Analysis of residues of veterinary drugs – nitroimidazoles in rare honey species

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Abstract

Honey is a natural product produced by honeybees valued for its nutritional and health benefits. However, contamination with veterinary drug residues, such as nitroimidazoles (NMZ), poses a health risk to consumers. Nitroimidazoles, banned in the European Union due to their carcinogenic and genotoxic properties, have been misused in beekeeping to treat diseases such as nosemosis. The aim of this study was to detect nitroimidazole residues in rare unifloral honey species from Croatia, *Ailanthus altissima* (Mill) Swingle and *Mentha* spp., using a validated ultra-performance liquid chromatography-tandem mass spectrometry method. Although the applied method was originally developed for the determination of NMZs in poultry muscle and eggs, the results of the validation study show that it is also suitable for determination in honey with some modifications. For all analytes, the decision limit and detection capability values were between 0.33 and 0.71 µg/kg, which is below the minimum method performance requirement for NMZs in honey of 1 µg/kg set by the European Union Reference Laboratories. Ten nitroimidazoles were analysed in 11 honey samples and no residues were detected, confirming compliance with European regulations. These results are in line with broader studies showing rare NMZ contamination in the global honey market. However, they emphasise the need for continuous monitoring, especially as the possible use of banned NMZs in honey production cannot be completely ruled out. Future studies should also investigate the transfer of NMZs from beeswax to honey, emphasising the importance of good beekeeping practises to avoid contamination.

Key words: *nitroimidazoles; honey; prohibited substances; UHPLC/MS-MS*

Introduction

According to European Union Directive 2001/110/EC, honey is defined as a sweet, thick, viscous or crystallised product obtained by honeybees from the nectar of honey plants or from secretions of living parts of plants or secretions of sucking

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insects. As a natural food source, honey contains many nutrients, including sugars, organic and amino acids, enzymes, minerals, vitamins and flavour compounds (Bogdanov et al., 2008; Ajibola et al., 2012). It is highly valued in a healthy diet, not only as a substitute for sugar, but also for its numerous properties that benefit human health (Ajibola et al., 2012). Good beekeeping practises are of great importance in honey production. Bee diseases—viral, bacterial, fungal, invasive or non-infectious—can occur under certain conditions, as can bee mortality and bee poisoning. Proper hive care, brood management and attention to other factors affecting honey production significantly reduce the occurrence of such diseases. With proper disinfection and care, some diseases can even be prevented (Matašin, 2012).

The quality of honey must meet several standards, including the absence of veterinary drug residues (European Union, 2001; European Commission, 2010). The irresponsible use of chemical substances, unauthorised products and antibiotics banned in the European Union for the treatment of bees, such as nitroimidazoles (NMZ), can lead to antibiotic residues and other harmful substances entering honey and other bee products (European Commission, 2010; Matašin, 2012). NMZs belong to a group of antibiotics used to treat infections caused by anaerobic bacteria and parasites in both veterinary and human medicine. Together with their metabolites, they have carcinogenic, genotoxic and mutagenic properties, which is why their use has been banned in the European Union (Haagsma et al., 1990), and according to Commission Regulation (EU) 37/2010, their residues are not permitted in honey (European Commission, 2010).

NMZs are mainly used for the prevention and treatment of bacterial and protozoan diseases in poultry and pigs and for the prevention of bacterial infections. In recent years, however, their use has also been found in the prevention and control of *Nosema* in beehives (Li et al., 2018; Pasho et al., 2024). *Nosema apis* and *Nosema ceranae* are microsporidian parasites that infest the digestive tract of adult honey bees and potentially cause the disease nosemosis (Kralj and Fuchs, 2009). Given the suspected use of antibiotics in beekeeping, especially in the treatment of this disease, it is crucial to monitor bee products, primarily honey, for antibiotic residues (Bilandžić et al., 2018). In Croatia, the monitoring of residues of veterinary drugs, including NMZs, is part of the national residue monitoring programme of the Ministry of Agriculture.

