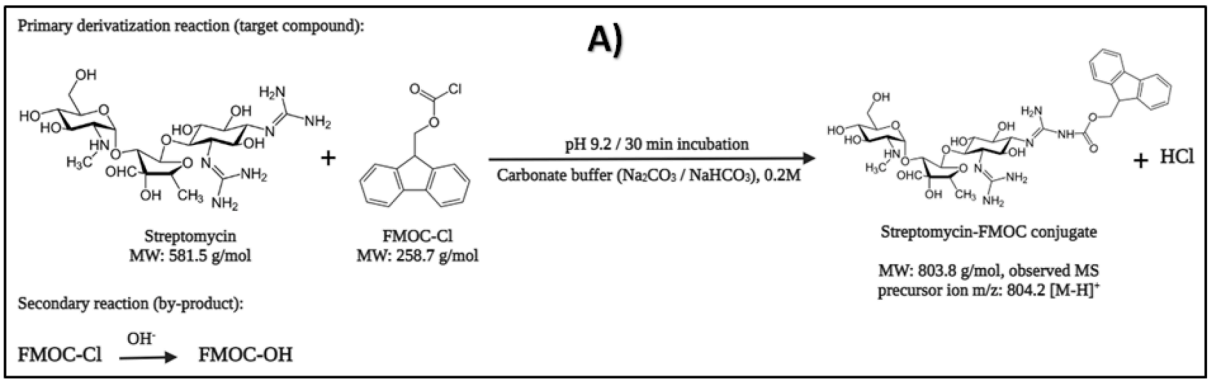
time was also deemed inappropriate. Owing to all these emphasized reasons, the desired sensitivity could not be achieved and these analytical approaches were also omitted. On the other hand, we know that the Fmoc-Cl reagent has an affinity merely for primary and secondary amines (Ziegler & Abel, 2014). The primary amines provided by the guanidium functional groups in STR and DSTR offer a high affinity for Fmoc-Cl. As an outcome of our preliminary experiments, it was observed that the Fmoc-Cl reagent was able to demonstrate rapid derivatization efficiency in aqueous solution. It can derivatize STR molecule at linear concentrations as uniform derivates at room temperature and in a short incubation period. It has been determined that there are no conditions such as the formation of more than one product in comparison to other derivatizing agents, and the necessity of additional organic solvent extraction. In another experiment using Fmoc.Cl, we also observed that the signal of the Fmoc-STR conjugate increased linearly up to 30 min and longer durations did not contribute to the signal. Comparison results are given as LC chromatograms in Fig. 1.

We also thought that Fmoc.Cl is the only reagent in our hands to make this reaction at a relatively low cost and without toxic effects. In this regard, all optimization tests were carried out by selecting solely Fmoc-Cl as the adequate derivatization agent. While Fmoc-Cl is generally used as a derivatization agent to impart ultraviolet or fluorescent properties to the related molecules by presenting chromophore groups (Mohammadi et al., 2013), in this study, it was chosen to bring to STR and DSTR the ability to be retained in RP conditions due to its highly apolar structure. Reversed-phase chromatography conditions provide higher ionization in MS than in HILIC conditions. In this respect, for the sensitive measurement of STR and DSTR molecules, analysis under RP conditions instead of HILIC became possible after derivatization, and hence, a high-throughput sensitive analysis was able to be performed. In addition, due to the high ionization capability of Fmoc-Cl, the method we developed provided the opportunity to measure low level concentrations of STR and DSTR-linked Fmoc conjugates. In the next steps of the method development, we continued by incorporating the honey matrix and by monitoring the reaction in the MS system. We deeply investigated the optimum conditions to get maximum yield when the honey matrix was also included in the reaction pot.

Optimization of the single pot in situ aqueous derivatization and dilute & shoot protocol Honey has lots of UV active compounds, hence transferring the optimization experiments to an MS-based platform and searching for Fmoc-linked STR and DSTR would have been a more specific, non-sophisticated, and prominent way to make data assessment. Therefore, we first performed the Fmoc.Cl-STR reaction without the honey matrix. After Quadrupole 1 (Q1) scanning to find precursor ions and followed by Quadrupole 3 (Q3) scanning to find daughter ions formed by collision-induced fragmentation, we built an SRM acquisition table that enabled the sensitive and selective monitoring of the labeled AMGs during optimization studies. The derivatization scheme and mass spectrum of the non-optimized STR derivatization reaction are depicted in Fig. 2.

By using generated SRMs, we tested and compared the incubation times, buffering pHs, homogenization buffer compositions, derivatizing agent molarity and optimum reaction stoichiometry, conditions for reaction quenching, amount of sample used, and volumes of added reagents.

