Development and validation of an analytical method for tetracycline antibiotic residues in honey using OPT-SPE and LC-MS/MS detection

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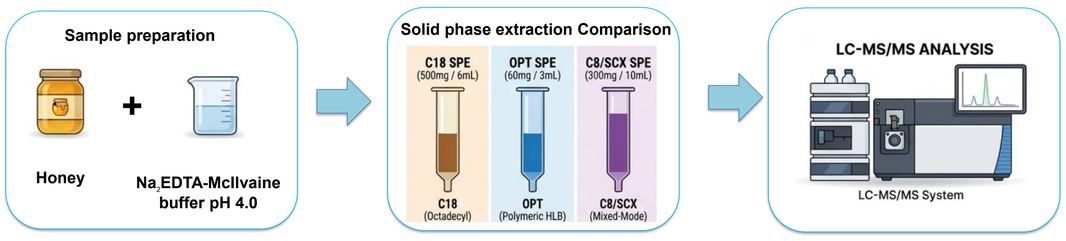
Background

The use of tetracycline antibiotics to control bee diseases can lead to residues in honey, posing risks to consumer safety and creating trade barriers, particularly with the European Union market, which enforces a zero-tolerance policy for such residues. Therefore, it is necessary to develop and validate an analytical method for detecting residues of oxytetracycline, tetracycline, chlortetracycline, and doxycycline in honey using liquid chromatography–tandem mass spectrometry (LC–MS/MS), a technique known for its high sensitivity, specificity, and reliability.

Methods

1. Sample extraction



2. LC-MS/MS optimization

Determination of precursor ions and retention time (Full scan)

1. Standard preparation

- **Standard :** oxytetracycline, tetracycline, chlortetracycline, doxycycline , sulfaphenazole (Internal standard)
- **Diluent**: 20% Acetonitrile

2. LC conditions

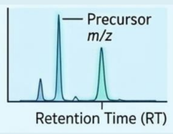
- **Column type:** C18
- **Elution mode:** Gradient

Table 1. Mobile phase gradient system

Time (min)	Flow rate (mL/min)	0.2% Formic acid (%)	0.1% Formic acid in 80% ACN (%)
0.00	0.30	95	5
4.00	0.30	40	60
5.50	0.30	40	60
6.00	0.30	5	95
6.50	0.30	5	95
7.00	0.30	95	5
9.00	0.30	95	5

3. MS conditions

Analysis mode: Full scan



Determination of product ions and collision energy (MRM builder)

1. Ion source parameter

Ion source type	HESI
Spray voltage positive	3,500 V
Probe temperature	450 °C
Cone pressure	20 psi
Probe pressure	45 psi
Nebulizer probe pressure	50 psi

2. Software and data acquisition

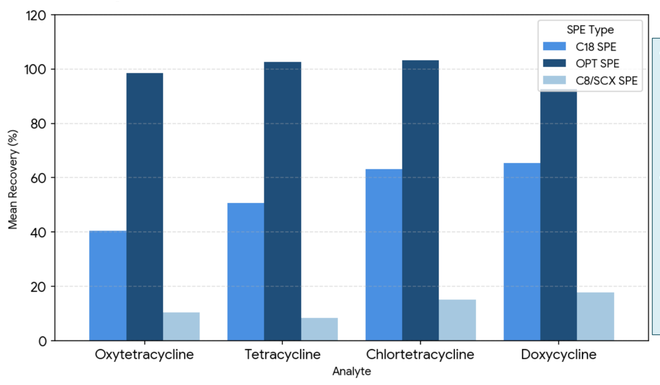
- **Software**: TQ control program
- **Acquisition mode**: MRM builder
- **Objective**: Optimize product Ions and collision energy (CE) for MRM transitions

3. Method validation (Eurachem Guide 2025)

- Matrix effect
- Linearity & Range
- Accuracy
- Precision (repeatability & reproducibility)
- Decision limit (CCα)
- Selectivity/specificity
- Robustness
- Measurement uncertainty

Results and Discussions

Sample extraction



- The OPT cartridge exhibited the highest extraction efficiency with percentage recoveries ranging from 92.5 to 103.2%, demonstrating its suitability for sample preparation in quantitative analysis.
- These results suggest that the physicochemical characteristics of the OPT cartridge provide favorable interactions with tetracycline compounds in the honey matrix. Consequently, analyte loss during the washing step was minimized, resulting in improved retention and extraction efficiency.

Figure 1. Comparison of mean recovery (%) of tetracyclines spiked in honey at 10 µg/kg using three different SPE cartridges (C18, OPT, and C8/SCX)

LC-MS/MS optimization

Table 2. Precursor ions and retention times of oxytetracycline, tetracycline, chlortetracycline, doxycycline, and sulfaphenazole.