There are several published methods for the determination of NMZs in honey, differing in extraction, purification, concentration and instrumentation (Zhou et al., 2007; Lei et al., 2018; Li et al., 2018; Plotnikova et al., 2022; Melekhin et al., 2024). Rare honeys are rarely sampled for the official control of veterinary drug residues. The aim of this study was therefore to determine whether NMZs were misused in the production of unifloral honeys of rare botanical origin from Croatia: *Ailanthus altissima* and *Mentha* spp. honey. For this purpose, the method of ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was validated and used for the determination of ten NMZs in these honeys.

Materials and methods

Samples

The botanical origin of the honeys analysed in this study was previously determined according to the method described by Louveaux et al. (1978) and identified as *Ailanthus altissima* by Saftić Martinović et

al. (2024) and as *Mentha* spp. by Pavlešić et al. (2022). To be categorised as *Mentha* spp. honey, the sample must contain at least 20% *Mentha* pollen grains, and to be categorised as *A. altissima* honey, it must contain at least 45% pollen grains of the species (Anonymous, 2009).

Four samples of *A. altissima* honey were collected between April and July 2021 in the Ičići region of Croatia. *A. altissima* is an invasive plant that belongs to the Simaroubaceae family and is commonly known as the Tree of Heaven. It is native to eastern Asia, but is also widespread in urban areas (Kowarik and Säumel, 2007). *Mentha* spp. belongs to the Lamiaceae family (mint) and is a nectar-producing plant. Mint honey is rare, and for this study seven samples were collected from different regions of Croatia between 2015 and 2020.

The samples were delivered in jars sealed with metal caps and properly labelled, with each sample weighing 100 g.

Certified reference materials and chemicals

Dimetridazole (DMZ), ornidazole (ORZ) and ronidazole (RNZ) (Sigma-Aldrich) were provided by the European Union Reference Laboratory of the Federal Office of Consumer Protection and Food Safety (BVL, Berlin, Germany), as well as hydroxymetronidazole (MNZOH), hydroxy-dimetridazole (HMMNI), ternidazole (TRZ), metronidazole (MNZ), ipronidazole (IPZ), hydroxy-ipronidazole (IPZOH), secnidazole (SNZ) and the corresponding internal standards DMZ-d3, IPZ-d3, RNZ-d3, MNZ-$^{13}C_2$,$^{15}N_2$, HMMNI-d3, MNZOH-d3 and IPZOH-d3 (WITEGA, Berlin, Germany). Acetonitrile (ACN) of LC-MS quality, and formic acid, acetone, glacial acetic acid, sodium sulphate anhydrous and ammonium hydroxide 30-33% were purchased from Honeywell (Seelze, Germany). Ultrapure water was obtained from a Milli-Q water purification system (Millipore S.A.S., Molsheim, France).

Sample preparation

Extraction and purification of samples were performed according to the method published by Sams et al. (1998), with some modifications in the sample preparation and the dissolution of the residues after evaporation. Before extraction with ACN, 2 g honey was completely dissolved in 2 mL ultrapure water. The second modification was the omission of ethylene glycol/methanol from elution mixture. Finally, the residues were dissolved in 200 µL aqueous mobile phase. Purification by solid phase extraction (SPE) was performed using Phenomenex SCX Strata 500 mg/3 mL cartridges (Torrance, USA). A mixture of internal standards at a concentration of 1.5 µg/kg was added to all samples prior to extraction.