In the optimization experiments using honey in different weighings, it was figured out that high amounts created a detrimental matrix suppression effect in the MS system and also dramatically decreased the yield by adversely affecting the stoichiometry of the derivatization reaction. Apart from STR and DSTR, there are many components in honey that derivatizing agents can show affinity and react with. This situation negatively affected and disrupted the stoichiometry required for optimum derivatization reaction. The *N*-terminus presented by the amine moieties of amino acids, vitamins, enzymes, major royal jelly proteins, and other unknown xenobiotics in the honey matrix pose suitable functional groups for described reaction and can alter the derivatization of target molecules with high recovery. For this reason, 200 mg as starting sample weight, which can meet the required method sensitivity



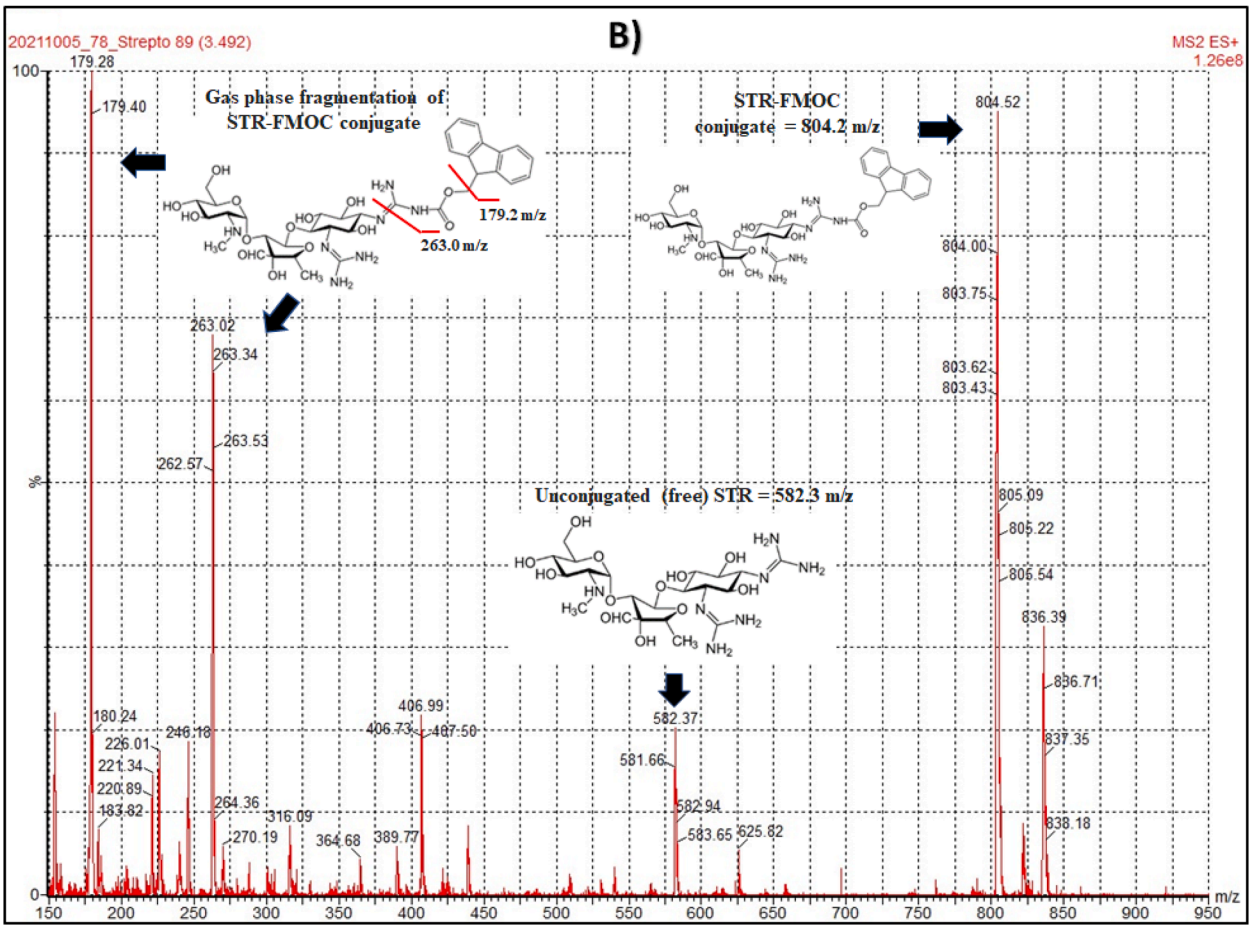


Fig. 2. A) Derivatization reaction scheme; B) Mass spectrum of the non-optimized STR-Fmoc derivatization reaction.

and gives affirmative recovery values was chosen in line with the experimental observations.

Additionally, excess derivatizing reagent must be present in the reaction pot for complete tagging of the AMGs in a short period. Thus, to get optimum stoichiometry, the linear concentration between 0.05 M and 1 M of the derivatization reagent was tested and appropriate molarity was decided empirically as 0.2 M. It was observed that higher concentrations did not change the STR and DSTR responses but lower concentrations showed weakened signals which were indicating the derivatizing agent should be in higher molarity in the reaction pot.

Many antibiotics are in conjugated form and may require enzymatic or chemical hydrolysis to liberate the target prior to extraction. Sulfation, glucuronidation, or conjugation interactions such as binding propensity to proteins or metals can alter the structure of drugs (Barbosa et al., 2007). Amine-bearing compounds are prone to adhere to silica surfaces. Hence using glass materials such as glass LC vials can cause non-specific binding and analyte loss at AMGs analysis. Derivatization chemistry within this research prevented the binding issue and this drawback was also eluded and a satisfactory extraction yield has been obtained.

Incubation temperature and duration were also optimized. We performed the reaction at room temperature and under-emphasized conditions. Empirical outputs indicated that 30 min incubation time is sufficient for the complete derivatization after a survey that was employed at stepwise increasing incubation time. We have deeply investigated the determined reaction conditions by applying different concentrations of STR and DSTR residues. Within this experiment, we have tried to figure out if the higher concentrations of residues disarrange the reaction stoichiometry. Up to 2000 μg/kg concentrations of both STR and DSTR were tested concomitantly and the dynamic range of

Table 2
Comparison results of the derivatization reaction yields in the presence of honey matrix carried out under different conditions using Fmoc.Cl agent.

