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Determination of nitroimidazole and fumagillin residues in honey employing Liquid Chromatography-Tandem Mass Spectrometry: An insight in the 2023-2024 Greek honey production

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Summary Honey bees (*Apis* sp.) are vital to ecosystems, enhancing agricultural productivity and preserving biodiversity. However, the observed decline of their populations, caused by, among other, Nosema infections has led to the use of the antibiotics fumagillin and nitroimidazoles. Nonetheless, due to increasing concerns over their genotoxic, mutagenic, and carcinogenic properties, the presence of fumagillin residues in honey is prohibited, while similar is the case for the parent nitroimidazoles and their metabolites in animal-derived food. Within this context and considering the limited availability of pertinent data on Greek honey, we have developed and applied robust analytical methods for the detection of fumagillin and nitroimidazole residues in honey, employing liquid chromatography-tandem mass spectrometry (LC-MS/MS). The developed protocols, based on solid phase extraction, proved fit for the purpose of detecting fumagillin and nitroimidazoles in honey samples with substantial sensitivity (detection capability, CCβ not exceeding 0.78 µg/kg), comparable to recent literature, and could be applied in routine analyses to ensure consumers' safety. Application of the methods in 30 Greek honey samples did not unveil residues above the CCβ of the analytes, and the developed pipeline can be further exploited in future large-scale monitoring studies to investigate Greek apiculture and its adherence to regulatory obligations.

Additional keywords: Dimetridazole, LC-MS/MS, Metronidazole, Ronidazole, Solid Phase Extraction, Veterinary antibiotics

Introduction

Honey bees is a subset of bees in the genus *Apis* that are indispensable to ecosystems and agriculture, mainly due to their exceptional pollination abilities, which not only enhance agricultural productivity and yield, but also safeguard biodiversity conservation (Galajda *et al.*, 2021). Nonetheless, over the last two decades, a decline in honey bee populations has been observed worldwide, a multifaceted issue driven by several biotic and abiotic factors, including, among others, the reduction of their natural habitats, climate change, exposure to pesticides, as well as various diseases caused by pests and pathogens (Goulson *et al.*, 2015).

Microsporidian parasites of the genus *Nosema*, and more specifically the species *Nosema apis*, *Nosema ceranae*, and the recently detected *Nosema neumanni*, cause one of the most devastating honey bee diseases, nosemosis (Mazur and Gajda, 2022). These parasites infect the gut epithelial cells of adult bees causing symptoms such as dysentery, reduced lifespan, and decreased foraging efficiency. Infected bees often show signs of lethargy, poor coordination, and inability to return to their hive. The disease can result in colony collapse, diminished honey production, and increased winter mortality (Grupe and Quandt, 2020; Hristov *et al.*, 2020). Prevention and treatment of nosemosis include regular monitoring and a combination of hive management practices

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and chemical treatment, with the most used antibiotics being fumagillin and nitroimidazoles (NIs) (Formato *et al*., 2022).

Fumagillin is a mycotoxin initially reported in 1949 by Hanson and Eble, who isolated it from the filamentous fungus *Aspergillus fumigatus* (Hanson and Eble, 1949). It is a meroterpenoid of low molecular weight (458.54 g·mol^{-1}), with poor water solubility, whereas it is soluble in organic solvents (Guruceaga *et al*., 2019). In the early 1950s, not long after its discovery, fumagillin was recognized as a bioactive compound against *Nosema* infections and has been used extensively since (Peirson and Pernal, 2024; Stanimirovic *et al*., 2007; van den Heever *et al*., 2015a). Its mode-of-action involves its binding to the active site of the methionine aminopeptidase type 2 (MetAP-2) protein through the epoxide located on its cyclohexane moiety (Van den Heever et al., 2014). Since the MetAP-2 enzyme plays a key role in protein synthesis, this inhibition disrupts essential cellular processes, leading to impaired growth and replication of the *Nosema* spores within the honey bees' guts (Guruceaga *et al*., 2019).

On the other hand, NIs is a group of antibiotics discovered in the early 1950s when researchers isolated azomycin, a 2-nitroimidazole compound, from a *Streptomyces* extract (Nakamura, 1955). This discovery spurred the synthesis of an array of derivatives with remarkable bioactivity, mainly against anaerobic bacteria, protozoa, and parasites (Ang *et al.,* 2017). Their chemical structure is characterized by the presence of an imidazole ring with a nitro group attached to it in different positions (Guo *et al*., 2017). Synthetic 5-nitroimidazole (5-NI) derivatives, such as dimetridazole, metronidazole, ronidazole, and ipronidazole, have been extensively exploited in the prevention and management of nosemosis in honey bees (Zhou *et al*., 2007; Mitrowska *et al*., 2014). These metabolites seem to exert their antimicrobial properties due to the presence of the nitro group in their molecule, which generates reactive nitro radical anions, inhibiting microbial nucleic acid synthesis and leading to cell death (Ang *et al*., 2017).

Despite the effectiveness of fumagillin and NIs in combating Nosema disease in honey bees, the potential presence of their residues in honey can pose a potential risk to consumer health. Fumagillin is believed to be genotoxic, which can be a threat to both beekeepers and consumers of honey-containing residues (Stanimirovic *et al*., 2007), and therefore, it is no longer authorized in most European Union (EU) member states (European Commission 2009; Kunat-Budzyńska *et al*., 2022). Since no maximum residue limit (MRL) has been established for honey, any detectable fumagillin residue levels are prohibited, adhering to a zero-tolerance policy (Nozal *et al*., 2008). Likewise, NIs are considered mutagenic and carcinogenic to humans and thus, they are classified at the group A6 of prohibited substances (European Commission 1996). Consequently, their use in food-producing animals is not allowed in the EU under Regulation 2377/90, depicted also for dimetridazole, metronidazole, ronidazole and other NIs in EU Regulation 37/2010 (European Commission 2010). Also, metabolites formed by hydroxylation of the main NIs (e.g., hydroxy metronidazole and hydroxy ipronidazole) could display similar mutagenic properties and, as a result, EU Reference Laboratory's recommended concentration for analytical methods to determine parent NIs and their metabolites in animal-derived food is suggested at 3 μg·kg-1 (CRL 2007).

Based on the abovementioned, there is an increasing need for the generation of analytical data on contaminants in honey due to its broad consumption and acknowledgment of health benefits. In this context, the development and application of sensitive analytical methods for the detection of a wide range of levels of NIs and fumagillin in honey, for the discovery of any illegal uses that may occur, is of high importance. Such a task requires the development and validation of sophisticated sample preparation and analytical protocols. Within this context, and focusing on consumers' safe-

ty, here, enhanced experimental protocols and robust analytical methods were developed for the detection of fumagillin and NI residues in honey based on Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and Solid Phase Extraction (SPE). The methods were applied to Greek honey samples collected during the period 2023-2024. Greek honey has exceptional quality with pronounced biological properties (Patouna *et al*., 2023), which is attributed to the unique plant diversity of the country and respective chemical constituents (Karabagias *et al*., 2014; Kasiotis *et al*., 2022). Therefore, the prevalence of these compounds in this valuable food commodity should be contemplated. To our knowledge, there is a shortage of data on monitoring the presence of such chemicals in Greek apicultural products.