LC-MS/MS instrumentation and quantification

The LC-MS/MS system used was a 1290 Infinity UPLC (Agilent Technologies, Singapore) coupled to a G6460 Electrospray Ionisation Triple Quad Mass Spectrometer (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed on a Poroshell 120 SB-Aq 3 × 100 mm, 2.7 µm column (Agilent Technologies, Santa Clara, USA) set to 12°C. The mobile phase A consisted of water acidified with 0.1% formic acid and the mobile phase B was pure ACN. A gradient elution was performed at a flow rate of 0.475 mL/min, starting with 5.0% B, increasing to 10.0% in the first two minutes, followed by an increase to 11.7% over the next 7 minutes. Within 0.5 minutes, the organic phase B was increased to 95.0% and held for 2.5 minutes for column rinsing, then reduced to 5.0% within 0.5 minutes, followed by

equilibration for 1.5 minutes. The injection volume was 5 µL. The instrument was controlled with MassHunter Workstation Software LC/MS Data Acquisition for 6400 Series Triple Quadrupole, version 10.1 B.10.1.67. Data processing was performed with MassHunter Quantitative Analysis, version 10.2 B.10.2.733.8 (Agilent Technologies, Waldbronn, Germany). The mass spectrometric parameters were optimised using NMZ standards as shown in Table 1. The product ion used for quantification is printed in bold. The analysis was performed in Multiple Reaction Monitoring

Table 1. Mass spectrometric parameters for NMZs

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	FV (V)	CE (V)	CAV (V)
DMZ	142.1	**96.1** 81.1	100 100	16 28	1 1
HMMNI	158.1	**140.2** 55.4	80 80	6 16	1 1
IPZ	170.2	124.2 **109.2**	100 100	14 24	1 1
IPZ-OH	186.1	**168.1** 122.1	80 80	8 18	1 1
MNZ	172.1	**128.2** 82.3	90 90	10 24	1 1
MNZOH	188.1	**126.1** 123.3	80 80	14 8	1 1
ORZ	220.1	**128.2** 82.2	90 90	10 30	1 1
RNZ	201.1	143.2 **140.2**	70 70	2 2	1 1
SNZ	186.1	**128.1** 82.3	80 80	8 24	1 1
TRZ	186.2 186.2	**128.2** 82.2	90 90	10 26	1 1
DMZ-d3	145.1	99.3	90	12	1
MNZ-$^{13}C_2$,$^{15}N_2$	176.1	132.2	90	10	1
HMMNI-d3	161.1	143.1	80	6	1
IPZ-d3	173.2	127.2	100	14	1
IPZOH-d3	189.2	171.2	90	8	1
MNZOH-d3	190.1	125.2	80	8	1
RNZ-d3	204.2	143.2	70	2	1

DMZ dimetridazole; HMMNI hydroxy-dimetridazole; IPZ ipronidazole; IPZOH hydroxy-ipronidazole; MNZ metronidazole; MNZOH hydroxymetronidazole, ORZ ornidazole, RNZ ronidazole, SNZ secnidazole, TRZ ternidazole; FV fragmentor voltage; CE collision energy; CAV cell accelerator voltage; *m* mass; *z* charge number

(MRM) mode with positive electrospray ionisation. The interface parameters for electrospray ionisation were set as follows: drying gas temperature 300°C, gas flow 11L/min, nebuliser pressure 50 psi, sheath gas temperature 375°C, sheath gas flow 11 L/min and capillary and nozzle voltages 2500 V and 500 V, respectively.

The quantification was carried out using an external calibration curve in a solvent (10% aqueous methanol solution) in the range from 2 to 25 ng/mL, which corresponds to 0.2 to 2.5 µg/kg. The ratio of intensities between qualifier and quantifier ions had to be consistent with those of the calibration standards, within the tolerances specified in Commission Decision 2002/657/EC (European Commission, 2002). As this regulation was in force at the time of method validation, we refer to it and not to its replacement, Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021). For quality control purposes, both negative (blank) and positive (spiked) control samples were analysed with each batch of study samples.

Method accuracy was ensured by regular inter-laboratory comparisons organised by the European Reference Laboratory in Berlin, Germany (Figure 1).

Method validation was carried out in accordance with Commission Decision 2002/657/EC (European Commission, 2002). The experimental design and the calculation of the validation parameters were performed with the InterVAL Plus software version 3.4.0.4 (QuoData, Gesellschaft für Qualitätsmanagement und Statistik GmbH, Dresden, Germany).