Experiment	Variable	Relative STR-Fmoc[a] Peak Area (TIC[b] at 20 μg/kg conc.)
Honey weighings	1000 mg	9212
	500 mg	14,230
	200 mg[c]	15,617
	100 mg	8102
Fmoc.Cl concentrations	1 M	16,021
	0.5 M	15,418
	0.2 M[c]	15,617
	0.1 M	12,170
	0.05 M	6229
Incubation times at room temperature	60 min	15,625
	50 min	14,985
	40 min	15,755
	30 min[c]	15,617
	20 min	14,733
	10 min	11,621
	Initial	4825
Homogenization buffer compositions and pHs	0.2 M Carbonate buffer (pH 9.2)[c]	15,617
	0.2 M Carbonate buffer (pH 8.5)	12,350
	0.2 M Carbonate buffer (pH 8.0)	11,922
	0.2 M Borate buffer (pH 9.0)	4871
	0.2 M Borate buffer (pH 8.5)	4988
	0.2 M Borate buffer (pH 8.0)	3717
Volumes for terminating the reaction	600 uL FA additive	13,560
	300 uL FA additive[c]	15,617
	150 uL FA additive	14,920

Each variable was evaluated by keeping the other optimization parameters constant under predefined ideal conditions by UHPLC-PDA analysis. UHPLC-PDA preliminary test confirmed predefined analytical conditions as; 30 min incubation time, 0.1 M carbonate buffer at pH 9.2, and 0.1 M Fmoc.Cl. [a]STR-Fmoc; 9-Fluorenylmethoxycarbonyl linked Streptomycin molecule as monitored derivatization product, [b]TIC; Total Ion Chromatogram, [c]Determined (ideal/final) conditions.

the reaction was also validated for extended concentrations.

The proper volume, pH, composition, and molarity of the extraction & dilution (homogenization) reagent to be used for the dilute and shoot method were also investigated. Honey has a pH between 3.5 and 5.0 and this acidic nature might mitigate the purposed derivatization. Buffering of the honey in alkaline conditions is urgent for high yielded *in situ* Fmoc-Cl derivatization since we know that at alkaline pH, the Fmoc reaction is rapid and robust (El-Enany et al., 2011). For this, we have tested 2 different alkaline buffer compositions to adjust the pH of the honey solutions. In the preliminary tests we performed with UHPLC-PDA, it was observed that carbonate buffer provided higher efficiency for all derivatizing agents in experiments using 3 different buffer systems (carbonate, borate, and TEA). For this reason, 0.1 M carbonate buffer at pH 9.2 was evaluated as the predefined conditions in the optimization tests to be carried out in the MS system. However, since this buffer concentration could not buffer the pH of the honey solution to the desired level, the concentration was increased to 0.2 M and compared with different pH values in the equivalent molarity of borate buffer. Reagents were interpreted based on maximum signals (peak areas) and recoveries. Besides, the homogenization reagent volume should be at a convenient volume to adjust the viscosity of the honey solution, which can pass through the syringe filter without backpressure and can be easily injected. On the other hand, determining volume should be sufficient to tolerate the detection limit. Higher dilutions can prevent the detection of the desired amounts. According to these assumptions, the volume of the 4 mL reagent was found to be the most appropriate. Peak areas and recoveries of AMGs pinpointed that the best extraction yields could be gained at pH 9.2 with carbonate buffer. 0.2 M of carbonate buffer successfully adjusted the pH of the final honey solution to 9.2 when 200 mg honey and 4 mL of homogenization buffer were preferred.

During the Fmoc-Cl derivatization reaction, Fmoc-OH as a by-product may be formed because of the alkaline environment created by the sodium carbonate buffer. A high concentration of the by-product suppresses the ionization and may cause a carry-over problem. To address this issue, after the incubation process, the derivative reaction should be stopped by lowering the alkaline pH. The stopping reagent was chosen as formic acid (FA), due to its MS-compatible volatile nature and the low amount of it was able to decrease the pH for quenching the derivatization reaction. The added amount of formic acid was gradually tested to find out the optimum volume and concentration which can reduce the pH but not too dilute the sample. For this 300 uL MS grade FA was determined as a sufficient amount. The overall comparison results as the calculated peak areas are given in Table 2.