Materials and methods

1. Chemicals and Reagents

All chemicals and reagents used in the sample preparation and analysis were of the highest commercially available grade. More specifically, the organic solvents, methanol (MeOH) and acetonitrile (ACN) (purity ≥ 99.9%) were purchased from Fisher Chemical (Thermo Fisher Scientific, Waltham, MA, USA). Ultrapure water was obtained by an Ultra-Clear® TWF UV ultra-clear water system (Evoqua Water Technologies, Pittsburgh, PA, USA). Acetic acid (purity ≥99.9%) was purchased from Merck (Merck KGaA, Darmstadt, Germany), and formic acid (purity ≥99.9%) from Honeywell Research Chemicals (Morris Plains, NJ, USA). Magnesium sulfate and sodium chloride were obtained from Fisher Chemical (Thermo Fisher Scientific, Waltham, MA, USA). The ammonium hydroxide solution that was used in the sample preparation for nitroimidazole analysis was prepared by diluting the desired amount of ammonia (Merck KGaA, Darmstadt, Germany) in ultrapure water.

For method development and analyses, the analytical standards fumagillin (purity 93%, Toronto Research Chemicals, North York, ON, Canada), metronidazole (MNZ) and dimetridazole (DMZ) (purity ≥ 98.0%, Sigma-Aldrich Solutions, St. Louis, MO, USA), ronidazole (RNZ) (purity ≥ 95%, Sigma-Aldrich Solutions, St. Louis, MO, USA), ipronidazole (IPZ) (purity 99.5%, Dr. Ehrenstorfer Reference Materials, LGC Standards, Teddington, Middlesex, UK), hydroxymetronidazole (MNZ-OH) (purity ≥ 99.0 %, Sigma-Aldrich Solutions, St. Louis, MO, USA), hydroxydimetridazole (DMZ-OH) (purity 98%), hydroxyipronidazole (IPZ-OH) (purity 99.14%), and ternidazole hydrochloride (TRZ) (purity 86.0%) (Dr. Ehrenstorfer Reference Materials, LGC Standards, Teddington, Middlesex, UK), were used. Ipronidazole D3 (IPZ-D3) (purity 99.47%) (Dr. Ehrenstorfer Reference Materials, LGC Standards) served as the internal standard for the LC-MS/MS analysis of NIs.

2. Preparation of standard solutions

Stock solutions were prepared using the analytical standards listed in section 2.2, in ACN for fumagillin and in MeOH for NIs, which were stored at –20° C until further use. Appropriate dilutions of the stock solutions of the analytical standards were performed to obtain the corresponding working solutions for experimentation. For the construction of calibration curves, solutions of the analytical standards were prepared to final concentrations ranging from 0.5 to 250 ng/mL in ACN for fumagillin, and MeOH for NIs.

3. Honey sample preparation

The developed experimental protocols for the detection of fumagillin and NIs in honey samples were based on Solid Phase Extraction (SPE), applying minor modifications to previously published protocols (Dmitrovic and Durden, 2013; Mitrowska and Antczak, 2017; Li *et al.,* 2018), such as the use of mixed-mode SPE (but not in dispersion), the incorporation of salts in the extraction step for NIs, and the Biotage VacMaster 20 device (Biotage, Uppsala, Sweden).

For fumagillin, initially, a portion of hon-

ey (5 g) was weighed in 50-mL falcon tubes and 10 mL of ultrapure water were added. Samples were then extracted using an automatic axial extractor (Agytax®, AgytaxLab, Madrid, Spain) for 10 min, taking advantage of the magnetic-axial movement under controlled conditions, and centrifuged (Hermle Z446K, Hermle Labortechnik GmbH, Wehingen, Germany) for 15 min at 4500 rpm, at ambient temperature. SPE was performed employing Phenomenex Strata-X cartridges (60 mg, 3 mL) (Phenomenex, Torrance, CA, USA), which were conditioned with 5 mL MeOH and 5 mL ultrapure water prior to sample loading. Afterwards, the cartridges were washed with 10 mL of a water-MeOH mixture (60:40, v/v), and were dried under vacuum. The elution step was conducted using 2 portions of 2 mL of ACN, and the solutions were evaporated to dryness in a rotary evaporator (Heidolph WB 2001, Schwbach, Germany). Dried samples were reconstituted in 1 mL ACN and filtered (13 mm, 0.22 μm, Membrane Solutions, Seattle, Shanghai) into 2 mL amber glass autosampler vials (Macherey-Nagel, Düren, Germany). All handling was performed under a low light regime to avoid potential fumagillin degradation.

For NIs, 2 g of honey were weighed in 50-mL falcon tubes and the internal standard (IS) ipronidazole D3 was added. Samples were then extracted with 15 mL of a 2% (v/v) acetic acid solution in an ultrasonic bath (Isolab Laborgeräte GmbH, Eschau, Germany) for 10 min. Afterwards, 0.5 g of magnesium sulfate and sodium chloride (4:1 w/w) were added to the sample, the mixture was hand-shaken for 1 min, placed again for 2 min in an ultrasonic bath (Grant Instruments, Shepreth, Cambridgeshire, UK), and the resulting extract was centrifuged for 5 min at 4000 rpm (Hettich Universal 32 R Centrifuge, Tuttlingen, Germany) with constant temperature at 10°C. Then, the supernatant was passed through Oasis MCX (Mixed Mode Cation Exchange, for interaction with MNZ see Figure 1, and for similar interaction Dugheri *et al*., 2017) cartridges (6 mL) (Waters Corporation, Milford, MA, USA). The cartridges had been previously conditioned with 5 mL MeOH followed by 5 mL of the 2% acetic acid solution. The same solvent system in reverse order was used for cartridge washing. Elution was performed with 5 mL of an ACN-ammonium hydroxide (25% v/v) mixture in a 95:5 v/v ratio, and evaporation followed in a SpeedVac vacuum concentrator (HyperVac VC2124, Gyrozen, Gimpo, South Korea). Reconstitution was performed with 0.5 mL of the following system: 0.1% (v/v) aqueous solution of formic acid: 0.1% (v/v) ACN solution of formic acid. The reconstituted solutions were filtered into inserts (Macherey-Nagel, Düren, Germany) and placed into glass autosampler vials (2 mL).

