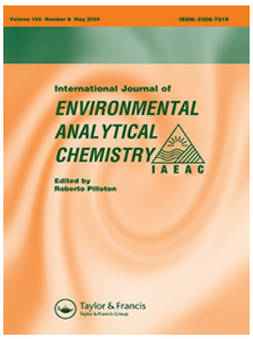

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Development and validation of a multiclass method for the determination of veterinary drug residues in honey

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ABSTRACT

This study developed and validated an analytical method for simultaneous identification and quantification of 41 veterinary drugs in honey using liquid liquid extraction (LLE) and liquid chromatography coupled to quadrupole-Orbitrap high-resolution mass spectrometry (LC-Q-Orbitrap HRMS). The method was validated in accordance with Commission Implementing Regulation (CIR) EU 2021/808 at five different concentrations ranging from 0.075 to 50 µg/kg. The mean recoveries ranged from 70 to 105, while repeatability values were all below 17%. The linearity, as correlation coefficients (R^2) ranged from 0.994 to 1. The limits of detection (LOD) and limits of quantification (LOQ) were in the range of 0.006–3.92 µg/kg and 0.011–6.54 µg/kg, respectively. The decision limit (CCα) and detection capability (CCβ) ranges were 0.0759–5.33 µg/kg and 0.0804–6.08 µg/kg, respectively. Of the 263 honey samples that were collected from local markets in Egypt, 47.5% had antibiotic residues. The mean concentration (µg/kg) and detection frequency (%) of the five most frequently detected antibiotics in the honey samples were as follows: trimethoprim (143.25 µg/kg and 39.9%), sulfamethoxazole (136.69 µg/kg and 30.7%), sulphadiazine (77.19 µg/kg and 18.6%), tylosin (184.37 µg/kg and 18.2%), and ciprofloxacin (185.33 µg/kg and 7.60%). The applicability of the developed method was proven through successful three proficiency testing (PT). The proposed method was demonstrated to be reliable for the simultaneous analysis of multi-class veterinary drugs in honey.

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Antibiotic residues; honey; LC-Q-Orbitrap HRMS; food safety

1. Introduction

The extensive availability of bioactive components and distinctive physicochemical features of honey have played a significant role in its wide utilisation as both a medicinal component and a prevalent substance for food and flavour enhancement [1,2]. The medicinal properties of honey are largely attributed to plant-derived chemicals, such as flavonoids and polyphenols, which are contingent upon certain floral sources and types of bees [3]. Honey contains phytochemicals that have antioxidant, anticancer, anti-inflammatory, immunomodulatory, cardiovascular, and antibacterial properties, making it

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a valuable natural product [4]. Honey can be susceptible to various chemical contaminants, including inorganic pollutants such as lead, cadmium, mercury, and arsenic, which can compromise its quality and safety for consumers [5]. However, concerns about the safety of honey have arisen due to the presence of various harmful organic pollutants, including pesticides and antibiotics, as documented in numerous studies [4,6,7]. Antibiotics are commonly employed in animal husbandry to manage infections and encourage growth [8]. In beekeeping, antibiotics are also frequently used to control infectious diseases among honeybees. Protozoa, fungi, viruses, and bacteria pose a hazard to bee populations. American and European foulbrood are caused by the gram-positive bacteria *Penibacillus larval* and *Melissocccus plutonius* [9]. The antibiotics penicillin, streptomycin, chloramphenicol, and aureomycin were among the most effective against *P. larvae*. Even while *P. larvae* only infect larvae, the antibiotic is consumed by non-target adult bees following preventive treatment [10]. In addition to endangering honeybee health, antibiotic use in beekeeping can promote the emergence and enrichment of antibiotic resistance in the microbiome of honeybees and their environments [11,12]. Consequently, the irrational use of antibiotics in beekeeping may result in the presence of antibiotic residues in bee-derived products, including honey. Residual antibiotics in honey can have negative consequences on human health, such as toxic responses and allergies, promote antibiotic resistance in the human microbiome, or disturb the human microbiome, leading to a variety of immunological and metabolic problems [8]. To assess and reduce the potential hazards to human health, an urgent investigation of antibiotic residues in honey is required. The conventional techniques employed for the detection of antibiotic residues in honey exhibit several limitations, including time-consuming procedures, labour-intensive processes, and the inability to concurrently detect a variety of antibiotics [13]. Thus, it is essential to develop a rapid, highly responsive, and cost-effective method for the quantification of several categories of antibiotics present in honey [7]. Several techniques such as liquid – liquid extraction (LLE), QuEChERS, and solid-phase extraction (SPE) have been used to prepare samples for antibiotic analysis [13]. On the analytical side, Alcántara-Durán et al. [14] point to ultra-performance liquid chromatography coupled to tandem mass spectroscopy (UPLC-MS/MS) as the method of choice for fast, targeted analysis in high-throughput environments. Yang et al. [15] developed a rapid and sensitive method for detecting antibiotic residues in honey using a modified QuEChERS extraction and UPLC-MS/MS analysis. The authors suggested that their method could be used for routine monitoring of honey quality and safety. However, when considering the broader applications of mass spectrometry techniques in food safety, Orbitrap mass spectrometry has a substantial advantage over UPLC-MS /MS due to its remarkable high-resolution mass spectra capabilities when combined with a variety of chromatography techniques. Accurate mass measurements, structural information, and the determination of previously identified chemicals in complicated combinations are its strong suits [16]. Additionally, quadrupole Orbitrap (Q-Orbitrap) and high-resolution mass spectrometry (HRMS) have enhanced the data acquisition speed and resolution significantly [7,17–20]. These techniques have also improved MS detection, allowing for the identification and quantification of more analytes in complex matrices. When compared to traditional unit-mass-resolution tandem mass spectrometry, HRMS has a number of advantages. This technology enables the acquisition of full-scan spectra, which allows for a thorough analysis of the sample's composition. Moreover, it enables the detection of compounds without requiring any prior tuning specific to each compound. Monitoring