3.3. Optimization of the mass spectrometry acquisition

An LC/MS-MS methodology is a sensitive technique for the simultaneous detection of AMGs in honey (Farouk et al., 2015). It has been experienced that the precursor ions are predominantly found as [M + H] + and they were chosen and fragmented by collision-induced dissociation (CID) to generate derivate-specific daughter ions under MS/MS conditions. The formation of at least 2 daughter ions for each compound under MS/MS conditions leads to the acquisition of SRM transitions. The compound-specific MS parameters were determined by directly infusing the (100 μg/L) derivatized standard solution of each compound using the fluidics mechanism at LC combined flow status. Mobile phase A and B (95:5, v/v) at initial condition was used to combine with standard infusion, and optimization of the SRM was conducted in positive ESI mode. Product ions were obtained by adapting the cone voltages and collision energies from the obtained precursor ions. The most abundant transitions from precursor ions were used for quantification, whereas other transitions were used for identification. STR derivatization assay was conducted initially to see Fmoc.Cl labeling efficiency under the non-optimized reaction conditions. The base peak at the MS spectrum can be seen as STR-Fmoc conjugate at 804.5 m/z. Unconjugated STR (582.3 m/z) and gas-phase fragments (179.2 m/z and 263.0 m/z) of the resulting Fmoc-linked STR product were also monitored which could be stated as a confirmative mass spectrum illustration of the predicted derivatization reaction. During the derivatization survey, excess STR and non-optimized conditions were used to see only the Fmoc efficient conjugation, and this situation was assessed as the reason to see free (unconjugated) STR in the MS spectrum. Identical reaction patterns were observed for DSTR and trimethoprim (IS) molecules (data not shown). We have chosen trimethoprim due to its guanidium-bearing structure. In the case of the occurrence of the trimethoprim residue in honey samples, alternative alkaloid-based guanidine functional group-bearing molecules such as guanadrel, guanoxan, guanethidine, or phenformin was also confirmed as surrogate standard (IS) alternatives. The illustration of the conspicuous masses obtained from the preliminary STR derivatization reaction is given as a mass spectrum in Fig. 2. As global parameters for MS analysis, desolvation temperature, gas flows (cone gas and desolvation gas), and positive ESI capillary needle voltage was optimized carefully. ESI capillary voltage, values at 3 kV, 2 kV, and 1 kV were tested, and the best signal was observed at 1 kV. Since the desolvation temperature and gas flows did not affect the signal intensity, to efficiently form gas-phase ions, we opted to use 450 °C desolvation temperature and kept the desolvation and cone gas flow rates at 850 L/h and 50 L/h respectively. A scheduled SRM acquisition was established according to the experimental retention times. Additionally, to overcome the matrix effects, we retained the analytes for more than 2 min, which gave us enough time to send

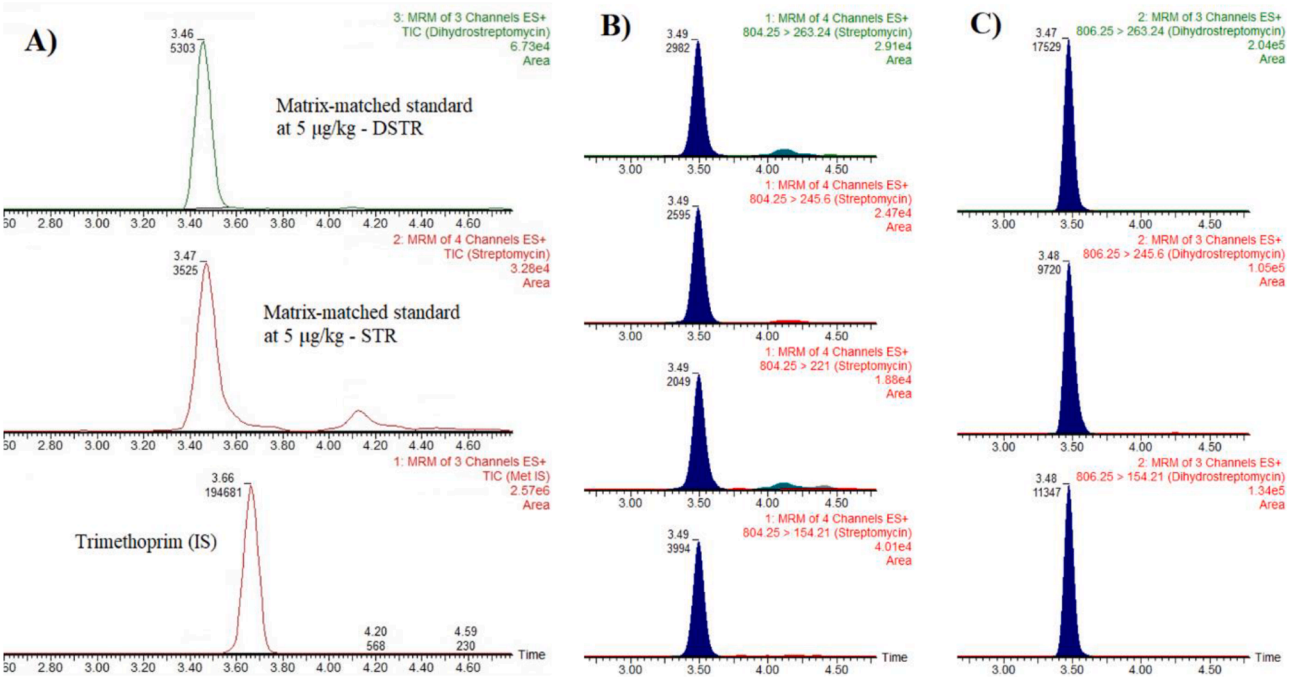


Fig. 3. Illustration of the matrix-matched calibration standard as chromatograms of STR and DSTR analysis at 5 µg/kg concentration. **A)** Total Ion Chromatograms (TICs) for STR, DSTR, and IS, **B)** Chromatograms of each mass transition for STR, **C)** chromatograms of each mass transition for DSTR.

hydrophilic to waste using the switching valve. Hence, we kept the divert valve at the waste position for 2 min and switched to the load position before compound elution.