4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis

For method development and honey sample analysis for the detection of fumagillin and NIs, two instruments were employed, with the key parameters for their LC-MS/MS analyses being demonstrated in Table 1. Regarding fumagillin analysis, elution was performed in a mobile phase (A):(B) 30:70 (v/v) ratio, whereas for NIs, the following gradient elution was applied: 5% of the mobile phase B was held for 1 min, increased to 40% at 1 min and held for 4 min. Then, linearly decreased within 0.5 min to initial conditions and held for 10 min for equilibration of pump pressure. The precursor and productions of the analytes are presented in Ta-

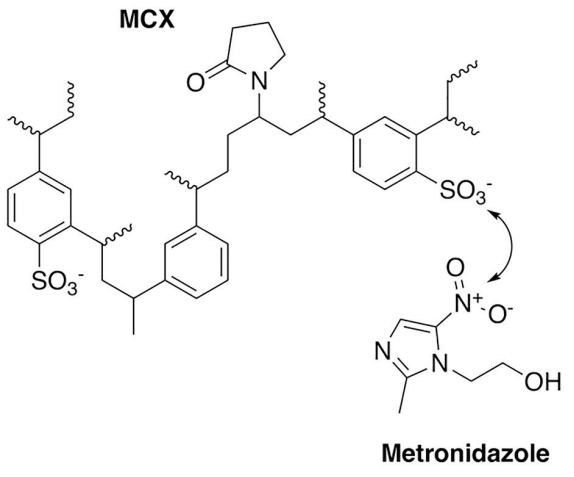

Figure 1. Metronidazole interacting with MCX sorbent.

Table 1. LC-MS/MS equipment and parameters employed for fumagillin and nitroimidazole analysis (AF: Ammonium formate, FA: Formic acid, MRM: Multiple Reaction Monitoring, ESI^+: positive Electron Spray Ionization).

Instrument & Parameters \ Analyte	Fumagillin	Nintroimidazoles
Instrument	Agilent Technologies 6410 Triple Quad LC/MS system	Shimadzu 80/60NX Triple Quad LC-MS/MS system
Column Type	Agilent reversed phase Zorbax Eclipse XDB-C18	reversed phase Kinetex
Column Dimension	150 mm x 2.7 mm, 3.5 μm	150 mm x 2.1 mm, 2.6 μm
Column Temperature	30°C	35°C
Injection volume	10 μL	10 μL
Flow rate	0.3 $mL{\cdot}min^{-1}$	0.25 $mL{\cdot}min^{-1}$
Mobile phase composition	(A) H2O 2mM AF (B) ACN: H2O 90:10 (v/v) 2mM AF	(A) H2O 0.1% FA (B) ACN0.1% FA
Elution mode	Isocratic	Gradient
MS/MS parameters	MRM mode ESI^+	MRM mode ESI+
Nebulizer and collision gas	Nitrogen	Nitrogen
Data acquisition	Agilent Mass Hunter data acquisition Triple Quad B.01.04	LabSolutions LCMS software v. 4.52
Data processing	Agilent MassHunter Workstation Qualitative Analysis B.01.04	LabSolutions LCMS software v. 4.52

bles 2 and 3, for NIs and fumagillin, respectively. In Figure 2, MRM chromatograms of fumagillin in control and fortified control samples, along with fumagillin standards, that were analyzed for method validation purposes, are displayed. In Figure 3, the elution order of NIs is displayed (in Figure S1, indicative MRM chromatograms for DMZ and IPZ are presented).

5. Analytical Method Validation

Analytical methods' validation is a key element that determines their robustness and validity. Here, the two chromatographic analytical methods were validated according to Commission Decision 2002/657/EC (Commission-Decision-2002/657/EC, 2002) and based on the guidelines for screening methods for veterinary medicines (CRLs-20/1/2010, 2010), which complements the aforementioned EC Decision. A previous work of our group dealing with the determination of antibiotics in honey was also advised for the establishment and determination of respective validation criteria (Kasiotis *et al.,* 2023). The latter were linearity, specificity, matrix effect (ME), decision limits (CCα), detection capabilities (CCβ), limit of quantitation (LOQ), precision, trueness and uncertainty. CCα and CCβ were calculated using the calibration plots metrics in agreement with suggestions by ISO 11843-2 (ISO-11843-2, 2000), considering also the work by Van Loco and colleagues (Van Loco *et al.*, 2007). Linearity was assessed in an overall range of 0.5-250 μg/L, incorporating eight calibration levels using standard solutions of all analytes and corresponding matrix-matched extracts prepared from blank honey. Trueness was studied by performing recovery experiments for all substances at three concentration levels utilizing twenty replicates at CCβ, and six replicates for two additional levels (3×CCβ, 10×CCβ). Precision was determined by evaluating repeatability

Table 2. Retention time and mass spectrometer parameters for nitroimidazoles (MNZ; Metronidazole, RNZ; Ronidazole, DMZ; Dimetridazole, IPZ; Ipronidazole, IPZ-D3; Ipronidazole D3; MNZ-OH; Hydroxymetronidazole, DMZ-OH; Hydroxydimetridazole, IPZ-OH; Hydroxyipronidazole, TRZ; Ternidazole).

Analyte	Retention time (min)	Precursor Ion (m/z)	Product Ion (m/z)*	Q1 Pre Bias (V)	CE (eV)	Q3 Pre Bias (V)	Ratio**
MNZ	5.92	171.9	**82.2**	22	24	15	0.95
			128.2	19	15	13	
RNZ	6.54	200.8	**140.1**	22	12	24	0.24
			98.05	10	17	21	
DMZ	6.04	141.9	81.1	16	27	14	0.51
			96.1	16	17	17	
IPZ-D3	8.48	169.9	124.2	21	20	23	0.59
			109.2	20	24	21	
IPZ	8.43	172.9	**127.3**	20	21	24	
MNZ-OH	4.46	187.9	126.2	20	25	23	0.63
			123.2	22	17	29	
DMZ-OH	6.56	157.9	**140.2**	17	13	12	0.07
			55.3	19	16	27	
IPZ-OH	7.3	186	**168.2**	17	19	22	0.67
			122.2	12	14	17	
TRZ	5.87	185.9	**128.2**	12	21	22	0.58
			82.2	14	15	23	
				10	26	14	

*in bold quantitation ion, **(area qualification ion/area quantitation ion)

Table 3. Retention time and mass spectrometer parameters for fumagillin.

Analyte	Retention time (min)	Precursor Ion (m/z)	Product Ion (m/z)*	Fragmentor voltage (V)	CE (eV)	Collision Cell Accelerator Voltage (V)	Ratio**
Fumagillin	1.82	459.2	**177**	50	-12	4	0.54
			131	50	-33	4	

*in bold quantitation ion, **(area qualification ion/area quantitation ion)

(intra-day) and reproducibility (inter-day) at the same concentration levels (with an identical number of replicates) of the trueness study and expressed through the % relative standard deviation (RSD). ME was extrapolated by juxtaposing the slope of standard and matrix-matched calibration curves. Methods' expanded measurement uncertainty (U') was determined following the approach and equations described in the SANTE document (SANTE, 2021) and in the recently published work of our group (Kasiotis *et al.*, 2023), encompassing recovery data as the main driver of uncertainty.

