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Analytical Assessment of Nitroimidazoles in Honey Samples from South eastern Albania, utilizing SupelMIP™ SPE Columns with LC-MS/MS

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Abstract **-** The global issue of colony collapse disorder (CCD) has led to substantial losses in bee populations, affecting both apiculture and agriculture reliant on bees for pollination. Despite extensive investigations into potential causes, the precise reasons behind honey bee decline, particularly related to nitroimidazoles such as metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ), and ipronidazole (IPZ), remain elusive. This study explores the impact of nitroimidazoles on honey bee health, emphasizing the need for effective analytical methods. The investigation focuses on the utilization of SupelMIP™ SPE columns in conjunction with LC-MS/MS for evaluating nitroimidazoles in honey. The method shows high sensitivity and selectivity, addressing challenges associated with matrix effects. The study includes honey samples collected from the southeastern region of Albania, employing a meticulous sample preparation protocol. The analytical methodology, employing SupelMIP™ SPE columns, demonstrates excellent extraction efficiency, minimal matrix interference, and adherence to regulatory detection limits. The research reveals the presence of nitroimidazoles in certain honey samples, with concentrations exceeding regulatory standards. Chromatograms obtained through LC-MS/MS confirm the reliability of the method. The results emphasize the significance of monitoring nitroimidazole residues in honey to safeguard bee health and ensure the integrity of honey as a consumable product. Furthermore, the study delves into the optimization of mass spectrometer conditions, presenting detailed MS/MS parameters for target analytics. Conclusions highlight the effectiveness of the SupelMIP™ SPE column extraction method, offering advantages over alternative approaches. The research also discusses the broader implications of nitroimidazole residues in honey for human health, underscoring the importance of stringent monitoring practices. In conclusion, the combination of SupelMIP™ SPE columns with LC-MS/MS emerges as a robust and efficient approach for evaluating nitroimidazoles in honey, contributing valuable insights to the ongoing discussions on honey bee health and food safety.

Keywords**:** Nitroimidazoles, honey, nosema, antimicrobials, LC-MS/MS, SupelMIP™.

Introduction

The global phenomenon of colony collapse disorder (CCD) has inflicted severe damage on bee populations, impacting not only apiculture but also the broader spectrum of agricultural activities reliant on bees for pollination. Despite extensive investigations into potential causes such as pests, pathogens, pesticides, antibiotics, environmental stress, and malnutrition, the precise etiology of honey bee decline remains elusive.1,2]

Nosemosis, attributed to Nosema apis and N. ceranae, has been identified as a potential primary contributor in the disappearance of bees. The colonization of Apis mellifera by N. cerenae can trigger sudden colony collapse, and fumagillin has traditionally been used to mitigate such losses. [3-6] However, the high cost of fumagillin has led beekeepers to explore alternatives, including dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ). Despite their efficacy in preventing and controlling N. apis, the presence of MNZ residues in honey raises concerns, given their reported mutagenic, genotoxic, and carcinogenic effects. [7]

The regulatory landscape governing these compounds emphasizes the importance of rigorous detection limits. The European Union, the United States, Canada, China, and Japan have collectively banned the use of nitroimidazoles in food-producing animals. Japan, in particular, has set stringent "not detected" standards for DMZ, MNZ, and RNZ in foods, emphasizing the need for precise and reliable analytical methods to ensure compliance. [8-11]

The investigation into nitroimidazoles in honey stems from the necessity to understand their impact on honey bee health. To this end, an effective and precise method for their extraction and analysis is crucial. This study focuses on the use of SupelMIP™ SPE columns in tandem with LC-MS/MS for the evaluation of nitroimidazoles in honey.

Our Honey samples were subjected to a robust sample preparation protocol, involving the utilization of SupelMIP™ SPE columns. These molecularly imprinted polymers exhibit specificity for nitroimidazoles, enhancing the extraction efficiency. Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) was employed for the separation and quantification of nitroimidazoles. The system offered high sensitivity and selectivity, crucial for the accurate detection of these compounds in honey matrices.