Results and discussion

The validation of the method, carried out in accordance with the procedures described in Commission Decision 2002/657/EC (European Commission, 2002), showed satisfactory specificity, linearity of the calibration curve and accuracy. The decision limit (CCα), detection capability (CCβ), limit of detection (LOD), limit of quantification (LOQ) and recovery rate for each analyte as determined in the validation study are shown in Table 2.

For all analytes, the CCα values ranged from 0.33 to 0.71 µg/kg, which is below the minimum method performance requirement (MMPR) of 1 µg/kg for NMZs in

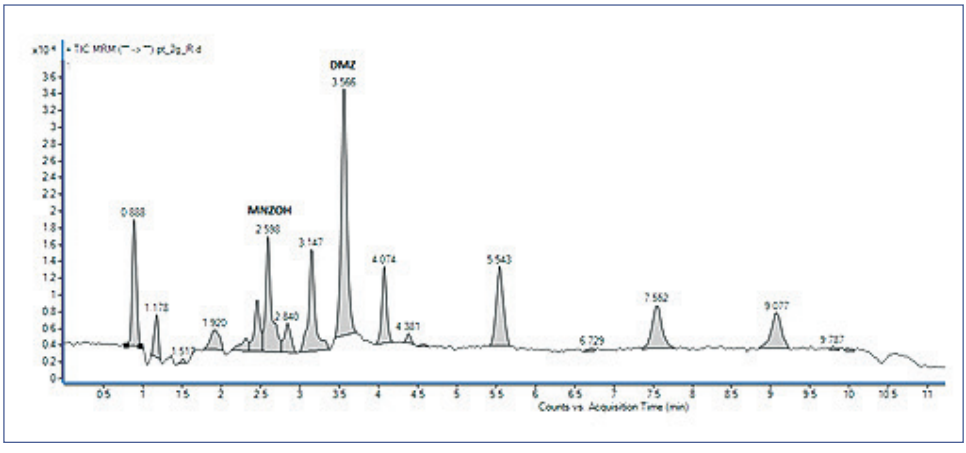

Figure 1. Chromatogram of the proficiency test honey sample with 2.64 µg/kg MNZOH and 4.84 µg/kg DMZ.

Table 2. Validation parameters

Analyte	CCα (µg/kg)	CCβ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)
DMZ	0.33	0.39	0.03	0.09	101.90
HMMNI	0.67	0.78	0.16	0.54	91.10
IPZ	0.38	0.47	0.02	0.07	96.90
IPZ-OH	0.37	0.46	0.01	0.04	90.60
MNZ	0.70	0.84	0.04	0.14	104.30
MNZOH	0.38	0.48	0.05	0.18	98.60
ORZ	0.71	1.02	0.04	0.12	92.40
RNZ	0.34	0.40	0.03	0.08	96.00
SNZ	0.41	0.52	0.07	0.23	96.50
TRZ	0.43	0.56	0.03	0.11	92.60

DMZ dimetridazole; HMMNI hydroxy-dimetridazole; IPZ ipronidazole; IPZOH hydroxy-ipronidazole; MNZ metronidazole; MNZOH hydroxymetronidazole; ORZ ornidazole; RNZ ronidazole; SNZ secnidazole; TRZ ternidazole; CCα decision limit; CCβ detection capability; LOD limit of detection; LOQ limit of quantification.

honey set by the EURL (EURL, 2022). The LOD values ranged from 0.01 to 0.16 µg/kg, while the LOQs were determined to be between 0.04 and 0.54 µg/kg. The recoveries ranged from 90.6% for IPZ-OH to 104.3% for MNZ. By applying these chromatographic parameters, an acceptable peak resolution was achieved for SNZ and TRZ, analytes with the same *m/z* values for precursor and product ions, allowing their indisputable identification and quantification.