3.4. LC method considerations

Particular attention has to be paid to combining the convenient LC conditions with MS detection to get improved ionization. Signal enhancement or suppression effects of the selected LC conditions were investigated and compared. Injection volume, column chemistry, column oven temperature, flow rate, and mobile phase compositions were evaluated and optimized to get the best responses. It was figured out that AMG-Fmoc conjugates tend to be ionized at positive polarization and in positive ESI the predominant adduct was in all cases the protonated molecule $[M + H] +$. Formic acid and acetic acid were tested at different concentrations as organic modifiers in the mobile phase to favor protonation. 0.005% of acetic acid additive was selected to increase the ionization signal. Various gradients using MeOH, ACN, and a mixture of them (50:50, v/v) were tested. It was deduced that ACN was decreasing the bandwidth and increasing the efficiency of the peaks. Whereas, ACN did not present a positive effect on ionization. Shortening the analysis time effect of ACN also seemed to be an advantage but ACN limited the time required to send the unwanted co-extracts to waste. For these reasons, we decided to combine MeOH and ACN at 50:50 (v/v) at mobile phase B composition is more reasonable. The injection volume was determined as 50 µL. Larger volume injections caused band broadening and tailing and also did not provide a linear peak area increment. Higher injection volumes were not preferred also due to the absence of pre-clean-up steps with the risk to introduce more matrix components into the column. 0.35 mL/min as flow rate was chosen which did not extend the analysis time much and generated higher theoretical plate numbers. In this study, a reversed-phase UHPLC column containing the C18 ligand was used as the selected chemistry. In terms of column physical properties, we used 2.1 mm i.d, 50 mm column length, and 1.7 µm silica particle size. Waters® UHPLC Acquity BEH C18 was determined as the most effective column among the UHPLC columns we tested in terms of retention time, peak shape, response, and run time. Column temperature at 40 °C presented appropriate separation resulting in the most ideal peak shapes at plausible backpressure. Representative chromatograms of quantitation and confirmation ions as a result of optimized LC conditions are given in Fig. 3.

3.5. Method performance

The selectivity of the methods was evaluated by analyzing honey samples from different botanical origins. SRM method allowed us to monitor each molecule with at least 2 fragments and this prevented false positiveness. Analytes had a good chromatographic resolution and matrix components did not cause any interference. The sensitivity of the method was established by determining the CCα and CCβ which were calculated from the lowest detectable limits. Mean CCα and CCβ values were 5.7 µg/kg and 6.2 µg/kg for STR and 5.8 µg/kg and 6.4 µg/kg for DSTR, respectively. For the analysis of AMGs in honey without any MRL value, these concentrations met the required sensitivity. These values are able to be improved by using a more sensitive MS instrument and the resulting MRPL for the analysis can be updated. Using matrix-matched standards, 6-points (5 µg/kg, 10 µg/kg, 15 µg/kg, 20 µg/kg, 50 µg/kg and 100 µg/kg) calibration curves (including blank) were prepared for the given method. To confirm the linearity of the curve, prepared concentrations of AMGs were plotted against the areas we obtained and regression equations were defined. It was seen that MS signals were linear over the range of 5 µg/kg to 100 µg/kg for the method and the correlation of coefficient values (R^2) was consistent (greater than0.99). Seven repetitions were made in 3 different concentrations for recovery/accuracy tests. It showed that both AMGs had acceptable recoveries with mean values of 102.6% and 101.3%. A precision study of the method was conducted as repeatability (intra-day repeatability) and reproducibility (inter-day repeatability). Relative standard deviations (RSD %) were between 1.5% and 9.7% for intra-day repeatability for all compounds including three concentration levels, while the RSD % values assessed at inter-day reproducibility studies were between 1.5% and 9.9%, covering the three spiking levels. In the ruggedness study, recoveries found were all above 99%. As for the robustness study, the method conditions were deliberately altered, and the reliability of the

Table 3
Summary of the method validation results.

Analytes	Sensitivity		Accuracy	Precision		Linearity	Robustness		Ruggedness
	CCα (μg/kg)	CCβ (μg/kg)	Mean recovery, % ($n = 7$), (5.0/20.0 /100.0 μg/kg)	Intra-day RSD_r %, ($n = 7$), (5.0/20.0 /100.0 μg/kg)	Inter-day RSD_R %, ($n = 7$), (5.0/20.0 /100.0 μg/kg)	R^2	Column temperature alteration (RSD%)	Flow rate alteration (RSD%)	Mean recovery (%) of condition, 1/2*
STR[a]	5.7	6.2	102.6	8.1/4.1/1.6	9.9/5.8/1.5	0.997	1.8	1.1	97.9/99.3
DSTR[b]	5.8	6.4	101.3	9.7/9.6/1.5	6.0/6.4/2.2	0.998	0.8	1.6	99.2/99.9

*Condition ½: analyst-1, column-1 and instrument-1/analyst-2, column-2 and instrument-2, [a]STR; Streptomycin, [b]DSTR; Dihydrostreptomycin.

methods was confirmed. Alterations of the column oven temperature values ± 10 °C (30 °C and 50 °C) and flow rates ± 10% (0.315 mL/min and 0.385 mL/min.) were tested and the deviation in results was reported. It was determined that method was robust and remained stable according to obtained insignificant RSD% values. The maximum RSD value occurred at STR with a value of 2.0%. According to the stability test we organized, prepared samples could remain stable at 4 °C for up to 1 week in amber glass vials, while at room temperature they could maintain their stability for a maximum of 24 h. We have also tested the method's accuracy by participating in inter-laboratory comparison (proficiency test scheme) studies in 2021. Two proficiency test materials from BIPEA were supplied and our findings were compared with conventional methodologies (ELISA & SPE-LC/MS). 109.2 μg/kg STR and 54.7 μg/kg STR residues were recorded respectively for the samples according to the novel method in which their assigned values were 103 μg/kg and 55.7 μg/kg consecutively. On the other hand, for DSTR residue, 145.2 μg/kg DSTR and 43.4 μg/kg DSTR were monitored for the samples with assigned values of 158 μg/kg and 52.4 μg/kg respectively. These values for each molecule were in the range of acceptance limits (z scores < 2.0) which were established according to the assigned values of the samples and method accuracy was confirmed by this approach. Results of the method performance study are listed as a whole in Table 3.