The developed methodology demonstrated excellent extraction efficiency for nitroimidazoles in honey samples. SupelMIP™ SPE columns exhibited minimal matrix interference, allowing for the accurate determination of target compounds. LC-MS/MS analysis further confirmed the reliability of the method, meeting or exceeding regulatory detection limits.

The use of SupelMIP™ SPE columns coupled with LC-MS/MS represents a robust and efficient approach for the evaluation of nitroimidazoles in honey. This method overcomes challenges associated with matrix effects, ensuring accurate quantification and compliance with stringent regulatory standards. The findings contribute to the ongoing discussions on safeguarding honey bee health and maintaining the integrity of honey as a consumable product. [12-13]

Methodology

Nitroimidazoles, antibacterial and antiprotozoal veterinary drugs, have been previously utilized for the prophylactic and therapeutic treatment of diseases such as histomoniasis in poultry, hemorrhagic enteritis in pigs, parasitic infections in fish, and sporidian parasites like Nosema. Examples of these compounds include metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ), and ipronidazole (IPZ). They are rapidly metabolized within animals a few hours after administration. These metabolites (MNZ-OH, RNZ-OH, DMZ-OH, IPZ-OH, HMMNI) containing the imidazole ring may have mutagenic and carcinogenic potential similar to their parent compounds. MNZ, DMZ, and RNZ are prohibited, but other nitro-5-imidazoles are not licensed for use in food-producing animals within the European Union. [14,15] Consequently, monitoring these compounds is now required within the EU and by exporting countries to the EU. The European Union Reference Laboratory provides technical recommendations for analytical methods, with the "recommended concentration" laboratories should aim to measure for the most significant nitroimidazoles and their hydroxy metabolites being 1 μg/kg = 1 ppb. [16]

a. Collection of honey samples

Our sample collection extended across the southeastern region of Albania, including various cities, such as Permet, Korca, Leskovik, and Pogradec. A total of 24 samples were meticulously acquired from this geographical area. Each sample was obtained directly from local beekeepers on their respective farms, ensuring a domestic and immediate collection process.

The sampling period extended from June to September 2022, aligning with the harvest season. The honeys predominantly originated from multi-flower sources, reflecting the diverse floral composition. Additionally, the majority of bee hives were strategically positioned in mountainous areas, contributing to the distinct characteristics of the collected samples in correspondence with the geographical characteristics of their origin.

b. Instruments and equipment

The instruments and equipment utilized as operational components during the analysis in this scientific work are up-to-date, reflecting contemporary advancements. Each part of equipment is accompanied by a corresponding "Memo" and relevant manuals that attest to the authenticity and efficiency of each device.

a. Spatula (Lind Kitchen)
b. Micropipette 10 ml (Eppendorf)
c. Beaker (Pyrex), Erlenmeyer flask, and cylinder (IWAKI)

d. Centrifuge (iStock)
e. SPE Column (Isolute C18)
f. Volumetric flask (Iwaki)
g. Thermostatic Incubator (Chennai)
h. Vortex (Uxi)
i. Tubes 50 and 15 ml (SPL Life Sciences)
j. Analytical balance (Sagar Alyar)
k. Syringe with filter 2 ml (ADAMAS-BETA)
l. LC Column (GL Science)
m. Vial 1 ml (Alwsci)

c. Reagents and Chemical Components

All chemical compounds and solvents used were calibrated and suitable for liquid chromatography. Acetonitrile, acetone, methanol, formic acid, acetic acid, anhydrous sodium sulfate, and 25% ammonium hydroxide met the required purity for analysis. Ultra-pure water with a quality and resistance of 18.2 MΩ·cm was obtained from a Milli-Q plus system.