pollutants in honey is essential to provide quantitative data on dominant pollutants, comply with regulations, contend with antibiotic resistance human health issues. Most studies in Egypt to date [21–23] have focused on the analysis of limited numbers and few classes of veterinary drugs with infrequent sampling. Our study addresses these gaps by developing and validating a novel analytical method capable of identifying and quantifying 41 veterinary drugs in honey. This extends the scope of contaminants beyond what has been traditionally monitored, thus contributing to a more comprehensive understanding of the potential risks associated with veterinary drug residues. The method's robustness, coupled with its ability to detect low levels of residues, positions it as a crucial tool for regulators and public health officials tasked with safeguarding food quality and protecting ecosystem health. By implementing the latest Commission Implementing Regulation (CIR) EU 2021/808 guidelines for validation, our method sets a new standard for reliability and compliance in residue analysis. We introduce a pre-treatment method based on the liquid liquid extraction (LLE) procedure. This method is complemented by the development and validation of a quantitative technique for the detection and quantification of forty-one veterinary drugs in honey from different nine families. Our approach utilises liquid chromatography coupled with quadrupole-Orbitrap high resolution mass spectrometry (LC-Q-Orbitrap HRMS).Furthermore, our research makes a contribution to honey safety assurance by gathering a significant quantity of samples from the Egyptian market. The findings contribute to evaluating the health implications of antibiotic residues in a widely consumed natural product renowned for its nutritional and therapeutic benefits. The presence of these residues raises concerns regarding consumer health, which is of particular importance given the scarcity of studies conducted in this specific area within the Egyptian context.

2. Materials and methods

2.1. Chemicals and reagents

All the analytical standards of veterinary drugs utilised in this work were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and were of high purity (95%). The LC-MS grade acetonitrile (ACN) and methanol (MeOH) were purchased from CARLO ERBA (Milan, Italy) and Merck (Darmstadt, Germany), respectively. The chemicals used in this study were citric acid monohydrate, ammonium hydroxide, ammonium acetate, pure formic acid (99%), and ethylenediaminetetraacetic acid di-sodium salt (Na_2EDTA), all obtained from Sigma-Aldrich (Darmstadt, Germany). The acquisition of ultra-pure water was accomplished by the utilisation of a MilliQ UF-Plus system, manufactured by Millipore in Germany. Stock solutions of individual compounds were prepared in ACN (1000 µg/mL), which were then stored in glass bottles at a temperature of −18°C, within their designated validity period. The mixed standard of working solution used in the experiment was diluted and then stored at a temperature of −4°C.

2.2. Apparatus

The centrifuge was obtained from Hermle (Gosheim, Germany). Similarly, the rotary evaporator was supplied by Heidolph (Schwabach, Germany). The pH-metre was obtained

from Mettler Toledo (Greifensee, Switzerland) and calibrated before being used with certified calibration standards, including pH 4, pH 7, and pH 10.

2.3. Chromatographic conditions

The chromatographic separation procedure was performed utilising a Thermo Scientific Vanquish High Performance Liquid Chromatography system (Thermo Scientific, Bremen, Germany). The separation technique employed a reversed-phase ZORBAX Eclipse Plus C18 column (150 mm length × 4.6 mm inner diameter; 5 µm particle size, Agilent Technologies, Santa Clara, CA, USA). Thermo Hypersil GOLD aQ (100 mm length × 2.1 mm inner diameter; 1.9 µm particle size), Thermo Accucore VDX (100 mm length × 2.6 mm inner diameter; 1.9 µm particle size). In the positive mode of HPLC, eluent A consisted of an aqueous solution containing 0.1% formic acid, while eluent B comprised ACN with 0.1% formic acid. The gradient process started with 5% eluent B for 0.3 minutes, followed by a linear increase to 100% over 7 minutes. This condition was maintained for 4 minutes. The system then reverted to 5% eluent B in 0.1 minute and was re-equilibrated for 3 minutes, resulting in a cumulative run time of 14 minutes. A flow rate of 0.5 mL/min was used. In the negative mode for HPLC, eluent A was an aqueous solution containing 2 mM ammonium acetate, and eluent B was ACN with 0.1% formic acid. The gradient procedure started with 20% eluent B for 0.2 minutes, then increased linearly over the course of 3 minutes to 100% B. This state persisted for a minute. After 2.5 minutes of re-equilibration, the system returned to 20% B (total run time: 6.5 minutes). The sample temperature was held at 25°C, the injection volume was 10 µL, and the column temperature was kept at 40°C. The flow rate was set at 0.8 mL/min in the negative mode.

2.4. MS conditions

The Orbitrap mass spectrometer utilised in this study was the Q-Exactive model (Thermo Scientific, Bremen, Germany). It was equipped with a heated electrospray ionisation (HESI) source, capable of operating in both positive and negative ionisation modes. The HESI temperature was set to 350°C, while the capillary temperature was set to 325°C. The electrospray voltage was set to 3.75 kV for positive ionisation mode and 3.20 kV for negative ionisation mode. The value of the S-lens was adjusted to 50 volts. The sheath and auxiliary gas flows were adjusted to 50 and 12 arbitrary units, respectively. The automatic gain control (AGC) was configured to 3.10^6, and the maximum injection time (IT) was set to 200 ms. Full scan data were acquired in both the positive and negative ionisation modes at a mass resolving power of 70,000 full width at half maximum (FWHM), with an m/z scan range of 120–2000 and Full MS/vDIA as the scan type. The resolution was set at 70,000 for Full MS and 35.000 for vDIA. Data acquisition and processing were performed using TraceFinder (version 4.1) software (Thermo Fisher Scientific, Bremen, Germany).