6. Honey samples

The developed experimental and analytical protocols for the determination of

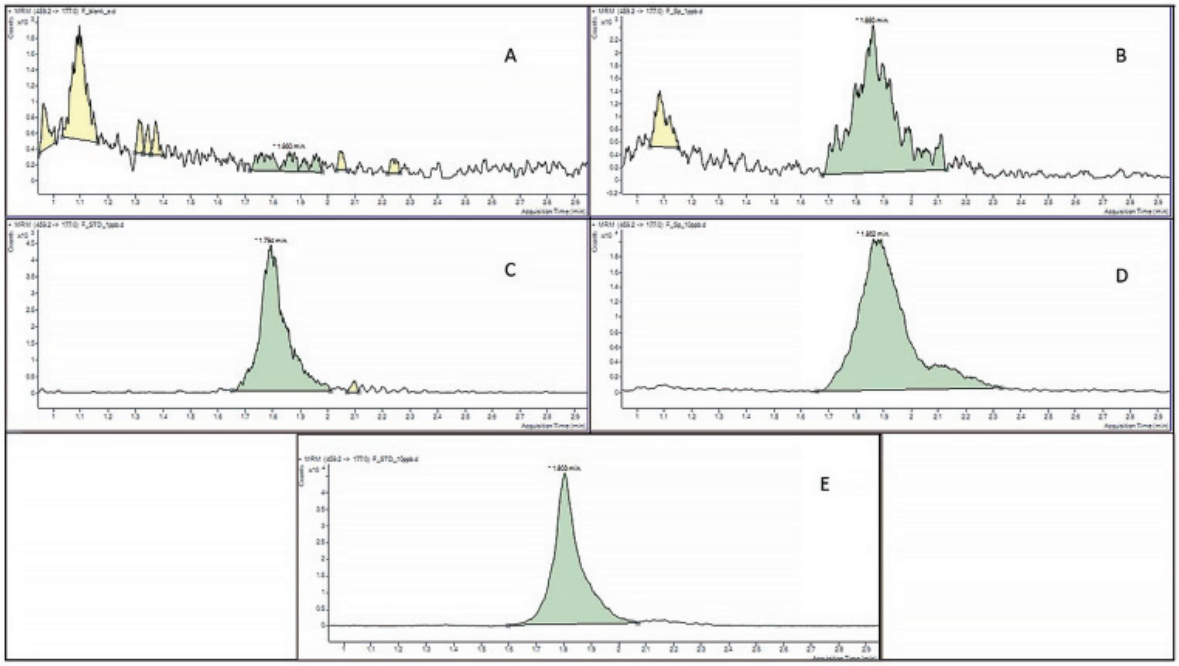

Figure 2. Multiple Reaction Monitoring (MRM) chromatograms (quantitation transition) of Fumagillin in A: control sample, B: Fortified control sample at 1 µg/kg, C: Standard solution (STD) 1 µg/L, D: Fortified sample at 10 µg/kg and E: STD 10 µg/L.

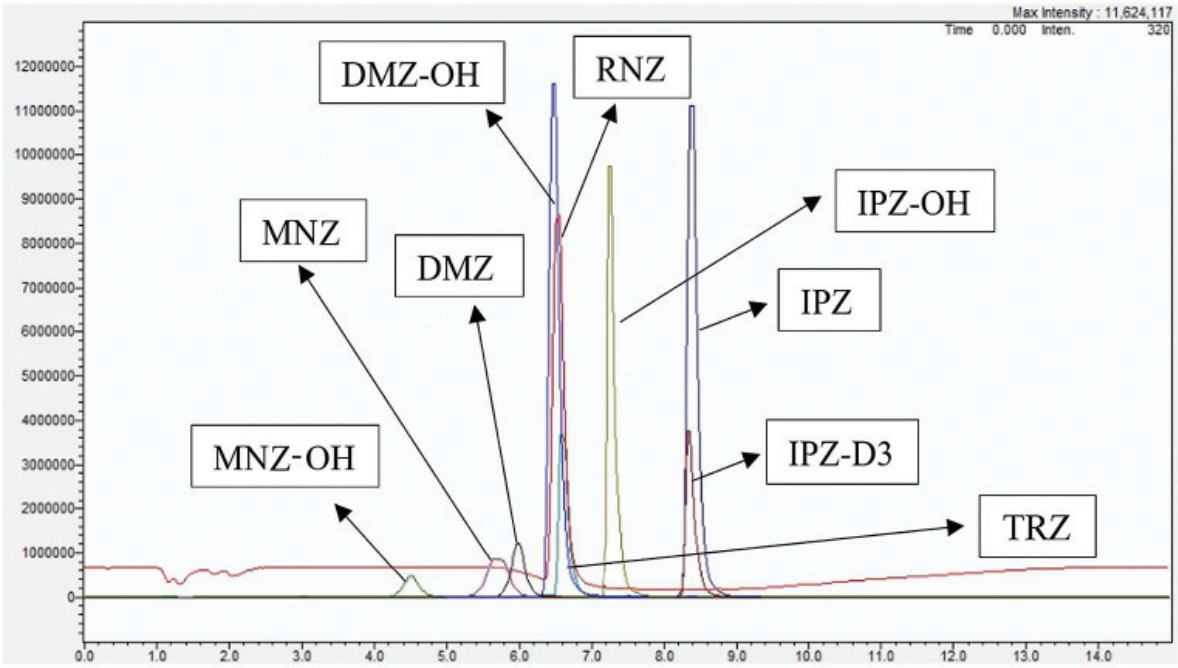

Figure 3. TIC MRM chromatogram of a standard mix solution at 100 µg/L showing the elution order of NIs (MNZ; Metronidazole, RNZ; Ronidazole, DMZ; Dimetridazole, IPZ; Ipronidazole, IPZ-D3; Ipronidazole D3; MNZ-OH; Hydroxymetronidazole, DMZ-OH; Hydroxydimetridazole, IPZ-OH; Hydroxyipronidazole, TRZ; Ternidazole).

fumagillin and NI residues in honey were applied, as a proof-of-concept, to honey samples, collected and sent to Benaki Phytopathological Institute, Laboratory of Pesticides' Toxicology by public authorities and individual beekeepers. In total, thirty honey samples of different floral origins, consistencies, and colors, were collected from

different beehives from various Greek prefectures (Attica, Evia and Aegean islands, Macedonia, Central Greece, Crete, and Peloponnese) (Table 4) during the period 2023-2024, to include as much variability as possible in terms of geographical distribution (yet, it was out of the scope of the work to proceed to melissopalynological analysis for exact pollen identification and botanical source attribution). The samples were stored in boxes following the completion of sampling to avoid light-induced fumagillin degradation, and they were immediately cooled at 0-4°C using ice packs. When received at the laboratory, the samples were stored at –20°C until further processing. For the validation of the analytical methods, blank monofloral honey samples from the repository of the laboratory (routine samples) were used.