Analytical standards of MNZ, DMZ, RNZ, IPZ, MNZ-OH, HMMNI, IPZ-OH, $MNZ - {}^{13}C_2$, ${}^{15}N_2$, DMZ-D_3, RNZ-D_3, IPZ-D_3, MNZOH-D_2, HMMNI-D_3 and IPZOH-D_3 used in the analysis were accompanied by respective purity certificates (Albania). All isotopically labeled nitroimidazoles were of chemical and isotopic purity >98% and were utilized as internal standards (IS). SupelMIP™ SPE (solid-phase extraction) columns (molecularly imprinted polymers) (500 mg^3/ml) were employed for extraction.

d. Standard Solutions

The establishment of calibration curves for each nitro-5-imidazole compound necessitates the creation of standard solutions tailored to each respective component. Consequently, for metronidazole and other nitroimidazoles, distinct calibration curves are crafted at varying concentrations. The formulation of these calibration solutions involves individual standard solutions for each compound, employing suitable diluents to ensure optimal storage conditions. However, due to the impracticality of preparing each solution individually, a pragmatic approach involves working with mixed standard solutions.

Mixed standard solutions, originating from mother solutions with a concentration of 0.1 ppm (0.1 μg/ml) and a volume of V = 10 ml (1000 μl), were meticulously prepared by dilution with methanol. All standard solutions were meticulously stored at -20°C to maintain their integrity and stability.

e. Calibration Standard Preparation

For the extraction method with SupelMIP™ SPE columns, the calibration curve in organic solvent HCOOH 0.1% was used (Table 1).

Table 1: Solvent-based calibration curve

C1–7(ppb)	*Mixed standard (μl)*	*Internal standard (μl)*	*HCOOH 0.1%*
0 ppb	0 μl	150 μl	850 μl
0.3 ppb	45 μl	150 μl	805 μl
0.5 ppb	75 μl	150 μl	775 μl
1 ppb	150 μl	150 μl	700 μl
1.5 ppb	225 μl	150 μl	625 μl
2 ppb	300 μl	150 μl	550 μl
3 ppb	450 μl	150 μl	400 μl

f. Sample Preparation for the Extraction of Nitro-5-imidazole Analytes

Following the collection of samples from beekeepers, meticulously stored under optimal conditions, the samples are transported to the "Organic Chemistry" laboratory for weighing at the Faculty of Natural Sciences. To detect nitroimidazoles in these samples, less than 10 grams of honey are required, based on scientific studies regarding veterinary drug residues in honey.

Due to the varied physical properties of honey, such as viscosity, precise weighing of each sample, particularly in quantities as low as 5 grams, becomes increasingly challenging. To address this issue, each sample is preheated in a thermostat at temperatures not exceeding 50°C until they achieve a viscosity lower than 12.2 Pa·s.

Subsequently, the samples are sent for weighing on an analytical balance with a sensitivity of 4 digits after the decimal point. Using a spatula, the honey is transferred from the container to laboratory tubes of 50 ml. After weighing 24 samples (each at 5 grams), they are then submitted for analysis at the ISUV (Institute of Food and Veterinary Safety).

Extraction Method with SupelMIP™ SPE Columns for Nitro-5-Imidazole Analytics from Honey Samples and Their Preparation for LC-MS/MS Analysis

Molecularly imprinted polymers (MIPs) represent a highly cross-linked class of polymers with well-defined elements, designed to selectively bind a targeted compound or a class of analytics (with structurally similar features) with high specificity. The selectivity is demonstrated during the synthesis of MIPs, where a template molecule, designed to mimic the analytic, aids in the binding of specific sites that are sterically and chemically capable of interacting with the targeted analytic(s). It is crucial for analysts to adhere to a specific methodology when utilizing this phase. The following method is defined for nitroimidazoles, optimized for matrices such as honey.

Analysis of Samples Using LC-MS/MS Technique

All vials prepared for analysis in LC-MS/MS are placed in the vial room of the instrument. However, before LC-MS/MS begins the chromatographic reading of the 24 vials from the SupelMIP™ SPE column extraction method, it is essential to acquaint ourselves with the LC-MS/MS parameters.

a. Liquid Chromatography (LC) Parameters

The chromatographic analyses were conducted on the Agilent Technologies 6460 Triple Quadrupole LC/MS system (provided by ISUV), and the chromatographic separation was achieved using the Agilent Eclipse XDB-C18 column (fig.1,2).