2.5. Targeted compounds

The 41 analytes are listed in Table S1, which contains detailed information such as compound names, exact precursor masses, characteristic fragment ions, mass accuracy, and molecular formula. The mass differences that were measured experimentally were

consistently less than 2 mg/kg, and all 41 analytes showed either protonated molecules ([M + H]+) in the positive ion mode or deprotonated molecules ([M-H]-) in the negative ion mode.

2.6. Sample preparation

Honey samples were obtained from local markets. For a typical test, 2.00 ± 0.02 g of homogenised samples were placed into 50 mL polypropylene centrifuge tubes. Subsequently, 1.0 mL each of 1.0 M sodium citrate ($Na_3C_6H_5O_7$) and 0.5 M sodium EDTA (Na_2EDTA) were added. The samples were then extracted twice with 20 mL of ACN (divided into two 10 mL portions); Then mechanically shaken for 3 minutes at 700 rpm. a 30-minute ultrasonication period was necessary. Following a 10-minute centrifugation at 4500 rpm at 4°C, the combined supernatants were transferred to a 50 mL flask and evaporated at 40°C using a rotary evaporator. The residue was reconstituted into 2.0 mL of 10 mM ammonium formate at pH 2.78. The final solution was filtered through a disposable 0.45 µm PTFE membrane filter into an amber glass vial and then injected into the HPLC-Orbitrap HRMS for analysis.

2.7. Method validation

The method validation was conducted in accordance with the CIR EU 2021/808 [24]. Linearity was comprehensively assessed in both the solvent and matrix. The validation process included a thorough examination of key parameters, such as precision (encompassing repeatability and within-laboratory reproducibility), recovery (trueness), decision limit (CCα), detection capability (CCβ), as well as LOD, LOQ, and matrix effects. The CCα concentration levels established for our target analytes are as follows: 2.5 µg/kg for most compounds, 0.75 µg/kg for the nitroimidazole group, and 5 µg/kg and 0.075 µg/kg for florfenicol and chloramphenicol, respectively. CCβ was calculated based on the determined CCα values. Five concentration levels were applied across honey matrices. The spike range for most compounds is 2.5 to 50 µg/kg, enabling accurate quantification. In some cases, the nitroimidazole group has a narrower spike range (0.75 to 15 µg/kg) due to lower concentrations. While honey samples may vary, florfenicol has a wider spike range of 5 to 100 µg/kg. In contrast, chloramphenicol, typically found in low concentrations, to reflect the sensitivity of this method, the spike range for chloramphenicol was precisely established from 0.075 to 1.5 µg/kg, which is well within the ultra-trace concentration levels of interest for honey safety compliance. For each spike level, six replicates were analysed on the same day, with the use of matrix-matched calibration curves. This process was repeated over three different days, introducing variations in time, operator, and the calibration status of the LC-HRMS/MS equipment.

According to the CIR 2021/808 [24], for analytes with no specified MRLs, CCα should be calculated by analysing at least 20 representative blank materials for honey to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. For CCβ, each concentration level of 20 fortified blanks from honey shall be analysed in order to ensure a reliable basis for this determination. The concentration level, where only ≤ 5% false compliant results remain, equals the detection capability of the method.

Matrix effects (ME) were conducted to measure the extent of ion suppression or enhancement. The calculation of the ME involved the division of the slopes of the matrix-matched calibration curves (Slope M) by the slopes of the calibration curves produced using ACN solvent (Slope S), as expressed by the following equation: ME% = 100 × ((Slope M)/(Slope S)) − 1

This quantifies how the matrix affected the analyte analysis. A result of 100% indicates the absence of any matrix influence. On the other hand, outcomes over 100% indicate ion enhancement, while results below 100% indicate ion suppression. Positive values boost the ion signal, while negative values suppress it. The importance of addressing the ME to accurately measure multiple veterinary drug classes in different matrices. Thus, matched matrix calibration was used in this study. The ruggedness, or robustness, of an analytical procedure reflects its ability to withstand minor intentional changes in its parameters without affecting the outcome. Ruggedness provides an indication of the method's reliability during normal usage. The ruggedness of the method was mainly tested through systematic variation of shaking time, sonication temperature, and ammonium formate pH, which demonstrate the method's reliability in the face of experimental fluctuations.

3. Results and discussion

3.1. Optimization of LC conditions

The chromatographic analysis employed three different types of columns: Thermo Hypersil GOLD aQ (100 mm × 2.1 mm, 1.9 µm), Thermo Accucore VDX (100 mm × 2.6 mm, 1.9 µm), and the ZORBAX Eclipse Plus C18 column (150 mm × 4.6 mm, 5 µm). During the utilisation of Thermo columns for separation, some compounds displayed reduced response rates, and in some cases, the separation of isomers was challenging. Several isomers share identical precursor ions, with values of 311.08085 for sulfadimethoxine and sulfadoxine, 281.07029 for sulfamethoxypyridazine and sulfamonomethoxine, 268.07504 for sulfamoxol and sulphisoxazole, and 445.16054 for doxycycline and tetracycline. This similarity in precursor ions posed challenges in their separation, as illustrated in Figure S1.

Nevertheless, employing the ZORBAX column enabled the complete separation of these compounds, effectively resolving the isomeric mixture. After conducting a comparative analysis of the separation of isomeric compounds, achieving baseline separation, and determining retention times (RTs) across the three columns for the 41 compounds, the ZORBAX column consistently exhibited superior performance. The majority of these compounds showed sharper peaks and increased response levels when analysed with the ZORBAX column. Consequently, this column was selected for the present study due to its ability to provide high resolution, allowing for the effective separation of compounds with closely similar properties [25]. Figure 1 displays representative chromatograms.