When comparing the results of this validation study with published data on the validation of NMZs in honey matrix and its detection by LC-MS/MS, we encountered similar LOD/LOQ values to those reported by Li et al. (2018), despite differences in extraction and sample preparation procedures, and in chromatographic conditions. Those authors used a dispersive solid-phase extraction with a mixed-mode strong cation exchange sorbent (MCX), in contrast to our extraction method with ACN and SCX cartridges. The authors reported LOD/LOQ values of less than 0.1 µg/kg for all NMZs studied. The LOD was slightly higher for HMMNI in our validation study (0.16 µg/kg), as was the LOQ for MNZ, MNZOH and especially HMMNI (0.54 µg/kg) when considering the common analytes in both studies. They reported the highest recovery for MNZ (105.6%), similar to our study (104.3%).

In addition, Melekhin et al.(2024) found the highest recovery for MNZ (118%) using magnetic hyper-crosslinked polystyrene (HCP/Fe_3O_4) for solid-phase extraction and clean-up in the determination of several banned veterinary drugs in honey, including NMZs. Their reported LOQ values were below 1 µg/kg. In contrast, Lei et al. (2018) used a modified QuEChERS method and obtained the lowest recovery for MNZ in jujube honey (81%) and the highest for tinidazole in acacia honey (115.6%), with LODs be-

tween 0.64 and 1.58 μg/kg and LOQs between 2.13 and 5.27 μg/kg.

With regard to CCα, many authors report values below the MMPR of 1 μg/kg, as in this study. Cronly et al. (2010) achieved this by extraction with ACN, similar to our method, but with the addition of NaCl and without SPE clean-up. The only reported value above 1 μg/kg was for tinidazole, which was not included in our study. Galarini et al. (2015) applied acidic hydrolysis of honey before a double purification step (defatting and strong cation exchange solid phase extraction) and found CCα values below the MMPR for most of the NMZs analysed, with the exception of RNZ, HMMNI and MNZOH, where the values were between 1 and 2 μg/kg. Mitrowska et al. (2014) used molecularly imprinted solid-phase extraction followed by liquid chromatography-tandem mass spectrometry and reported lower CCα values for NMZs, consistent with our study, ranging from 0.11 to 0.19 μg/kg (IPZ and TRZ).

Although the method of Sams et al. (1998) was originally developed for NMZ determination in poultry muscle and eggs, the results of our study indicate that it is also suitable for NMZ determination in honey with minor modifications. This was confirmed by the successful application of the method to honey samples in a proficiency test organised by Fera's Food Analysis Performance Assessment Scheme in 2020/2021. Of the seven potentially present analytes, we identified and quantified the levels of DMZ and MNZOH, with z-scores of -0.1 and -0.3 respectively (Figure 1).

Residues of the NMZs analysed in this study were not detected in any of the honey samples tested, which means that the results are compliant with Commission Regulation (EU) 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (European Commission, 2010). As no maximum residue level for NMZs or their metabolites in honey are defined by EU regulations, the CCα values determined by the validation study are the measure of the significance of the results. If the concentrations are equal or above the CCα, the results are non-compliant. A review of published data on the occurrence of NMZs in honey from European and global sources showed that misuse of these antibacterial agents is rare and that, as in our study, most samples analysed comply with European standards. Petcu et al. (2020) analysed NMZs (MNZ, DMZ, RNZ) among different antimicrobial agents in 15 samples of acacia honey using the LC-MS/MS technique, and all results were below the established LOQ. A study conducted by Galarini et al. (2015) on 74 honey samples of different botanical origin also showed that NMZs were not present in the Italian honey market. However, non-compliant samples were found in some countries. In Albania and China, honey samples with NMZs were found several times. Of 41 samples collected in Albania, Pasho et al. (2024) detected NMZs in six samples. In another study from the same country, Vaso et al. (2024) detected NMZs in three of 24 samples. In China, Li et al. (2018) reported NMZs in three of 42 samples with concentrations between 0.3 and 1.1 μg/kg, while Lei et al. (2018) found NMZs in four of 46 samples with significantly higher concentrations between 5.87 and 66.95 μg/kg.