Figure 1: LC MS/MS Aparatus

Figure 2: Agilent Eclipse XDB-C18 column

Eclipse XDB-C18 employs super dense bonding technology (XDB) of organic-silane ligands and double-end capping (at both ends of the column) to protect the ultra-pure silica support (Type B) from dispersion in the mobile phase for intermediate and high pH. Eclipse XDB-C18 is particularly useful for separating acidic, basic, and other highly polar compounds through reversed-phase liquid chromatography. The packaging of Eclipse XDB-C18 involves initially chemically bonding a densely packed monolayer of dimethyl-n-octadecylsilane stationary phase to a specially prepared ultra-pure silica support, ZORBAX Rx-SIL porous silica support. This special Type B silica support is designed to reduce or eliminate strong adsorption of highly polar compounds. This densely covered column packing, with non-active components, can be used for acidic and neutral samples, but is particularly suitable for separating basic components that form poorly resolved peaks on most columns.

b. Characteristics of the Agilent Eclipse XDB-C18 Chromatographic Column

1. Brand: ZORBAX
2. Carbon Loading: 10%
3. End-Capping: Both ends of the column
4. Column Guardian: N/A
5. Inner Diameter (ID): 4.6 mm
6. Maximum Temperature: 40 °C for pH 6-9 and 60 °C for pH 2-6
7. Lower Molecular Weight Limit: 0 Da
8. Upper Molecular Weight Limit: 3000 Da
9. Particle Size: 3.5 µm
10. Particle Type: Fully porous
11. Pore Size: 80Å
12. Maximum Pressure Limit: 600 bar
13. Separation Mode: Reversed phase
14. pH Range: 2-9

A. Type of Column: Eclipse XDB-C18, 80Å, 4.6 x 250 mm, 3.5 µm
B. Mobile Phase:
   a. Eluent A: 0.1% formic acid in LC-MS water
   b. Eluent B: LC-MS grade methanol
C. Analysis Time: 25 minutes according to the gradient
D. Flow Rate: 0.6 mL/min for the column
E. Injection Volume: 20 µL

The Agilent Eclipse XDB-C18 chromatographic column was used for the analysis of samples extracted using the SupelMIP™ SPE column. Below you can find the time table for each solvent (Table 2).

Table 2: Time Table

Time (min)	0.00 min	1.00 min	7.00 min	15.00 min	16.00 min	25.00 min
Solvent A (%)	95 %	95 %	5 %	5 %	95 %	95 %
Solvent B (%)	5 %	5 %	95 %	95 %	5 %	5 %

c. The parameters of the mass spectrometer/tandem mass spectrometer (MS/MS)

The parameters for the mass spectrometer are as follows:
1. Gas Temperature (°C): 325°C; 2. Gas Flow (l/min): 10 l/min; 3. Nebulizer Pressure (psi): 20 psi; 4. Sheath Gas Temperature (°C): 400°C; 5. Sheath Gas Flow (l/min): 12 l/min; 6. Capillarity (V): 4000 V; 7. Continuous Electric Current (V): 500 V.

Results and Discussion:

The statistical processing of data following the parameter identification and their application in the liquid chromatography software connected to the Mass Spectrometer MassHunter detector were analyzed according to the extraction method using "SupelMIP™ SPE" columns.

The suspicious samples for nitro-5-imidazoles were analyzed and read by the LC-MS/MS instrument using two extraction methods, totaling 24 samples, out of which only 3 samples yielded positive values for nitro-5-imidazole class analytics.

Extraction analysis with "Supel$MIPTM$SPE" columns

The calibration curve in organic solvent HCOOH 0.1% shows good reliability and precision ($R2 > 0.95$).
The following chromatograms of positive samples were obtained using the 6460 Triple Quadrupole LC/MS Agilent Technologies instrument with the operating chromatographic column Eclipse XDB-C18, 80Å, 4.6 x 250 mm, 3.5 µm.