In high-performance liquid chromatography (HPLC), the column packing material plays a central role. It improves HPLC performance and affects chromatographic separation by interacting with the analyte [26]. Thus, carefully selecting and packing this material is crucial for the best separation results. The ZORBAX C18 column is composed of silica particles with C18 (octadecyl) hydrocarbon chains [27]. These chains produce

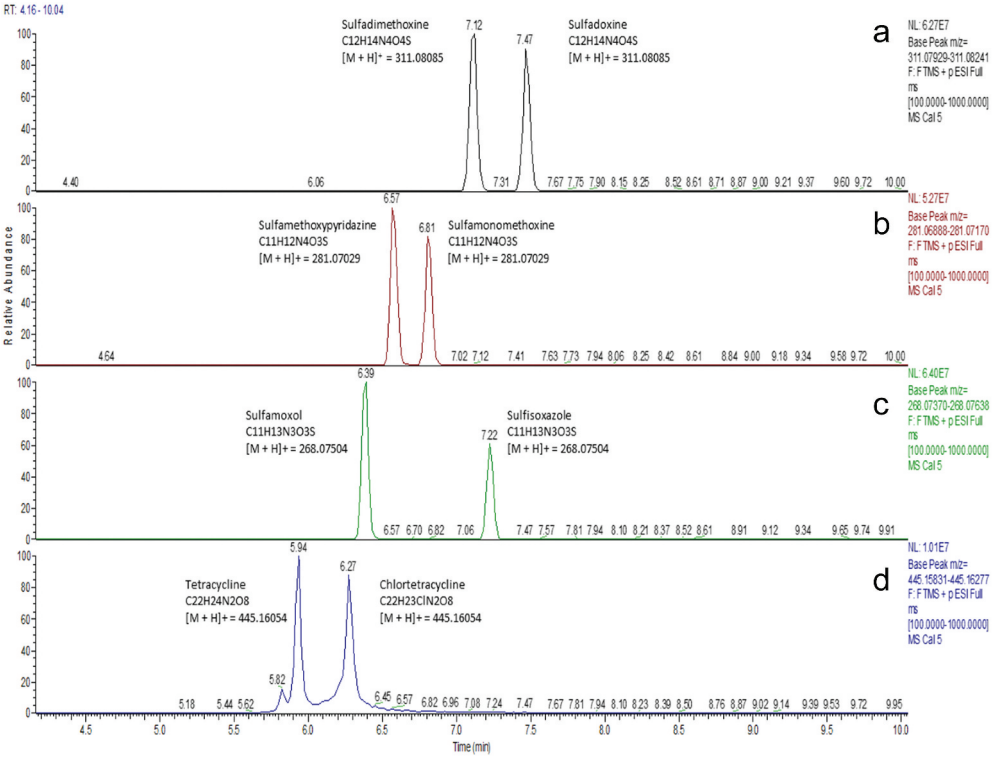

Figure 1. The ZORBAX column enables effective separation of antibiotic isomers having the same m/z a) Sulfadimethoxine and sulfadoxine (311.08085), b) Sulfamethoxypyridazine and sulfamonomethoxine (281.07029), c) Sulfamoxol and sulphisoxazole (268.07504), and d) Doxycycline and tetracycline (445.16054).

a hydrophobic column. Non-polar molecules interact more strongly with the non-polar stationary phase (C18 chains) and spend more time attached to the column, resulting in prolonged retention. However, polar molecules travel through the column faster and interact less with the stationary phase. The polarity of sulphonamides, tetracyclines, penicillins, macrolides, nitroimidazoles, cephalosporins, and amphenicoles ranges from modest to high. When these various groups are placed into a ZORBAX C18 column with a polar mobile phase, each molecule interacts differently with the stationary phase. This separation process achieves the best precision in a short time.

3.2. Optimization of mobile phase

For our optimisation procedure, we tested ACN, MeOH, and a 50:50 (v/v) mixture of the two. ACN proved to be more sensitive and had better peak shapes. ACN is employed for the extraction of compounds with moderate polarity and non-polar compounds that possess a high degree of lipophilicity [28]. To improve ionisation, we added 0.1% formic acid to ACN. Previous studies have also employed formic acid in ACN as a modifier for the mobile phase to enhance the ionisation efficiency and sensitivity of analytes that undergo positive ionisation [17,29].

We explored the addition of ammonium formate and ammonium acetate to the mobile phase, aiming to improve peak shape and intensity for each target compound. However, chromatographic profiles demonstrated negligible changes to the peak shapes of the target compounds following the addition of these salts. To streamline the experimental process, we decided to exclude salts from the mobile phase. Penney et al. [30] and Barreto et al. [31] employed ammonium acetate in the mobile phase to achieve optimal peak shapes and enhance ionisation efficiency. Furthermore, we noticed that 2 mM ammonium acetate improved the peak shapes and ionisation efficiency of chloramphenicol and florfenicol in negative ionisation mode. This decision was critical for identifying and quantifying chloramphenicol and florfenicol residues. Formic acid and ammonium acetate buffered the pH of the mobile phase. Since pH changes impact antibiotic ionisation states and chromatographic behaviour, consistency is essential for ionisation and separation. To better separate isomers, gradient elution was chosen. Our LC gradient was fine-tuned to efficiently separate all substances. For hydrophilic interference elution, we started the gradient with a 95% aqueous phase. The aqueous phase was then gradually decreased from 95% water with 0.1% formic acid to 100% ACN with 0.1% formic acid to clean the column and prevent carry-over. For continuous sample analysis, we returned the aqueous phase to 95%.