3.6. Sample analysis

A total of 110 honey samples from different botanical origins were analyzed. In those cases, in which a high concentration of AMG was detected out of the linearity range, the sample extract was properly diluted and reinjected, considering the dilution factor applied. The confirmation of the identification was accomplished on the basis of the retention times and the ratio of the intensities of at least the two most abundant product ions for AMGs. The areas of the most intensive product ions were used for the quantification. The ion ratios were also calculated and the reference values were set as an average of ion ratios of matrix-matched calibration standards. Each sample was analyzed in triplicate to get statistically verified results and the obtained data were expressed as means ± the standard deviation ($n = 3$). According to the analysis results acquired by means of the novel and validated method, STR residues were able to be detected above the CCα value in 5 samples, while elevated DSTR residues were observed above the CCα value in 27 samples. The highest residue level was determined as 1131.8 μg/kg DSTR. AMG residues were found in 29 out of 110 samples and this corresponded to 26.3% of the analyzed samples. Concurrent (STR with DSTR) residues were detected in 3 samples. It was seen that DSTR ($n = 27$) was predominantly found in honey rather than STR ($n = 5$) residues. The results are given in Table 1**S (S.I)**. The actual AMG residue problem in honey was enlightened with the use of novel method. Results proved that strict quality control approaches should be applied in terms of the determination of AMG residues and the described method in this study presents ease of use along with unbiased results.

4. Conclusion

In summary, the actual work presents a UHPLC-ESI-MS/MS based method which was developed and validated for concomitant monitoring of the STR and DSTR residues. By using UHPLC-MS/MS system, a much shorter, versatile analysis method has been developed. During method development, chromatographic conditions were optimized to get the best analysis efficiency. With the use of optimized, derivatization involving dilute and shoot protocol, sample preparation processes are minimized. Cost-effective and, straightforward analysis was also provided by means of a one-pot *in situ* aqueous derivatization approach. Moreover, a high-precise and simplified sample pretreatment protocol accompanied by high sensitivity gave superior monitoring capability in comparison to existing methods. The developed methodology was validated in terms of specificity, linearity, sensitivity, precision, accuracy, robustness, and ruggedness. The promising method met the criteria of the legislation in terms of method performance. Novel method could be successfully applied for the analysis of honey. Herein, STR and DSTR antibiotics were investigated in 110 samples, and the risks that they could pose in terms of food safety and public health were unveiled. 26.3% of the samples were labeled as AMG contaminated. This revealed that AMG residue is getting a worth noting obstacle to establishing honey quality. Hence, more susceptibility and improved beekeeping culture are necessary to circumvent this issue. Consequently, with the method proposed in this study, residue analysis of two main AMGs in honey can be carried out practically, rapidly, and precisely. Concordantly, this method will make a great contribution by serving as a template workflow for many laboratories in the determination of other drugs which have resembled molecular structures in various matrices.

CRediT authorship contribution statement

İsmail Emir Akyıldız: Writing – original draft, Methodology, Conceptualization. **Sezer Acar:** Methodology, Validation, Writing – original draft. **Ece Kök Yetimoğlu:** Conceptualization, Project administration. **Sinem Raday:** Investigation, Writing – review & editing. **Özge Erdem:** Resources, Visualization. **Dilek Uzunöner:** Investigation, Validation. **Emel Damarlı:** Supervision.

Declaration of Competing Interest

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

Data availability

The data that has been used is confidential.

Acknowledgments

The authors would like to thank Altıparmak Food Co. (İstanbul, Turkey) for the donation of the honey samples besides sharing their scientific experiences.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134826.

References

Aksoy, A. (2019). Simultaneous screening of antibiotic residues in honey by biochip multi-array technology, 6240-2019 *Medycyna Weterynaryjna, 75*(9). https://doi.org/10.21521/mw.6240.

Asakawa, D., Uemura, M., Sakiyama, T., & Yamano, T. (2018). Sensitivity enhancement of aminoglycosides in hydrophilic interaction liquid chromatography with tandem mass spectrometry by post-column addition of trace sodium acetate in methanol. *Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment, 35*(6), 1116–1126. https://doi.org/10.1080/19440049.2017.1388543

Barbosa, J., Moura, S., Barbosa, R., Ramos, F., & da Silveira, M. I. (2007). Determination of nitrofurans in animal feeds by liquid chromatography-UV photodiode array detection and liquid chromatography-ionspray tandem mass spectrometry. *Analytica Chimica Acta, 586*(1–2), 359–365. https://doi.org/10.1016/j.aca.2006.11.053

Chang, X., & Yu, Z. (2011). Determination of etimicin in rat plasma using 9-fluorenylmethyl chloroformate precolumn derivatization by HPLC with fluorescence detection. *Journal of the Brazilian Chemical Society, 22*, 1246–1252. https://doi.org/10.1590/S0103-50532011000700008

Chen, L., Mei, M., & Huang, X. (2017). Development of multiple monolithic fiber solid-phase microextraction and liquid chromatography-tandem mass spectrometry method for the sensitive monitoring of aminoglycosides in honey and milk samples. *Journal of Separation Science, 40*(21), 4203–4212. https://doi.org/10.1002/jssc.201700795

Commission, E. (2002). Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Communities, 50*, 8–36.