1. City: Përmet

Sample number "11", using the SupelMIP™ SPE extraction method, showed the presence of the analytic metronidazole (MNZ, Fig.3). We had a very good extraction of the MNZ analytic from the matrix using the SupelMIP™ SPE method, thereby enhancing the method's selectivity. The extraction method with the Supel$MIPTM$SPE column confirms the presence of the MNZ analytic in this sample.

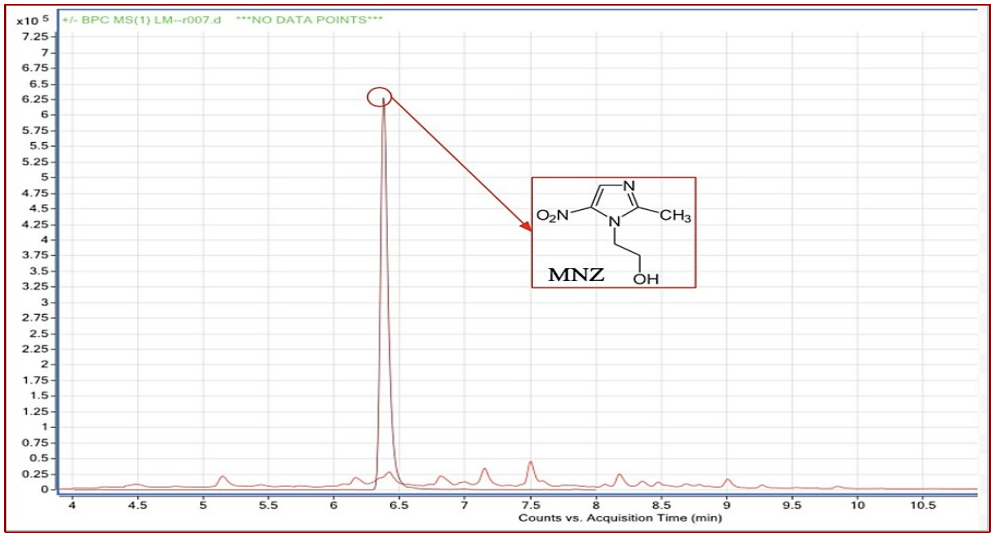

Figure 3: Chromatogram of the characteristic peak of the analytic metronidazole (MNZ)

Below, two chromatograms will be presented, where one shows the peak of the sample $LM7$ (which serves as the calibration curve point), and the other shows the peak of MNZ in the sample $M11$ (Fig.4).

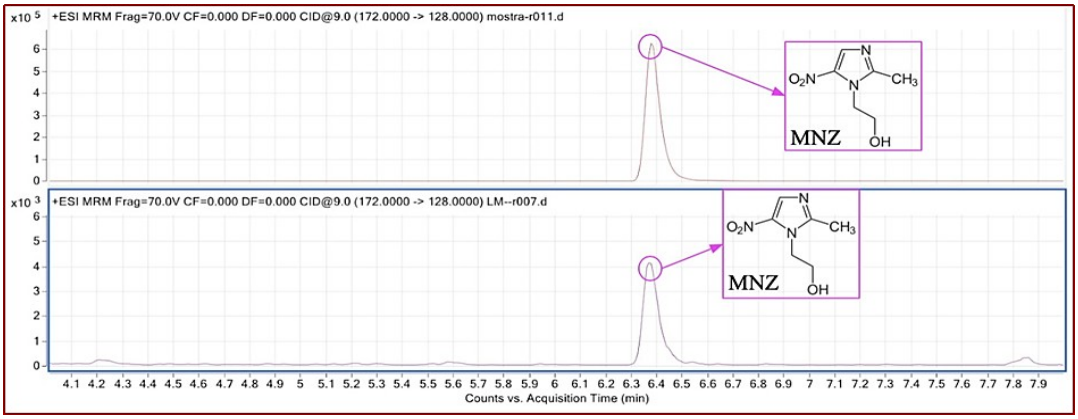

Figure 4: Comparison of the peak parameters of $LM7$ with the peak of $M11$

The comparison of the $LM7$ peak with the $M11$ peak demonstrates a very high concentration of MNZ in $M11$ in Figure 4. Since the $M11$ sample differs significantly from the matrix sample $LM7$ (with about 102 times difference), it is quoted as unnecessary to calculate its concentration. The reason is that if we want to find the concentration of sample "11", we would need to dilute it by a factor of 102. However, excessive dilution of the sample reduces reliability and chromatogram resolution as it would be excessively diluted, affecting the internal standard. Also, given that $C\_M11 > C\_LM7$, we conclude that the sample has $C\_M11 > 3ppb$ (thus qualifies as a positive sample for nitro-5-imidazoles).