3.3. Optimization of Q-Orbitrap HRMS parameters

The Q-Exactive Orbitrap/MS instrument was operated in Full MS/vDIA scanning mode, utilising both positive and negative ion modes. The initial full mass scan was employed for screening and quantifying target compounds, as well as for retrospective analysis of unknown substances. To confirm the identity of the target compound, the generation of fragment ions was crucial. When a target compound was identified and its signal intensity surpassed the predefined threshold, it was selected using the quadrupole and directed to the higher-energy collision dissociation collision cell through the C-trap for fragmentation [32]. All resulting fragments from the collision cell were gathered within the C-trap and subsequently introduced into the Orbitrap mass analyser [33]. This workflow allows for a comprehensive analysis of the compounds. It initiates with a full MS scan, followed by a series of data-independent scans focusing on fragment ions with applied fragmentation energy. Detailed information for the 41 analytes, including compound names, exact precursor masses, characteristic fragment ions, mass accuracy, and molecular formula, is presented in Table S1. All of the 41 analytes displayed either protonated molecules ($[M + H]^+$) in the positive ion mode or deprotonated molecules ($[M - H]^-$) in the negative ion mode, with experimentally measured mass differences of less than 2 ppm.

3.4. Optimization of the preparation procedure

In our multi-residue veterinary drug analysis, the extraction is critical due to the diverse characteristics of the analytes. We have optimised a modified QuEChERS method, eliminating sample clean-up while adding an evaporation step. This approach involves using an organic solvent, adding salt for partitioning analytes, and removing polar matrix compounds. Unlike methods that use additional clean-up stages like SPE after LLE [34–37], our method aimed to use LLE without extra clean-up stages to reduce both costs and

analysis time. However, to address the potential damage to the chromatographic column and contamination of the mass spectrometry system due to sample co-extractives, a potential solution to this issue was to use smaller injection volumes, a cost-effective and straightforward method that could potentially reduce matrix effects and co-extractives interference [38]. In this context, we investigated three injection volumes: 2.0, 5.0, and 10.0 µL. Notably, the injection of a 10.0 µL extract yielded the smallest coefficient of variation (CV) and provided a satisfactory response. This sample preparation method proved to be highly efficient in extracting target compounds, offering a swift and eco-friendly process with reduced solvent residues, distinguishing it from other approaches involving SPE. We evaluated the impact of different volumes of ACN – 5 mL, 10 mL, and 20 mL – on the recovery rates of the extracted samples. Using 5 mL of ACN led to low recoveries for most analytes. Increasing the volume to 10 mL improved the recovery of most analytes, but it remained below 60% for sulphonamides, nitroimidazoles, and penicillins. When the ACN volume was further increased to 20 mL, it resulted in stable and high recovery rates, with the mean relative standard deviation (RSD) for most analytes remaining below 20%. The improved recoveries can be attributed to the enhanced solvation capacity and the dilution of matrix effects, which are crucial for reducing ion suppression and enhancement during mass spectrometric analysis. The larger volume of ACN likely disrupts the analyte-matrix interactions more effectively, facilitating better solubilisation and extraction of the compounds. Given the balance between extraction efficiency and the economic and time costs associated with larger solvent volumes, 20 mL of ACN was selected as the optimal volume. This choice reflects a compromise that maximises recovery rates while maintaining reasonable processing times and costs (Figure S2).

To enhance our extraction process, we added a 0.5 M Na_2EDTA solution. Sodium EDTA boosts remarkable chelating properties, effectively preventing the formation of insoluble complexes involving divalent and trivalent metal ions. This, in turn, guarantees unhindered tetracycline extraction [39]. Notably, Na_2EDTA simultaneously preserves the stability of tetracyclines during extraction by shielding them from potential degradation in the presence of metal ions [40]. Hence, the use of 0.5 M Na-EDTA in the extraction process enhances the overall efficiency, resulting in higher recovery rates of tetracycline residues from honey samples. Despite its advantages, the use of Na_2EDTA was not without challenges, such as observed precipitation and clogging of the chromatographic system. The inclusion of Na_2EDTA in the extraction solvent was necessitated due to its powerful metal-chelating ability, which facilitates the complete release of tetracycline from samples [41]. As a practical solution to the issues encountered, we restricted the quantity of 0.5 M Na_2EDTA in the extraction solvent to 1 mL while still maintaining highly efficient tetracycline extraction. Based on the literature by Ye et al. [42], it was found that incorporating sodium citrate into the extraction process yielded the most favourable outcome in terms of recovery. Additionally, we carefully added 1 M sodium citrate to our extraction process. This choice was supported by sodium citrate's chelating, pH buffering, and antibiotic solubility properties. Sodium citrate, a mild acid, kept the extraction pH constant, enhancing antibiotic extraction efficiency. It also increased antibiotic solubility, improving extraction efficiency. This extraction used only 1 mL of 1 M sodium citrate. Sodium hydroxide and citric acid were carefully added to alter the pH of the sodium citrate solution. We avoided mixtures with a pH below 4.0 as they failed to generate dual phases within the concentration range of the phase-forming components [43]. According to

Frenich et al. [44], it was found that the primary-secondary amine (PSA) utilised in extraction has the ability to adsorb quinolones and tetracyclines, leading to decreased recoveries. As a result, we decided to exclude the use of PSA in our research. After adding sodium citrate, Na_2EDTA, sodium chloride, and ACN, the sample was agitated. We conducted three distinct trials to ascertain the optimal duration of shaking. The first attempt featured 700 rpm shaking for 1 minute. In the next trial, shaking lasted 2 minutes at the same speed. In the third trial, shaking at 700 rpm was increased to 3 minutes. We observed the highest recovery rate in the third trial. Thus, we concluded that shaking the sample for 3 minutes at 700 rpm yielded the most efficient extraction. Subsequently, the samples were subjected to centrifugation to facilitate the separation of the supernatant. This process was carried out for a duration of 10 minutes at a speed of 4500 rpm.

3.5. Method validation

According to CIR 2021/808 [24], the method was validated as a quantitative confirmatory method to test the concentration range in which the method would be applicable for quantitative determination.