Analyte	Precursor (m/z)	Retention time (min)
Oxytetracycline	461.0	3.33
Tetracycline	445.0	3.54
Chlortetracycline	479.0	4.18
Doxycycline	445.7	4.38
Sulfaphenazole	315.0	4.83

Table 3. Precursor ions, product ions, and collision enegy of oxytetracycline, tetracycline, chlortetracycline, doxycycline, and sulfaphenazole.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (volts)
Oxytetracycline	461.0	381.0	20
		426.0*	20
Tetracycline	445.0	154.0	25
		410.0*	10
Chlortetracycline	479.0	154.0*	30
		443.7	20
Doxycycline	445.7	154.0	30
		428.0*	15
Sulfaphenazole	315.0	156.0*	30
		92.0	35

Remark *= quantification transition

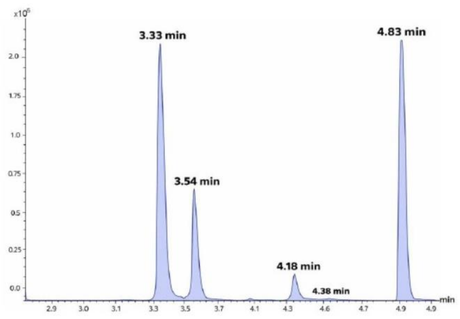


Figure 2. Total ion chromatogram (TIC) of oxytetracycline, tetracycline, chlortetracycline, doxycycline, and sulfaphenazole.

- The precursor ions were fragmented to optimize collision energies (CE), selecting the two highest intensity product ions as quantifier and qualifier transitions. Although tetracycline, chlortetracycline, and doxycycline shared a common fragment at m/z 154.0, which could potentially cause spectral interference, their distinct retention times allowed unequivocal identification and quantification of each analyte.
- This chromatographic separation provides high specificity and reliability for the quantitative determination of tetracycline antibiotics.

- The LC-MS/MS method was optimized using a C18 column and a gradient mobile phase of 0.2% formic acid in water and 0.1% formic acid in 80% acetonitrile at 0.3 mL/min. This configuration achieved rapid elution, with retention times ranging from 3.33 to 4.83 min for all analytes, which were shorter than those reported by Singh *et al.* (2015).
- The use of acetonitrile improved elution strength and reduced system backpressure owing to its lower viscosity. This modification improved peak resolution, ensured signal stability, and minimized column wear. Furthermore, increasing the formic acid concentration to 0.2% enhanced protonation efficiency in the positive HESI source, thereby improving signal intensity.
- The MS/MS optimization was performed under positive HESI and MRM modes by monitoring the highest intensity precursor ions. The precursor ions identified for oxytetracycline, tetracycline, chlortetracycline, and doxycycline were consistent with those reported by Singh et al. (2015). Additionally, the precursor ion for the sulfaphenazole internal standard was found at 315.0, in agreement with Guidi *et al*. (2017).

Method validation (Eurachem Guide 2025)

Table 4. Summary of validation data using OPT-SPE for LC-MS/MS detection.

Performance characteristics	Acceptance criteria	Oxytetracycline	Tetracycline	Chlortetracycline	Doxycycline	Result
Matrix effect	80-120%	119.7%	106.2%	104.9%	103.4%	Passed
Linearity& Working range	$R^2 \geq 0.995$	0.9985 (2.0-40.0 ng/g)	0.9993 (2.0-40.0 ng/g)	0.9989 (2.0-40.0 ng/g)	0.9978 (5.0-40.0 ng/g)	Passed
Accuracy (%recovery)	70-120%	97.41-99.86%	98.65-100.77%	99.61-102.95%	94.00-96.91%	Passed
Precision (Repeatability; %CV)	≤20%	1.42-6.50%	1.68-4.62%	1.96-3.43%	1.80-5.86%	Passed
Precision (Reproducibility; %CV)	≤30%	2.79-5.45%	3.18-4.43%	2.87-5.52%	2.97-6.48%	Passed

- Matrix Effect: No significant matrix effects were observed in the developed method.
- Linearity & Working Range: The developed method demonstrated a linear analytical range of 2.0-40.0 ng/g with a coefficient of determination (R2) ≥ 0.9978. Oxytetracycline, tetracycline, and chlortetracycline showed better sensitivity within this wider range (2.0-40.0 ng/g). Doxycycline exhibited relatively lower sensitivity and a narrower linear range (5.0–40.0 ng/g) compared with the other tetracyclines. This suggests lower sensitivity for doxycycline, meaning the LC-MS/MS requires a higher minimum concentration to quantify it accurately compared to the other three tetracyclines.
- Accuracy: The method exhibited good accuracy, with recoveries between 94.00 and 102.95%.
- Precision: The method exhibited satisfactory precision, with %CV values ranging from 1.42 to 6.50.
  - Repeatability was satisfactory, with %CV values below 20%, indicating excellent intra-laboratory precision.
  - Reproducibility showed minimal variation among analysts and analytical runs, with %CV values below 30%, confirming excellent intra-laboratory reproducibility.
- Selectivity/specificity: No detectable peaks were observed at the retention time of the target analyte.
- Robustness against variations in sample preparation conditions (McIlvaine buffer pH, centrifugation speed, elution volume, and reconstitution volume).
- The decision limits (CCα) were between 2.10 and 5.17 ng/g, which are below the minimum required performance limit (10 ng/g) established by the European Union.
- The measurement uncertainty was estimated in the range of ±6.4% to ±19.3%.

Conclusion

The developed LC-MS/MS method coupled with OPT-SPE is suitable, sensitive, accurate, and reliable for routine determination of tetracycline residues in honey.

Acknowledgements

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References

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