Commission, E. U. (2003). Commission Decision of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. Off J EU L, 71, 17-18.

Contin, M. D., Quinsaat, J. E., Negri, R. M., Tripodi, V. P., Opris, D., & Nb, d. a.. (2019). Development of carbohydrate functionalized magnetic nanoparticles for aminoglycosides magnetic solid phase extraction. *Analytica Chimica Acta, 1082*, 37–48. https://doi.org/10.1016/j.aca.2019.07.038

Driver, J. L., Thiex, N., Raynie, D., Ofitserova, M., & Pickering, M. (2009). Single-laboratory validation for the quantification of neomycin B and neomycin C in animal feeds by liquid chromatography fluorescence detection with post-column derivatization. *Journal of AOAC International, 92*(1), 34–41. https://www.ncbi.nlm.nih.gov/pubmed/19382560.

El Hawari, K., Daher, Z., Verdon, E., & Al Iskandarani, M. (2017). Impact of ion-pairs for the determination of multiclass antimicrobials residues in honey by LC-MS/MS. *Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment, 34*(12), 2131–2143. https://doi.org/10.1080/19440049.2017.1372641

El-Enany, N. M., Abdelal, A., & Belal, F. (2011). Spectrofluorimetric determination of sertraline in dosage forms and human plasma through derivatization with 9-fluorenylmethyl chloroformate. *Chemistry Central Journal, 5*(1), 1–8. https://doi.org/10.1186/1752-153X-5-56

European Commission. (2010). Commission Regulation 37/2010. *Official Journal of European Communities*, L15.

Farouk, F., Azzazy, H. M., & Niessen, W. M. (2015). Challenges in the determination of aminoglycoside antibiotics, a review. *Analytica Chimica Acta, 890*, 21–43. https://doi.org/10.1016/j.aca.2015.06.038

Glinka, M., Wojnowski, W., & Wasik, A. (2020). Determination of aminoglycoside antibiotics: Current status and future trends. *TrAC Trends in Analytical Chemistry, 131*, Article 116034. https://10.1016/j.trac.2020.116034.

Hubicka, U., Krzek, J., Woltyska, H., & Stachacz, B. (2009). Simultaneous identification and quantitative determination of selected aminoglycoside antibiotics by thin-layer chromatography and densitometry. *Journal of AOAC International, 92*(4), 1068–1075. https://doi.org/10.1093/jaoac/92.4.1068

Huidobro, A., Garcia, A., & Barbas, C. (2009). Rapid analytical procedure for neomycin determination in ointments by CE with direct UV detection. *Journal of Pharmaceutical and Biomedical Analysis, 49*(5), 1303–1307. https://doi.org/10.1016/j.jpba.2006.09.041

Husek, P., & Simek, P. (2006). Alkyl chloroformates in sample derivatization strategies for GC analysis. Review on a decade use of the reagents as esterifying agents. *Current Pharmaceutical Analysis, 2*(1), 23–43. https://doi.org/10.2174/157341206775474007

İsmail Emir, A., Ece, Y. K., Sinem, R., Sezer, A., & Özge, E. (2021). Validation of two UHPLC-MS/MS methods for fast and reliable determination of quinolone residues in honey. *Food Additives & Contaminants: Part A, 38*(5), 807–819. https://doi.org/10.1080/19440049.2021.1881621

Jiang, L., Wei, D., Zeng, K., Shao, J., Zhu, F., & Du, D. (2018). An enhanced direct competitive immunoassay for the detection of kanamycin and tobramycin in milk using multienzyme-particle amplification. *Food Analytical Methods, 11*(8), 2066–2075. https://doi.org/10.1007/s12161-018-1185-2

Jin, Y., Jang, J. W., Lee, M. H., & Han, C. H. (2006). Development of ELISA and immunochromatographic assay for the detection of neomycin. *Clinica Chimica Acta, 364*(1–2), 260–266. https://doi.org/10.1016/j.cca.2005.07.024

Khondker, A., Bider, R. C., Passos-Gastaldo, I., Wright, G. D., & Rheinstadter, M. C. (2021). Membrane interactions of non-membrane targeting antibiotics: The case of aminoglycosides, macrolides, and fluoroquinolones. *Biochimica et Biophysica Acta, Biomembranes, 1863*(1), Article 183448. https://doi.org/10.1016/j.bbamem.2020.183448

Kim, Y. R., & Kang, H.-S. (2021). Multi-residue determination of twenty aminoglycoside antibiotics in various food matrices by dispersive solid phase extraction and liquid chromatography-tandem mass spectrometry. *Food Control, 130*, Article 108374. https://doi.org/10.1016/j.foodcont.2021.108374

Liu, C., Jiang, Y., Xiu, L., Qian, R., Zhao, M., Luo, P., … Jiang, W. (2021). Ultratrace analysis of neomycin residues in milk at femtogram levels by flow-through immunoaffinity chromatography test. *Food Analytical Methods, 14*(11), 2298–2307. https://doi.org/10.1007/s12161-021-02058-5