3.5.1. Identification

During both the development and validation stages of this method, as well as the analysis of actual samples, the analytes were positively identified and confirmed when the retention time (RT), precursor ion, and product ion met the established criteria. Four pairs of epimers were successfully discriminated by their differing retention times, despite having identical molecular precursor ions and similar transition products, as shown in Table S1.

3.5.2. Selectivity

Analytical methods must distinguish analytes from closely similar compounds. This property depends on the measuring technique, the analyte class, and the matrix. The exact mass accuracy and low background noise of Orbitrap technology improve specificity. Twenty blank honey samples were analysed for interfering chemicals to verify the method's selectivity. Additionally, the retention times of the target compounds showed no interference peaks. While Figure S3 show that matrix effects are not substantial enough to significantly alter the quantification of analytes, matrix-matched calibration was employed to provide an additional layer of accuracy and to account for any minor matrix effects that may not be immediately apparent. This approach is in line with best practices for ensuring robust and reliable quantitative results in complex sample analysis.3.5.3 Linearity, limits of detection (LODs), and limits of quantification (LOQs)

The matrix-matched standard calibration curves displayed neat linearity across various concentration ranges for different antibiotic groups in honey. Calibration curves were designed in accordance with the Minimum Method Performance Requirements (MMPR) at five different concentration levels. The correlation coefficients (R^2) for all the matrix-matched standard calibration curves were found to be greater than 0.994. All compounds demonstrated a range of 2.5–50 µg/mL, except the nitroimidazoles group, which showed a range of 0.75–15 µg/mL; florfenicol ranged from 5 to100 µg/mL, and chloramphenicol had a range of 0.075 to 1.5 µg/mL, as shown in Table 1.

The LODs and LOQs were determined by analysing six blank samples spiked according to analyte sensitivity. The LODs ranged from 0.006 µg/kg for chloramphenicol to 3.92 µg/kg for ampicillin, while the LOQs ranged from 0.011 µg/kg for chloramphenicol to 6.54 µg/kg for ampicillin, as shown in Table 1, despite their complex matrices.

3.5.3. Matrix effects

High-viscosity honey contains sugars, proteins, and a variety of phytochemicals. Antibiotic residue extraction can be difficult due to honey's viscosity and heterogeneity. During sample preparation, these components may mix with analytes, affecting ionisation efficiency. Analysis can be skewed by the matrix effect, caused by endogenous substances. Using the standard calibration curve from a pure solvent to detect veterinary drugs highlights this deviation [45]. Our study quantitatively evaluates the ME, which compares solvent and matrix-matched standards. Within a honey matrix, all analytes showed

Table 1. Linearity, range, limits of detection (LOD), and limits of quantification (LOQ) of the 41 target analytes in honey.

Analyte	R^2	Range (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Sulfacetamide	0.9996	2.5–50	0.18	0.30
Sulfachloropyridazine	0.9997	2.5–50	0.45	0.75
Sulfadiazine	0.9987	2.5–50	0.35	0.58
Sulfadimethoxine	0.9995	2.5–50	0.30	0.51
Sulfadoxine	0.9960	2.5–50	0.47	0.79
Sulfaguanidine	1.0000	2.5–50	0.26	0.44
Sulfamerazine	0.9966	2.5–50	0.19	0.32
Sulfamethazine	0.9997	2.5–50	0.32	0.54
Sulfamethizol	0.9958	2.5–50	0.35	0.58
Sulfamethoxazole	0.9990	2.5–50	0.41	0.68
Sulfamethoxypyridazine	0.9957	2.5–50	0.47	0.79
Sulfamonomethoxine	0.9977	2.5–50	0.61	1.02
Sulfamoxol	0.9984	2.5–50	0.32	0.54
Sulfanilamide	0.9980	2.5–50	0.62	1.04
Sulfapyridine	0.9979	2.5–50	0.38	0.63
Sulfathiazole	0.9994	2.5–50	0.27	0.45
Sulfisoxazole	0.9985	2.5–50	0.32	0.54
Ciprofloxacin	0.9994	2.5–50	0.29	0.49
Enrofloxacin	0.9994	2.5–50	0.29	0.49
Flumequine	0.9984	2.5–50	0.28	0.47
Oxolinic acid	0.9990	2.5–50	0.27	0.45
Sarafloxacin	0.9974	2.5–50	0.35	0.59
Chlortetracycline	0.9999	2.5–50	0.35	0.58
Doxycycline	0.9946	2.5–50	0.48	0.81
Oxytetracycline	0.9971	2.5–50	0.46	0.77
Tetracycline	0.9946	2.5–50	0.81	1.36
Ampicillin	1.0000	2.5–50	3.92	6.54
Penicillin V	0.9940	2.5–50	0.41	0.69
Erythromycin	0.9981	2.5–50	0.45	0.75
Tylosin	0.9948	2.5–50	0.42	0.71
Dimetridazole	0.9996	0.75–15	0.10	0.18
Dimetridazole-OH	0.9999	0.75–15	0.69	1.15
Ipronidazole	0.9994	0.75–15	0.10	0.16
Ipronidazole-OH	0.9997	0.75–15	0.19	0.32
Metronidazole	0.9990	0.75–15	0.17	0.39
Ronidazole	0.9974	0.75–15	0.80	1.34
Tinidazole	0.9985	0.75–15	0.08	0.14
Ceftiofur	0.9988	2.5–50	0.35	0.72
Trimethoprim	0.9990	2.5–50	0.08	0.13
Florfenicol	0.9998	5–100	2.98	5.84
Chloramphenicol	0.9962	0.075–1.5	0.006	0.011

suppression between −43% and −2%. Ibronidazole and metronidazole showed enhancements of 3% and 11%, respectively, as shown in Table 2. The calibration curves were constructed using five concentration levels to ensure sufficient sensitivity and accuracy across the expected range of analyte concentrations in the samples. For most analytes, the calibration levels were set at 2.5, 5, 10, 20, and 50 µg/kg. For the nitroimidazoles group, levels were adjusted to 0.75, 1.5, 3, 6, and 15 µg/kg. For florfenicol, concentrations were at 5, 10, 20, 40, and 100 µg/kg. For chloramphenicol, the concentration levels were set at lower values of 0.075, 0.150, 0.3, 0.6, and 1.5 µg/kg. For precise quantification of multi-class veterinary drugs in honey, neutralising matrix effects are essential. Thus, this study used matched matrix calibration to account for these effects.