Results and Discussion

The introduction of salts in the extraction (partitioning) step and mixed-mode SPE (two modes of interaction, see also discussion below) in the sample preparation step proved efficient in recovering NIs from honey (recoveries depicted in Table 4) exploiting, in this way, the amphiphilic properties of NIs, and leading to slightly cleaner extracts (preliminary tests not shown, demonstrated this behavior compared to the sole use of MCX sorbent). The decision was also based on the intention to expand the scope of the method to other veterinary substances with different physicochemical properties than NIs that would benefit also from this combinatorial sample preparation. Calibration curves of both standards and matrix-matched solutions demonstrated very good linearity for each analyte in honey (0.5-250 μg/L) with a correlation coefficient above 0.9996. The analytical methods' specificity was evaluated by analysing a series (more than 20) of commercially available honey samples. The absence of major interferences in the retention time of the target compounds corroborated the high specificity of the method. The recoveries (as a measure of precision) were found to vary between 76.5% and 113.1% for all the analytes. The reproducibility and repeatability RSD% values of both methods were investigated at three concentration levels (CCβ, 3 × CCβ, 10 × CCβ) and were, in all cases, acceptable (≤20%). For NIs and ME, a negligible to slight suppression of the overall signal intensity was corroborated only when the IS was regarded, fluctuating from -0.8 to -7.8%, which is in line with the findings of Li et al. (2018). For fumagillin, a slight enhancement of ME was evidenced (ME, 8.2%). Lastly, the U' of the analytical methods was evaluated mainly from data of the precision (recovery) investigation (Table 5). For the three concentrations tested U' did not exceed 27.2% (for fumagillin was <16.9%).

Table 4. Geographical origin of honey samples.

No.	Origin	Number of samples
1	Attica	3
2	Macedonia	10
3	Crete	5
4	Peloponnese	4
5	Evia and Aegean islands	2
6	Central Greece	6

With regard to the sensitivity of the herein-developed methods, it was fit for purpose, considering both the pertinent literature (in terms of analytical method performance and actual findings) and the recommended concentration for NIs at 3 μg/kg (CRL 2007). Nevertheless, as mentioned in previous work of our group, particular attention should be given to the way of calculating these metrics (Kasiotis *et al.,* 2023). The values of CCα for NIs following the implementation of our protocol ranged between 0.17 and 0.54 μg/kg and those of CCβ were calculated in the range of 0.30 to 0.78 μg/kg for all NIs being analyzed (Table 5). In the same context, the LOQs for all NIs and fumagillin were established at 1 μg/kg, after spiking blank honey samples (n=5) at this concentration, considering the accept-

Table 5. Validation results for nitroimidazoles (MNZ; Metronidazole, RNZ; Ronidazole, DMZ; Dimetridazole, IPZ; Ipronidazole, MNZ-OH; Hydroxymetronidazole, DMZ-OH; Hydroxydimetridazole, IPZ-OH; Hydroxyipronidazole, TRZ; Ternidazole) and fumagillin determined in honey samples – decision limit (CCα) and detection capability (CCβ) values, recoveries (%), intra and inter-day (RSD%).

Analyte	CC*α* (µg/kg)	CC*β* (µg/kg)	Linear range (µg/L)	Recovery (%)			Intra-day RSD (%)			Inter-day RSD (%)		
				CC*β*	3 × CC*β*	10 × CC*β*	CC*β*	3 × CC*β*	10 × CC*β*	CC*β*	3 × CC*β*	10 × CC*β*
MNZ	0.44	0.63	0.5-250	89.2	93.7	99.2	8	3	6	10	8	7
RNZ	0.18	0.39		76.5	82.1	87.5	13	9	7	12	13	10
DMZ	0.37	0.56		88.4	93.7	101.9	6	7	2	10	8	5
IPZ	0.17	0.30		94.9	102.4	113.1	9	9	5	9	12	7
IPZ-OH	0.18	0.32		79.3	83.3	85.8	13	11	7	10	11	11
MNZ-OH	0.54	0.78		84.3	92.5	103.8	12	7	4	15	10	8
DMZ-OH	0.16	0.30		80.7	85.4	97.3	7	5	5	6	7	10
TRZ	0.34	0.51		97.6	95.9	108.4	11	9	6	13	10	9
Fumagillin	0.45	0.71		86.2	93.3	97.4	8	7	4	8	7	6

able precision (recovery in the range of 85.2 to 100.2%) and trueness of the method (RSD%<12.3). The latter was deemed necessary to avoid any confusion between CC*β* and LOQ values, since based on definition CC*β* is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of *β*. Following thorough research of the literature on the NI analysis in honey, these values lay between previously reported ones employing various sample preparation protocols and analytical platforms (Table 6) (Zhou *et al*., 2007; Cronly *et al*., 2010; Huang *et al*., 2011; Sakamoto *et al*., 2011; Kanda *et al*., 2012; Tölgyesi *et al*., 2012; Mitrowska *et al.,* 2014; Galarini *et al*., 2015; Shendy *et al*., 2016; Guo *et al*., 2017; Mitrowska and Antczak, 2017; Lei *et al*., 2018; Guo *et al*., 2021; Liu *et al*., 2022; Melekhin *et al*., 2024). Overall, the herein-developed method was improved in terms of the abovementioned values compared to the ones published by Cronly (Cronly *et al*., 2010), Guo (Guo *et al*., 2021), Huang (Huang *et al*., 2011), Lei (Lei *et al.,* 2018), Liu (Liu *et al*., 2022), Shendy (Shendy *et al.,* 2016), and Zhou (Zhou *et al.,* 2007) and their associates. Similarly, the performance of the presented method was similar to those of Galarini (Galarini *et al.,* 2015), Guo (Guo *et al*., 2017), Melekhin (Melekhin *et al*., 2024), and Mitrowska (Mitrowska *et al*., 2014; Mitrowska and Antczak, 2017) and their teammates. The only exceptions were the methods of Li and coworkers (Li *et al.,* 2018), Kanda (Kanda *et al*., 2012), Sakamoto (Sakamoto *et al.,* 2011), and Tölgyesi (Tölgyesi *et al.,* 2012), which displayed superior performance.