2. City: Përmet

Sample number "12" shows the presence of the analyte metronidazole (MNZ) and provides the chromatogram as in Figure 5. From the "Total Ion Chromatogram," we have $tRMNZ$ = 6.4 minutes and counts in the order of 103.

Also, since C_M11 and $C\_M12 > C\_LM7$, this means that the sample $M12$ does not comply with the European Union (EU) standard for the limit of nitroimidazoles in honey (C < 3 $\mu g/kg$).

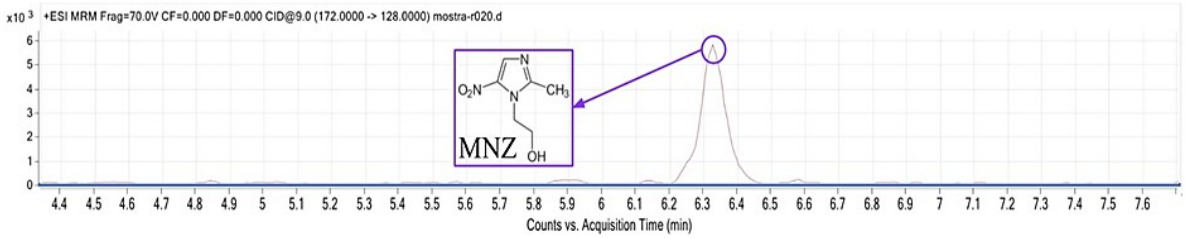

Figure 5: Chromatogram of the characteristic peak of the analytic metronidazole (MNZ).

The comparison of the peak $LM7$ with the peak $M12$ demonstrates well that $M12$ has a very high concentration of MNZ-OH (Figure 6).