Table 2. Recovery, coefficient of variation (CV) pooled, matrix effect (ME), decision limits (CCα), and detection capability (CCβ) in honey.

Analyte	Recovery (%)	CV, pooled (%) Repeatability	CV, pooled (%) Reproducibility	ME	CCα (µg/kg)	CCβ (µg/kg)
Sulfacetamide	99	12	7	−35	2.66	3.91
Sulfachloropyridazine	96	9	10	−31	2.69	3.89
Sulfadiazine	96	14	9	−31	2.62	3.93
Sulfadimethoxine	91	10	11	−13	2.70	3.81
Sulfadoxine	95	11	10	−35	2.72	3.99
Sulfaguanidine	77	13	12	−11	2.68	3.01
Sulfamerazine	89	13	11	−28	2.68	3.95
Sulfamethazine	94	13	13	−19	2.67	3.99
Sulfamethizol	94	11	8	−28	2.69	3.12
Sulfamethoxazole	98	13	7	−15	2.71	3.02
Sulfamethoxypyridazine	90	14	13	−23	2.73	3.89
Sulfamonomethoxine	97	11	12	−20	2.79	3.08
Sulfamoxol	89	8	12	−11	2.59	3.10
Sulfanilamide	96	15	13	−22	2.75	3.07
Sulfapyridine	92	13	9	−21	2.70	3.94
Sulfathiazole	90	10	8	−7	2.71	3.03
Sulfisoxazole	95	8	11	−13	2.59	2.97
Ciprofloxacin	81	11	9	−18	2.74	3.11
Enrofloxacin	94	10	8	−19	2.71	3.09
Flumequine	94	7	13	−21	2.78	3.14
Oxolinic acid	94	9	8	−39	2.64	3.19
Sarafloxacin	86	11	8	−28	2.60	2.98
Chlortetracycline	93	14	13	−43	2.80	3.33
Doxycycline	100	14	11	−12	2.85	3.24
Oxytetracycline	84	15	9	−40	2.71	3.28
Tetracycline	83	17	14	−23	2.78	3.17
Ampicillin	80	10	13	−13	2.69	3.07
Penicillin V	93	14	12	−42	2.78	2.97
Erythromycin	100	9	11	24	2.88	3.35
Tylosin	105	13	13	−27	2.62	2.99
Dimetridazole	95	12	14	−8	0.759	0.790
Dimetridazole-OH	98	12	13	−10	0.758	0.871
Ipronidazole	93	10	11	3	0.757	0.768
Ipronidazole-OH	99	8	10	−2	0.757	0.870
Metronidazole	94	9	11	11	0.760	0.779
Ronidazole	92	11	10	−31	0.755	0.780
Tinidazole	70	8	9	−28	0.761	0.799
Ceftiofur	78	12	11	−9	2.70	3.44
Trimethoprim	103	15	14	−18	2.64	2.99
Florfenicol	85	12	13	−2	5.33	6.08
Chloramphenicol	82	12	11	−16	0.0759	0.0804

3.5.4. Trueness and precision

The precision of this method, characterised in terms of repeatability and within-laboratory reproducibility, was evaluated using blank samples from the honey matrix. These samples were spiked at five different levels, with a total of 18 samples spiked per level. These samples were analysed across three different days, utilising standard solutions freshly prepared daily. Recovery rates ranged from 70% to 105%, and the coefficients of variation (CVs) did not exceed 17%. The data, consolidated in the corresponding Table 2, underscores the method's high degree of accuracy.

3.5.5. CCα and CCβ

In this work, the values of CCα and CCβ for every target compound were also estimated, and Table 2 summarises the results. The CCα values varied from 0.0759 to 5.33 µg/kg, whereas the CCβ values ranged from 0.0804 to 6.08 µg/kg. The ranges presented here illustrate the effectiveness of the developed method in detecting honey samples.

3.5.6. Ruggedness

In evaluating the ruggedness of our method, we conducted trials under varied conditions to ensure reliable performance. For shaking time, we tested durations of 2, 3, and 4 minutes, sonication temperature trials were carried out at 45°C, 50°C, and 55°C, and the pH of the ammonium formate dilution solvent was adjusted to 2.76, 2.78, and 2.80. The results, which are detailed in Table S2, demonstrated notable stability in the method's performance, with RSD measuring 8% for shaking time, 7% across temperature variations, and 9% in response to pH adjustments. These results underscore the method's ruggedness, effectively sustaining reliable operation through the tested parameter ranges. These findings affirm the method's robustness, withstanding variations within the tested ranges without significant impact on its efficacy.In Table S3, we provide a comparative analysis of our method relative to nine other established methods [24,45–52]. Our technique simplifies the extraction process and yields lower LOD and LOQ compared to those achieved by the other methods. Additionally, it produces recovery values that are closely aligned with the expected outcomes.

3.6. Real samples analysis

To verify the feasibility of the established technique, an analysis was conducted on a total of 263 samples of honey obtained from different local markets in Egypt to detect veterinary drug residues.