More specifically, the comparison with the literature disclosed comparable performance with the work by Melekhin and colleagues, who applied an ultrasonic-assisted derivatization – magnetic solid-phase extraction protocol for the determination of NIs and other antibiotics using LC-MS/MS (Melekhin *et al.,* 2024). The achieved LOQs were in the range of 0.3 to 1 µg/kg, therefore, comparable to the ones presented here. In another multiresidue LC-MS/MS-based method, developed by Galarini and fellows (Galarini *et al*., 2015) for the determination of 27 antibiotics in honey, the range in which CC*α* and CC*β* values fluctuated was relatively broader and, while detection of most NIs reached similar to our work levels, for some NIs those values were higher (Tables S1-S8). In other key works the LOQs for

Table 6. Comparison of the developed method with other analytical methods for the determination of nitroimidazoles in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CCα (µg/kg)	CCβ (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	94.7-108.9	3.5-12.4	0.38-1.16	0.66-1.98	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	84.8-122	6.2-23	0.15-1.70	0.21-2.50	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	79.7–110	8.2-13.2	ND	0.10-0.50	1.00	(Guo *et al.*, 2017)
SPE (T-POPs)[b]	HPLC-UV	1.5-1200	81.2-117	1-10	ND	0.50-1.50	1.50-4.50	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	2-200	71.1–114	1.4-9.1	ND	0.47–1.52	1.54–5.00	(Huang *et al.*, 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.005–5	76.1-98.5	2.8-14.2	ND	0.03-0.15	0.10-0.50	(Kanda *et al.,* 2012)
QuEChERS	LC-MS/MS	1–100	81-115.6	0.7-5.8	ND	0.64-1.58	2.13-5.27	(Lei *et al.*, 2018)
d-SPE (MCX)	LC-MS/MS	0-500	90.2-105.6	4.3-11.2	ND	ND	0.05-0.2	(Li *et al.*, 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1-200	93-111.3	1.9-7.1	ND	0.30-0.60	1.00-2.00	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	0.3-200	97-118	4-16	ND	ND	0.30-1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98-104.6	5.1-9.5	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	96-105.9	1-10.1	0.110-0.387	0.179-0.508	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	91.2-100.7	2.6-17.1	ND	0.05-0.20	ND	(Sakamoto *et al.,* 2011)
QuEChERS	LC-MS/MS	0.25-10	90.96-97	1.51-12.58	0.53–0.74	0.91–1.27	ND	(Shendy *et al.,* 2016)
SPE (SDB)[g]	LC-MS/MS	0-9	55.6-86.8	8.6-26.4	0.05-0.10	0.08-0.15	0.17-0.33	(Tölgyesi *et al.,* 2012)
SPE (amine)	LC-UV	10-500	71.5–101.4	2.4-17.2	ND	1.00-2.00	3.00-6.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	76.5-113.1	3-13	0.17-0.54	0.30-0.78	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

NIs varied from 0.1 to 0.5 μg/kg (Kanda *et al.*, 2012), and 0.05-0.2 μg/kg (Li *et al.*, 2018).

Interestingly, the work of Mitrowska and Antczak (2017), on which our experimental protocol was largely based, revealed similar to this study CC*α* values (0.5 μg/kg for all NIs), although the respective values for some of the NIs of our study were somewhat lower (Table 6). Minor modifications in Mitrowska and Antczak sample preparation protocol included the replacement of SCX (Strong Cation Exchange) SPE cartridges with the mixed-mode MCX ones, used effectively by Li and coworkers (2018). In our attempts, the clogging phenomena (Li *et al.,* 2018) were not so extensive to direct us to work in dispersive mode, yet this observation might be attributed to the type of honey analyzed in this work (both blank and actual samples). MCX cartridges combine both cation exchange and hydrophobic interactions, providing a comprehensive retention mechanism for NIs, which exhibit both hydrophilic and hydrophobic properties (see also Figure 1). This dual interaction mode could enhance selectivity, ensuring that NIs are retained, while minimizing the co-extraction of unwanted matrix components, such as sugars and proteins commonly found in honey. In this way, it is plausible to assume that MCX cartridges could handle the complex matrix of honey more effectively, resulting in cleaner extracts and higher recovery rates.

For fumagillin, the comparison of the presented findings with the work by Dmitrovic and Durden (2013) showed an analogous performance of CC*α*, CC*β* and LOQ. Nevertheless, when compared to the LOQ established in the work by Kanda and co-workers (Kanda *et al.*, 2011) the herein LOQ is one order of magnitude higher, yet not computed the same way. An elaborate stability study was not conducted since it is described in the literature for both fumagillin (van den Heever *et al.*, 2015b) and NIs (Mitrowksa *et al.*, 2014) and conditions contemplated in the presented study. Lastly, the runtime of both methods is relatively short, ensuring their applicability, especially for routine laboratories.

Overall, combining the results of our study with those of the literature, we can think about the use of SPE in sample preparation, together with the employment of an LC-MS/MS system, as the method of choice for the analysis of NIs and fumagillin in honey. Nonetheless, further experimentation can be considered to additionally improve the CC*α* and CC*β* values for some of the analytes. Moreover, after validating the analytical methods, analysis of thirty Greek honey samples of different floral and geographical origins, consistencies, and colors for NIs and metabolites and fumagillin showed that they were devoid of NIs or fumagillin residues. Considering the substantial sensitivity of the methods and the diversity of the analyzed samples, the study provides a first insight into the potential use of these chemicals in association with beekeeping practices in Greece, which seem to comply with the zero tolerance regulation regime regarding the application of such substances. In the same context, considering the approval of metronidazole for use in companion animals (EMA, 2019) the absence of residues in this work might also imply that improper use of veterinary medicines is not embraced by Greek beekeepers. The impact of such findings is particularly significant, especially considering similar studies conducted in other countries, such as those applied in Albanian (Pasho *et al.*, 2024) and Egyptian (Rabea *et al.*, 2024) honey, which have reported NI residues above EU standards. While the current evidence may not be sufficient to draw definite conclusions for the whole Greek apiculture sector, it provides a valuable first overview, which could be considered in future large-scale monitoring studies.

Conclusions

In this work, the first attempt for the determination of eight nitroimidazoles and fumagillin in Greek honey samples is reported, which, regardless of the banning of these chemicals in some countries, are still used by beekeepers on a global basis to control

honey bee diseases. Both analysis methods and protocols were optimized, validated, and successfully applied to a variety of honey samples (different flower origins, different geographical areas, different consistencies, and colors). The validation results and the relatively short analysis time propose that these methods are suitable for routine analysis. The actual samples analyzed were devoid of residues of all compounds investigated herein. Next steps, regard the incorporation of additional veterinary substances in the scope of the methods, and the consideration of analyzing honey samples with high-resolution mass spectrometry instrumentation to unveil additional related molecules.

Supplementary Material

Figure S1. MRM chromatograms of standard solutions of DMZ (A) and IPZ (B) at 100 μg/L.

Table S1. Comparison of the developed method with other analytical methods for the determination of metronidazole in honey published in the literature.

Table S2. Comparison of the developed method with other analytical methods for the determination of ronidazole in honey published in the literature.

Table S3. Comparison of the developed method with other analytical methods for the determination of dimetridazole in honey published in the literature.

Table S4. Comparison of the developed method with other analytical methods for the determination of ipronidazole in honey published in the literature.

Table S5. Comparison of the developed method with other analytical methods for the determination of hydroxymetronidazole in honey published in the literature.

Table S6. Comparison of the developed method with other analytical methods for the determination of hydroxydimetridazole in honey published in the literature.

Table S7. Comparison of the developed method with other analytical methods for the determination of hydroxyipronidazole in honey published in the literature.

Table S8. Comparison of the developed method with other analytical methods for the determination of ternidazole in honey published in the literature.