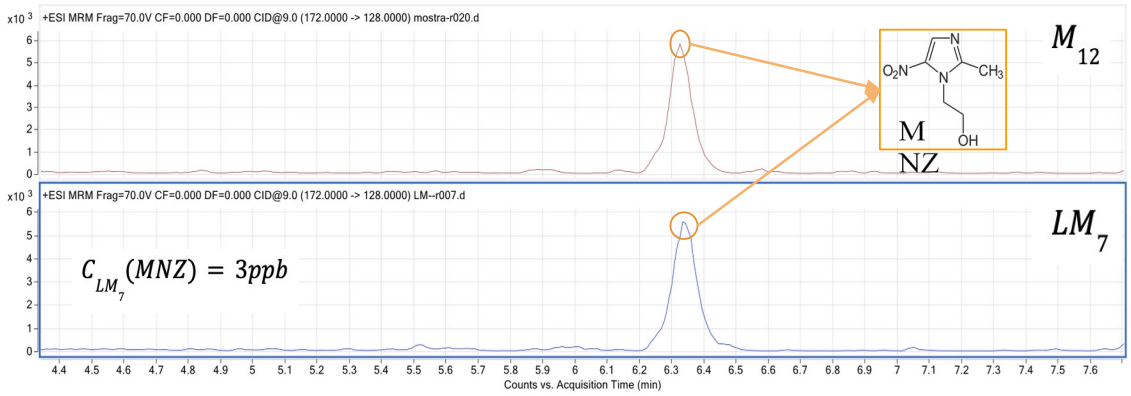

Figure 6: Comparison of the parameters of peak $LM7$ with the peak $M12$

Sample $M12$ does not deviate significantly from the values of the matrix sample $LM7$. From the calibration curve of metronidazole, its concentration has been calculated, resulting in C (MNZ) = 3.01 ppb, and they also have similar "counts" values. It should be considered that the calibration curves are constructed based on matrix samples with known concentrations and have a limit of detection (LOD) indicating the lowest measurable concentration. The peak $LM7$, which shows the maximum working concentration (the maximum level that can be measured), indicates that concentrations outside this range or linearity (outside the calibration curve) are statistically insignificant. However, since sample $M12$ is very close in concentration to the sample for the $LM7$ calibration curve, with extrapolation from the software, we have found its concentration. Sample $M12$ has the same results as sample $M11$ regarding some important chromatographic equations.

3. City: Leskovik

Sample "6" using the SupelMIP™ SPE extraction method shows the presence of hydroxymetronidazole (MNZ-OH, Figure 4.4.XVIII).

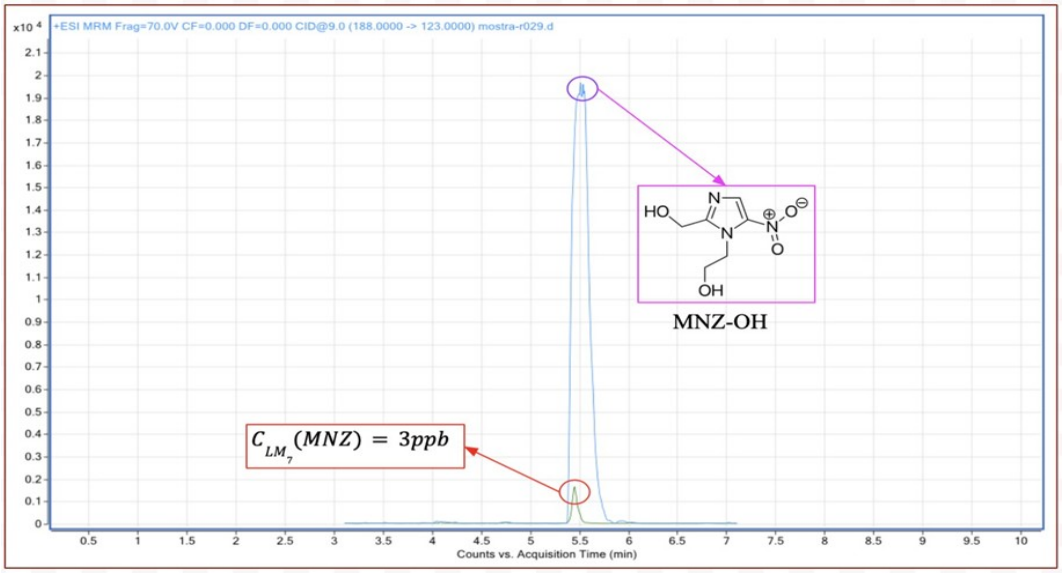

Figure 7: Chromatogram of the characteristic peak of the analytic metronidazole-OH (MNZ-OH)

From the "TIC," we have: $RTMNZ - OH = 5.5\ min$ and counts in the order of 104. Sample $M6$ has a concentration $CM6$ (MNZ − OH) > $CLM7$ for hydroxymetronidazole higher than what the European Union (EU) recommends. Also, in this case, similar to $M11$, it is not necessary to calculate the concentration since from the chromatogram, we see that the difference in counts from "$LM7$" to "$M6$" is extremely large. The problem of dilution consists of the fact that with the dilution of the sample, both the internal standard and the internal standard response in the MS detector are lowered, and from this error, C would be again incalculable. The calibration curve with LOD and the linear range (working zone, where beyond this zone, we enter the part of the dynamic zone) indicates that beyond the linear zone, the detector detects the highest concentration. Therefore, the fact that $M6$ has a higher concentration than what the detector can detect ($CLM7$), is sufficient to say that the sample has the presence of MNZ-OH.

Discussion

The detection of the class of antimicrobials was the objective of this scientific thesis, as they are recognized as agents (residues) of veterinary drugs, and when found in quantities beyond the limit set by the European Union (EU), they exhibit toxic effects.