It is important to emphasise that antibacterial substances can also be found in other bee products, such as beeswax. El Agrebi et al. (2020) detected 54 different pesticide and veterinary drug residues in four types of beeswax, stressing the im-

portance of good beekeeping practises, especially when reusing beeswax. This is crucial as NMZs can transfer from contaminated beeswax to honey (Mitrowska and Antczak, 2016). In view of these results, future studies on the occurrence of NMZs and other antibacterial agents in beeswax are necessary. Such studies could help to establish MRLs for these substances in beeswax, especially in view of the unregulated global trade in beeswax, which could pose a potential threat to honey consumers.

The misuse of antibacterial agents such as NMZs cannot be completely ruled out. Due to the health risks NMZs pose to consumers, continuous monitoring of these substances in different honey varieties, including rare varieties, is essential. To this end, reliable analytical methods with a detection capability of less than 1 μg/kg for NMZs must be developed in order to minimise the risks to the lowest possible level.

Conclusions

A sensitive analytical method has been developed for the analysis of NMZs in honey that fulfils the criteria set by the EURL for the MMPR of NMZs in honey. Its applicability was verified by a proficiency test on honey samples and it was successfully applied to the determination of NMZs in rare honey species from Croatia that were not previously included in monitoring plans. All analysed samples complied with the European standards for maximum residue limits for pharmacologically active substances in foodstuffs of animal origin in terms of NMZ content. However, as the possible use of banned NMZs in honey production cannot be completely ruled out, continuous monitoring of their presence in different types of honey is essential to ensure consumer safety.

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Analiza ostataka veterinarskih lijekova - nitroimidazola u rijetkim vrstama meda

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Med je prirodni proizvod kojeg proizvode medonosne pčele, cijenjen zbog svojih nutritivnih vrijednosti i dobrobiti za zdravlje. Međutim, njegova kontaminacija ostacima veterinarskih lijekova poput nitroimidazola, predstavlja zdravstveni rizik za potrošače. Nitroimidazoli, zabranjeni u Europskoj uniji zbog svojih kancerogenih i genotoksičnih svojstava, ponekad se zloupotrebljavaju u pčelarstvu za liječenje bolesti poput nozemoze. Cilj je ovog istraživanja bio utvrditi ostatke nitroimidazola u rijetkim jednocvjetnim vrstama meda podrijetlom iz Hrvatske - *Ailanthus altissima* (Mill) Swingle i *Mentha* spp. primjenom validirane metode ultraučinkovite tekućinske kromatografije spregnute s masenom spektrometrijom. Iako je primijenjena metoda izvorno razvijena za određivanje nitrominidazola u mišiću peradi i jajima, rezultati naše validacijske studije pokazuju da je uz određene modifikacije prikladna za njihovo određivanje u medu. Za sve analite, vrijednosti CCα bile su između 0,33 i 0,71 μg/kg, što je ispod 1 μg/kg - minimalnog zahtjeva za učinkovitost metode za određivanje nitroimidazola u medu utvrđene od strane Europskih referentnih laboratorija (EURL). Deset nitroimidazola analizirano je u 11 uzoraka meda. Svi uzorci bili su u skladu s europskim propisima, odnosno, nitromimidazoli u njima nisu detektirani. Ovi rezultati u skladu su s opsežnijim istraživanjima koja ukazuju na rijetku prisutnost nitroimidazola na globalnom tržištu meda. Međutim, postoji potreba za kontinuiranim nadzorom, budući da se mogućnost korištenja zabranjenih nitroimidazola u proizvodnji meda ne može u potpunosti isključiti. Buduća istraživanja trebala bi uključivati i ispitivanje mogućeg prijenosa nitroimidazola iz pčelinjeg voska u med, naglašavajući važnost dobre pčelarske prakse s ciljem sprječavanja kontaminacije.

Ključne riječi: *nitroimidazoli*, *med*, *zabranjene tvari*, *UHPLC/MS-MS*