Conflicts of Interest

The authors declare no conflict of interest.

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Προσδιορισμός υπολειμμάτων νιτροϊμιδαζολών και φουμαγγιλίνης στο μέλι με χρήση υγρής χρωματογραφίας συζευγμένης με φασματομετρία μάζας τριπλού τετράπολου (LC-MS/MS): Εφαρμογή σε δείγματα ελληνικού μελιού παραγωγής 2023-2024

Η. Μανέα-Κάργα, Φ. Καντέλη, Ε.Α. Παπαδοπούλου, Κ. Μαχαίρα και Κ.Μ. Κασιώτης

Περίληψη Οι μέλισσες (*Apis* sp.) αποτελούν αναπόσπαστο κομμάτι του οικοσυστήματος, ενισχύοντας τη γεωργική παραγωγή και διασφαλίζοντας τη διατήρηση της βιοποικιλότητας. Παρόλα αυτά, η παρατηρούμενη μείωση του πληθυσμού τους, η οποία οφείλεται, μεταξύ άλλων, σε λοιμώξεις από μικροσπορίδια του γένους *Nosema*, οδήγησε στη χρήση αντιβιοτικών όπως της φουμαγγιλίνης και των νιτροϊμιδαζολών. Ωστόσο, εξαιτίας των ολοένα αυξανόμενων στοιχείων για τις γονοτοξικές, μεταλλαξιογόνες και καρκινογόνες ιδιότητές τους, η παρουσία υπολειμμάτων φουμαγγιλίνης στο μέλι απαγορεύεται, ενώ παρόμοια είναι η κατάσταση και για τα μητρικά μόρια νιτροϊμιδαζολών αλλά και για τους μεταβολίτες τους σε τρόφιμα ζωικής προέλευσης. Στο πλαίσιο αυτό, και λαμβάνοντας υπόψη την περιορισμένη διαθεσιμότητα των σχετικών δεδομένων για το ελληνικό μέλι, αναπτύχθηκαν και εφαρμόστηκαν προηγμένες αναλυτικές μέθοδοι για την ανίχνευση υπολειμμάτων φουμαγγιλίνης και νιτροϊμιδαζολών στο μέλι, με εφαρμογή υγρής χρωματογραφίας συζευγμένης με φασματομετρία μάζας τριπλού τετράπολου (LC-MS/MS). Τα πρωτόκολλα βασίστηκαν στην εκχύλιση στερεής φάσης και αποδείχθηκαν κατάλληλα για την ανίχνευση φουμαγγιλίνης και νιτροϊμιδαζολών σε δείγματα μελιού, επιδεικνύοντας υψηλή ευαισθησία (ικανότητα ανίχνευσης, CCβ που δεν υπερέβη τα 0.78 μg/kg), συγκρίσιμη με την αντίστοιχη πρόσφατων μελετών της διεθνούς βιβλιογραφίας, και θα μπορούσαν να εφαρμοστούν σε αναλύσεις ρουτίνας για τη διασφάλιση της ασφάλειας των καταναλωτών. Οι μέθοδοι που αναπτύχθηκαν εφαρμόστηκαν σε 30 δείγματα ελληνικού μελιού, στα οποία δεν ανιχνεύθηκαν υπολείμματα που υπερβαίνουν την τιμή CCβ των αναλυτών, και η μεθοδολογία που αναπτύχθηκε μπορεί να αξιοποιηθεί περαιτέρω σε μελλοντικές μελέτες παρακολούθησης μεγάλης κλίμακας, για τη διερεύνηση του ελληνικού μελισσοκομικού τομέα σχετικά με την τήρηση των κανονιστικών ρυθμίσεων.

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Supplementary material

Figure S1. MRM chromatograms of standard solutions of DMZ (A) and IPZ (B) at 100 µg/L.

Table S1. Comparison of the developed method with other analytical methods for the determination of metronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	108.9	6.7	0.72	1.22	ND	(Cronly *et al.,* 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	84.8-105	6.9-16	0.43	0.52	ND	(Galarini *et al.,* 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	90.2-99.4	8.60-9	ND	0.50	1.00	(Guo *et al.,* 2017)
SPE (T-POPs)[b]	HPLC-UV	1.5-1200	90.8-115	1-8.3	ND	0.50-0.60	1.50-1.80	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	5-200	87.2-114	1.4-9.1	ND	1.23	4.06	(Huang *et al.,* 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.005–0.5	83.7-94.1	3.0-10.6	ND	0.03	0.10	(Kanda *et al.,* 2012)
QuEChERS	LC-MS/MS	1–100	81-100.7	2.0-5.8	ND	0.64-0.83	2.13-2.77	(Lei *et al.*, 2018)
d-SPE (MCX)	LC-MS/MS	0-500	90.8-105.6	4.3-8.9	ND	ND	0.05	(Li *et al.,* 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1-200	99.2-111.3	1-6	ND	0.30	1.00	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	1-200	115-118	6-10	ND	ND	1.00	(Melekhin *et al.,* 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98.1-101.7	5.1-6.6	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	97.8-103.2	1.4-2.2	0.15	0.20	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	96.5-100.7	2.6-8.7	ND	0.05	ND	(Sakamoto *et al.*, 2011)
SPE (SDB)[g]	LC-MS/MS	0-9	61.1–69.7	15.3–26.4	0.10	0.13	0.33	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	90.2-101.4	5.5-14.9	ND	1.00	3.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	89.2-99.2	3-8	0.44	0.63	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S2. Comparison of the developed method with other analytical methods for the determination of ronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	102.4	3.5	0.38	0.66	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	90.4-101	12-26	1.60	2.30	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	86.7-90.3	10.3-13.2	ND	0.50	1.00	(Guo *et al.*, 2017)
SPE (T-POPs)[b]	HPLC-UV	3-1200	89.1-116	1.9-10	ND	1.00-1.50	3.00-4.50	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	5-200	89-114	1.7-8.9	ND	1.52	5.00	(Huang *et al.*, 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.01–1	76.1-85.4	5.7-9.5	ND	0.06	0.20	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	93.8-98.2	7.9-11.2	ND	ND	0.05	(Li *et al.*, 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1.7-200	96-99.4	2.7-6.8	ND	0.50	1.70	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	1-200	97-100	5-10	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98.6-101.1	6.7-8	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	96.7-104.9	1.4-4.9	0.16	0.21	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	95.8-96.9	4.1-6.8	ND	0.20	ND	(Sakamoto *et al.*, 2011)
QuEChERS	LC-MS/MS	0.25-10	94.03-96.2	1.51-6.81	0.53	0.91	ND	(Shendy *et al.*, 2016)
SPE (SDB)[g]	LC-MS/MS	0-9	55.6–60.9	15.5–24	0.05	0.08	0.17	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	83.4-99.2	2.4-12.3	ND	1.00	3.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	76.5-87.5	7-13	0.18	0.39	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S3. Comparison of the developed method with other analytical methods for the determination of dimetridazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	94.7	9.4	0.73	1.24	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	92.1-105	12-20	0.48	0.64	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	89-90.8	8.9-12.5	ND	1.00	1.00	(Guo *et al.*, 2017)
SPE (T-POPs)[b]	HPLC-UV	4.5-1200	81.2-114	1.5-8.3	ND	1.00-1.50	4.50	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	2-200	71.1-87.3	2.3-9	ND	0.47	1.54	(Huang *et al.*, 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.01–1	86.8-95.1	3-8.1	ND	0.06	0.20	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	93.4-101.8	5.7-10.1	ND	ND	0.1	(Li *et al.*, 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1-200	95.7-104.2	1.9-5.9	ND	0.30	1.00	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	0.3-200	99-101	5-15	ND	ND	0.30	(Melekhin *et al.*, 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98.9-101.4	5.8-6.2	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	98.8-101.9	2-4.2	0.13	0.23	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	91.2-100.7	2.6-17.1	ND	0.20	ND	(Sakamoto *et al.*, 2011)
QuEChERS	LC-MS/MS	0.25-10	90.96-97	1.81-12.58	0.74	1.27	ND	(Shendy *et al.*, 2016)
SPE (SDB)[g]	LC-MS/MS	0-9	64.4–86.8	8.6–23.3	0.10	0.12	0.33	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	71.5-89.7	6.4-15.8	ND	2.00	6.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	88.4-101.9	2-7	0.37	0.56	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S4. Comparison of the developed method with other analytical methods for the determination of ipronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	97.8	4.3	0.40	0.68	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	84.9-108	8.8-21	0.44	0.54	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	90.4-102	8.6-9.9	ND	0.10	1.00	(Guo *et al.*, 2017)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.005–0.5	88.5-98.5	2.9-8.7	ND	0.03	0.10	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	90.2-96.9	5.8-9.9	ND	ND	0.1	(Li *et al.*, 2018)
UAD–MSPE[b]	LC-MS/MS	0.3-200	98-102	4-10	ND	ND	0.30	(Melekhin *et al.*, 2024)
SPE (SCX)[c]	LC-MS/MS	0.5-3000	98-102.3	6.6-8.6	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	99.4-100.9	2.2-7.3	0.11	0.19	ND	(Mitrowska *et al.*, 2014)
SPE (MCX)[d]	LC-MS/MS	0.5-250	94.9-113.1	5-9	0.17	0.30	1.00	This work