The method of extracting nitro-5-imidazoles with "SupelMIP™ SPE" columns (solid-phase extraction) demonstrated how this method extracted the target analytes from honey matrix with high selectivity. The "SupelMIP™ SPE" method was also fast, efficient, reliable, and could be used in monitoring antimicrobials in honey. This method is suitable for the purpose of the work, as it exhibits selectivity, sample recovery, and accuracy. Therefore, it was successfully applied to determine nitroimidazole residues.

The calibration standard matrix was successfully used to correct the matrix effect, and its linearity consisted of a correlation $R2 > 0.95$, making this linear regression reliable. The presence of nitro-5-imidazoles in these three samples with such high (alarming) concentrations indicates that this class of antimicrobials has widespread use in the honey business, so the control of antimicrobials in honey is crucial for "human health".

The equipment used, 6460 Triple Quadrupole LC/MS Agilent Technologies, and the chromatographic column Eclipse XDB-C18, enabled the successful detection of nitro-5-imidazole residues in honey. This indicates that LC-MS/MS is the most promising technique for analyzing nitroimidazoles in food due to its sensitivity and accuracy.

Conclusion

Scientific Research on New Routes for the Extraction and Detection of Antimicrobials "Nitro-5-Imidazoles"

Nitroimidazoles (NDZs) are effective antibiotics in the treatment of anaerobic infections. Due to their excellent antibacterial and anti-parasitic activities, they are often used for the management and prevention of infections and parasites. However, drinks and foods (honey, etc.) containing excessive amounts of NDZ residues can cause nausea, vomiting, and even cancer in consumers. To permanently prevent these substances, their use everywhere in consumed foods and by animals has been banned. Consequently, there is a need to create sensitive and fast analytical methods to determine NDZs in real samples. Due to the low concentration of NDZs in real samples and the complexity of the sample matrix, it is difficult to measure them directly on the instrument. In recent years, dispersive liquid-liquid micro extraction, liquid-phase micro extraction, solid-phase micro extraction, solid-phase extraction (SPE), magnetic dispersive micro-solid-phase extraction (MD-μ-MSPE), and magnetic solid-phase extraction (MSPE) have been developed for sample preparations. Due to simplicity and low environmental pollution, SPE is still a widely used sample preparation technique. However, compared to the number of targeted substances to be detected, the number and variety of new adsorptive materials are still limited. The development of new adsorbents for SPE is a current research topic.

Hypercrosslinked polymers (HCPs, new porous materials formed from organic monomers linked by covalent bonds) have been widely used in adsorbing non-target interferences and preserving energy due to their high efficiency, low density, large specific surface area, and micropores.

In this experiment, three HCPs (identified as OPD-HCP, MPD-HCP, and PPD-HCP) were prepared using phenylenediamine (OPD), MPD, and p-phenylenediamine (PPD) as monomers. Three HCPs were then studied as adsorbents for the first time. The adsorption performance of the prepared HCPs for five commonly used NDZs (MNZ, RNZ, DMZ, and IPZ) was investigated. NDZ in honey was successfully determined with the combined SPE-HCP method in high-performance liquid chromatography with ultraviolet detection (HPLC-UV). According to the experimental results, the calculation of theoretical density, hydrogen bonding, and electrostatic interactions can be the main driving forces for extraction.

The developed methodology we used in our study demonstrated excellent extraction efficiency for nitroimidazoles in honey samples. SupelMIP™ SPE columns exhibited minimal matrix interference, allowing for the accurate determination of target compounds. LC-MS/MS analysis further confirmed the reliability of the method, meeting or exceeding regulatory detection limits.

The use of SupelMIP™ SPE columns coupled with LC-MS/MS represents a robust and efficient approach for the evaluation of nitroimidazoles in honey. This method overcomes challenges associated with matrix effects, ensuring accurate quantification and compliance with stringent regulatory standards. The findings contribute to the ongoing discussions on safeguarding honey bee health and maintaining the integrity of honey as a consumable product.

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