Our study utilised a diverse collection of honey samples sourced from various local markets and apiaries throughout Egypt. The honey samples included a diverse range of floral sources commonly consumed in Egypt, including but not limited to clover, citrus, and cotton. The primary goal of our sampling approach was to broadly assess the presence of veterinary drug residues in Egyptian honey, rather than to link contamination levels to specific honey types or production areas. A total of 13 out of 41 antibiotics were found in 47.5% of the honey samples, as shown in Table 3. The detection frequencies varied from 0.38% to 39.9%. 47.5% of collected honey samples from local markets were contaminated with antibiotic residues. The mean concentration (µg/kg) and detection frequency (%) of the five

Table 3. Veterinary drug residues in honey samples ($n = 263$; positive samples = 125) collected from Egyptian retail markets.

	Range				Frequency[a]	
Analyte	Minimum	Maximum	Mean (µg/kg)	SD	No.	(%)
Chlortetracycline	8.51	61.9	27.94	21.45	5	1.90
Ciprofloxacin	5.68	606.67	185.33	243.26	20	7.60
Doxycycline	5.1	32.3	15.15	14.92	3	1.14
Enrofloxacin	12.48	250	90.56	108.21	4	1.52
Erythromycin	20.42	–	20.42	0.0	1	0.38
Oxolinic acid	<LOQ	<LOQ	–	–	1	0.38
Oxytetracycline	7.52	158.46	74.19	62.02	5	1.90
Sulfadiazine	5.93	1512.5	77.19	216.70	49	18.6
Sulfamethazine	8.34	354.35	70.15	102.59	15	5.70
Sulfamethoxazole	5.59	682.86	136.69	162.83	81	30.7
Tetracycline	10.34	47.52	26.97	15.02	5	1.90
Trimethoprim	2.83	864.29	143.25	188.03	105	39.9
Tylosin	7.67	1980	184.37	359.54	48	18.2

[a]Frequency (%) indicates the proportion of samples in which the compound was detected, out of the total number of samples analysed.

most frequently detected antibiotics in the honey samples were as follows: trimethoprim (143.25 µg/kg and 39.9%), sulfamethoxazole (136.69 µg/kg and 30.7%), sulphadiazine (77.19 µg/kg and 18.6%), tylosin (184.37 µg/kg and 18.2%), and ciprofloxacin (185.33 µg/kg and 7.60%).

The results obtained are in line with the study examining the Rapid Alert System for Food and Feed (RASFF) notifications for honey from 2002 to 2022 by Eissa and Taha [53]. Veterinary medicinal product residues made up 79.64% of all hazards, exclusively from beekeeping. Chloramphenicol, streptomycin, sulphathiazole, tylosin, and sulphadimidine were the most commonly reported contaminants. Furthermore, Ahmed et al. [54] introduced a validated multi-residue method to detect sulphonamides and tetracyclines in honey samples from Egypt, Libya, and Saudi Arabia using HPLC – MS/MS and HPLC – DAD. Egyptian, Saudi Arabian, and Libyan samples were 57.6%, 75%, and 77.7% positive, respectively. Sulphonamide antibiotics predominated in Egypt and Saudi Arabia, while tetracyclines dominated in Libya.

3.7. Application to a previously analysed PT sample

As part of the Food Analysis Performance Assessment Scheme (FAPAS), proficiency testing (PT) samples round 02463,02491 and 02499 were analysed to validate assay performance and calculation methodology. In round 02463, the optimised assay confirmed chloramphenicol and florfenicol concentrations of 0.33 µg/kg and 25.9 µg/kg, respectively. For the 02499 PT round for the nitroimidazole group and found dimetridazole and dimetridazole-OH concentrations of 1.12 µg/kg and 1.76 µg/kg, the 02499 round for the sulphonamide group found the three analyte concentrations of sulphamethazine, sulfamethoxypyridazine, and sulphathiazole at 11.72, 22.63, and 18.78, respectively. For the 02499 PT round focusing on the nitroimidazole group, we detected concentrations of 1.12 µg/kg for dimetridazole and 1.76 µg/kg for dimetridazole-OH. In the 02499 PT round targeting the sulphonamide group, the concentrations of sulphamethazine, sulfamethoxypyridazine, and sulphathiazole were found to be 11.72 µg/kg, 22.63 µg/kg, and 18.78 µg/kg, respectively. There were no findings for the other analytes, as shown in Table S4.

All results produced satisfactory outcomes within the acceptable range of Z-scores, where the absolute value of Z is less than 2. The Z-score shows the degree to which a particular value deviates from the standard deviation. The amount of standard deviations a given data point resides above or below the mean is known as the Z-score, or standard score. In simple terms, the standard deviation represents the degree of variability present in a certain data collection. The Z-score was computed for the obtained results and was found to be within the acceptable range of $|z| < (2, -2)$, as specified by the FAPAS reports.

4. Conclusions

The study presents LC-Q-Orbitrap HRMS technology to quantify 41 antibiotics in honey and a simplified pre-treatment method based on LLE. The developed method was highly reliable for the Full MS/vDIA scanning mode, with accurate masses of the parent ions and fragment ions. The procedure for analytical separation and detection significantly reduced the time needed for analysis and increased sample throughput. Ensuring high method sensitivity and the ability to determine residues in the range from 0.075 to 50 µg/kg. The validation procedure included selectivity, linearity, LOD, LOQ, trueness, repeatability, reproducibility, CCα, and CCβ. All validation parameter values met the intended use and established criteria. The developed method was successfully applied for the analysis of three proficiency testing (PT), and real honey samples, including 125 positive samples out of 263 samples, were detected. The method provides an integrated strategy for the screening and quantification of multi-class, multi-residue veterinary drugs in an effective manner. Continuous monitoring studies on the presence of veterinary drug residues in honey should be conducted on a regular basis to figure out their origins, particularly beekeeping practices, and to implement preventive and remedial strategies.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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