a: molecularly imprinted polymers
b: ultrasonic-assisted derivatization-magnetic solid-phase extraction
c: strong cation exchange
d: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S5. Comparison of the developed method with other analytical methods for the determination of hydroxymetronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	*CC*β (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	103.8	4.1	0.39	0.67	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	91.6-119	13-22	1.70	2.50	ND	(Galarini *et al.*, 2015)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.05–5	79.8-86.5	3.9-14.2	ND	0.15	0.50	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	92.3-95.0	4.7-10.3	ND	ND	0.2	(Li *et al.*, 2018)
UAD–MSPE[a]	LC-MS/MS	1-200	98-101	7-15	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[b]	LC-MS/MS	0.5-3000	100.2-102.5	7-8.1	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[c]	LC-MS/MS	0-10	97.6-103.6	1-1.7	0.16	0.21	ND	(Mitrowska *et al.*, 2014)
SPE (SDB)[d]	LC-MS/MS	0-9	<35	ND	0.10	0.13	0.33	(Tölgyesi *et al.*, 2012)
SPE (MCX)[e]	LC-MS/MS	0.5-250	84.3-103.8	4-12	0.54	0.78	1.00	This work

a: ultrasonic-assisted derivatization-magnetic solid-phase extraction
b: strong cation exchange
c: molecularly imprinted polymers
d: styrene-divinylbenzene
e: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S6. Comparison of the developed method with other analytical methods for the determination of hydroxydimetridazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	104.2	4.6	0.42	0.71	ND	(Cronly *et a*l., 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	86.6-115	15-20	1.50	2.00	ND	(Galarini *et al.*, 2015)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.05–5	80.4-88.5	2.8-9.4	ND	0.15	0.50	(Kanda *et al.*, 2012)
SPE (SCX)[a]	LC-MS/MS	0.5-3000	98.2-102.1	7.6-8.3	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[b]	LC-MS/MS	0-10	98.7-102	1.9-4.6	0.14	0.18	ND	(Mitrowska *et al.,* 2014)
SPE (SDB)[c]	LC-MS/MS	0-9	64.3–68.8	14.5–24.2	0.10	0.15	0.33	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	82.9-99.5	2.5-15.4	ND	1.00	3.00	(Zhou *et al.,* 2007)
SPE (MCX)[d]	LC-MS/MS	0.5-250	80.7-97.3	5-7	0.16	0.30	1.00	This work

a: strong cation exchange
b: molecularly imprinted polymers
c: styrene-divinylbenzene
d: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S7. Comparison of the developed method with other analytical methods for the determination of hydroxyipronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (μg/L)	Recovery (%)	RSD (%)	CC*α* (μg/kg)	CC*β* (μg/kg)*	LOQ (μg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	100.8	6.2	0.64	1.10	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	90-115	8.6-23	0.15	0.21	ND	(Galarini *et al.*, 2015)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.05–5	82.4-95	2.8-10	ND	0.15	0.50	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	92.7-104.2	4.8-7.9	ND	ND	0.2	(Li *et al.*, 2018)
UAD–MSPE[a]	LC-MS/MS	1-200	100-101	4-9	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[b]	LC-MS/MS	0.5-3000	98.9-104.6	7.8-9.5	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[c]	LC-MS/MS	0-10	96.9-104.6	2.3-4.5	0.16	0.21	ND	(Mitrowska *et al.*, 2014)
SPE (MCX)[d]	LC-MS/MS	0.5-250	79.3-85.8	7-13	0.18	0.32	1.00	This work

a: ultrasonic-assisted derivatization-magnetic solid-phase extraction
b: strong cation exchange
c: molecularly imprinted polymers
d: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S8. Comparison of the developed method with other analytical methods for the determination of ternidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (μg/L)	Recovery (%)	RSD (%)	CC*α* (μg/kg)	CC*β* (μg/kg)*	LOQ (μg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	102	9	0.78	1.34	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	87.3-106	6.2-12	0.38	0.43	ND	(Galarini *et al.*, 2015)
UAD–MSPE[a]	LC-MS/MS	1-200	108-111	4-9	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (MIPs)[b]	LC-MS/MS	0-10	98.3-102.7	1.9-5	0.19	0.33	ND	(Mitrowska *et al.*, 2014)
SPE (MCX)[c]	LC-MS/MS	0.5-250	95.9-108.4	6-11	0.34	0.51	1.00	This work

a: ultrasonic-assisted derivatization-magnetic solid-phase extraction
b: molecularly imprinted polymers
c: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)