Machado De-Melo, A. A., Almeida-Muradian, L. B.d., Sancho, M. T., & Pascual-Maté, A. (2018). Composition and properties of Apis mellifera honey: A review. *Journal of Apicultural Research, 57*(1), 5–37. https://doi.org/10.1080/00218839.2017.1338444

McGlinchey, T. A., Rafter, P. A., Regan, F., & McMahon, G. P. (2008). A review of analytical methods for the determination of aminoglycoside and macrolide residues in food matrices. *Analytica Chimica Acta, 624*(1), 1–15. https://doi.org/10.1016/j.aca.2008.05.054

Megoulas, N. C., & Koupparis, M. A. (2004). Enhancement of evaporative light scattering detection in high-performance liquid chromatographic determination of neomycin based on highly volatile mobile phase, high-molecular-mass ion-pairing reagents and controlled peak shape. *Journal of Chromatography A, 1057*(1–2), 125–131. https://doi.org/10.1016/j.chroma.2004.09.052

Mohammadi, B., Tammari, E., Fakhri, S., & Bahrami, G. (2013). Applicability of LC–MS/MS to optimize derivatization of topiramate with FMOC-Cl using reacted/intact drug ratio. *Journal of Chromatography B, 928*, 32–36. https://doi.org/10.1016/j.jchromb.2013.02.041

Moreno-Gonzalez, D., Lara, F. J., Jurgovska, N., Gamiz-Gracia, L., & Garcia-Campana, A. M. (2015). Determination of aminoglycosides in honey by capillary electrophoresis tandem mass spectrometry and extraction with molecularly imprinted polymers. *Analytica Chimica Acta, 891*, 321–328. https://doi.org/10.1016/j.aca.2015.08.003

Ortelli, D., Edder, P., & Corvi, C. (2004). Analysis of chloramphenicol residues in honey by liquid chromatography–tandem mass spectrometry. *Chromatographia, 59*(1), 61–64. https://doi.org/10.1365/s10337-003-0132-5

Pang, G.-F., Zhang, J.-J., Cao, Y.-Z., Fan, C.-L., Lin, X.-M., InlI, Z.-Y., & Jia, G.-Q. (2004). Evaluation of analyte stability and method ruggedness in the determination of streptomycin residues in honey by liquid chromatography with post-column derivatization. *Journal of AOAC International, 87*(1), 39–44. https://doi.org/10.1093/jaoac/87.1.39

Peru, K. M., Kuchta, S. L., Headley, J. V., & Cessna, A. J. (2006). Development of a hydrophilic interaction chromatography–mass spectrometry assay for spectinomycin and lincomycin in liquid hog manure supernatant and run-off from cropland. *Journal of Chromatography A, 1107*(1–2), 152–158. https://doi.org/10.1016/j.chroma.2005.12.057

Sachetelli, S., Beaulac, C., & Lagacé, J. (1998). Aminoglycoside detection using a universal ELISA binding procedure onto polystyrene microtiter plates in comparison with HPLC analysis and microbiological agar-diffusion assay. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1379*(1), 35–41. https://doi.org/10.1016/S0304-4165(97)00079-2

Saluti, G., Diamanti, I., Giusepponi, D., Pucciarini, L., Rossi, R., Moretti, S., … Galarini, R. (2018). Simultaneous determination of aminoglycosides and colistins in food. *Food Chemistry, 266*, 9–16. https://doi.org/10.1016/j.foodchem.2018.05.113

Shimanuki, H., & Knox, D. A. (2000). Diagnosis of honey bee diseases. *Agriculture Handbook (Washington)*, 690 rev.

Tölgyesi, Á., Barta, E., Sohn, M., & Sharma, V. K. (2018). Determination of antimicrobial residues in honey by liquid chromatography tandem mass spectrometry. *Food Analytical Methods, 11*(8), 2043–2055. https://doi.org/10.1007/s12161-018-1166-5

Tsikas, D., Sandmann, J., Holzberg, D., Pantazis, P., Raida, M., & Frolich, J. C. (1999). Determination of S-nitrosoglutathione in human and rat plasma by high-performance liquid chromatography with fluorescence and ultraviolet absorbance detection after precolumn derivatization with o-phthalaldehyde. *Analytical Biochemistry, 273*(1), 32–40. https://doi.org/10.1006/abio.1999.4209

Xu, N., Qu, C., Ma, W., Xu, L., Xu, L., Liu, L., … Xu, C. (2011). Development and application of one-step ELISA for the detection of neomycin in milk. *Food and Agricultural Immunology, 22*(3), 259–269. https://doi.org/10.1080/09540105.2011.569882

Yang, B., Wang, L., Luo, C., Wang, X., & Sun, C. (2017). Simultaneous determination of 11 aminoglycoside residues in honey, milk, and pork by liquid chromatography with tandem mass spectrometry and molecularly imprinted polymer solid phase extraction. *Journal of AOAC International, 100*(6), 1869–1878. https://doi.org/10.5740/jaoacint.16-0399

Young, M. S., van Tran, K., Goh, E., & Shia, J. C. (2014). A rapid SPE-based analytical method for UPLC/MS/MS determination of aminoglycoside antibiotic residues in bovine milk, muscle, and kidney. *Journal of AOAC International, 97*(6), 1737–1741. https://doi.org/10.5740/jaoacint.13-153

Ziegler, J., & Abel, S. (2014). Analysis of amino acids by HPLC/electrospray negative ion tandem mass spectrometry using 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) derivatization. *Amino Acids, 46*(12), 2799–2808. https://doi.org/10.1007/s00726-014-1837-5