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Abstract

The European Union operates a zero-tolerance policy for residues of prohibited substances, which have no maximum residue limits set. Reference Points for Action (RPA) have been established for prohibited substances when it is deemed necessary to ensure the functioning of controls of food of animal origin imported or placed on the market. Reliable analytical methods are needed for detection, quantification, and identification of residues of chloramphenicol (CAP) in various tissues, biofluids, and food products of animal origin. This application note describes the development of methodology, based on liquid chromatography-tandem quadrupole mass spectrometry, for the analysis of milk and honey. Extracts were prepared using solvent extraction, including a dispersive solid-phase extraction step for milk, followed by determination with LC-MS/MS (Xevo™ TQ-S cronos). The performance of the methods was successfully verified using acceptance criteria from Commission Implementing Regulation (EU) 2021/808. The results from analysis of the spikes were within the required tolerance for trueness and repeatability, respectively. The method is considered sensitive, accurate, and, after further validation, suitable for the determination of CAP in milk and honey for checking compliance with the RPA at 0.15 µg/kg.

Benefits

- Sensitive and selective methods for determination of CAP in milk and honey
 - Detection of CAP at lowest calibrated level (LCL) of 0.025 µg/kg, which is significantly lower than the RPA for CAP at 0.15 µg/kg
 - The performance of the method meets acceptance criteria for trueness and repeatability set out in (EU)
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Introduction

Veterinary medicine products are used to prevent and control disease, their residues may remain in animal tissues, and associated food products of animal origin, typically at very low levels. As such residues should not harm the consumer, many countries operate a system of registration and approvals for the use of veterinary medicines, underpinned by the setting of regulatory limits. In the European Union (EU), before a veterinary medicine is authorized for use on food-producing animals, the safety of its pharmacologically active substances and the residues formed needs to be evaluated and maximum residue limits (MRL) recommended. Prohibited substances, a group of substances for which MRLs cannot be established due to the risk to the consumer, are not allowed to be administered to food-producing animals. One example of this group is chloramphenicol (CAP), which is still being used illegally on commodities exported from some parts of the world into the EU. The EU operates a zero-tolerance policy to residues of prohibited substances. Reference Points for Action (RPAs) have been established for prohibited substances when it is deemed necessary to ensure the functioning of controls of food of animal origin imported or placed on the market. RPAs are set at the lowest level which can analytically be achieved by the official control laboratories. Commission Regulation (EU) 2019/1871 laid down the updated RPA for CAP at 0.15 µg/kg in food of animal origin.¹ Monitoring compliance with such a stringent regulatory requirement requires highly sensitive, selective, and accurate methodology. Previously we reported a method for the determination of CAP in chicken.² In the present study, the performance of methods based upon liquid chromatography-tandem quadrupole mass spectrometry (LC-MS/MS), using an ACQUITY™ H-Class UPLC System coupled with Xevo™ TQ-S cronos, is demonstrated for the determination of CAP in milk and honey.

Experimental

Sample Description

Homogenized samples of milk and honey, purchased from local retailers, were extracted using two protocols based upon solvent extraction. For milk, an acetonitrile-based extraction allowed precipitation of milk proteins. The organic layer was separated by salting out using sodium sulfate followed by centrifugation and subjected to

cleanup using dispersive solid-phase extraction (dSPE). For honey, a mixture of methyl tert-butyl ether (MTBE) and diethyl ether was used for extraction, followed by centrifugation and a concentration step. Full details are provided below in Figure 1.

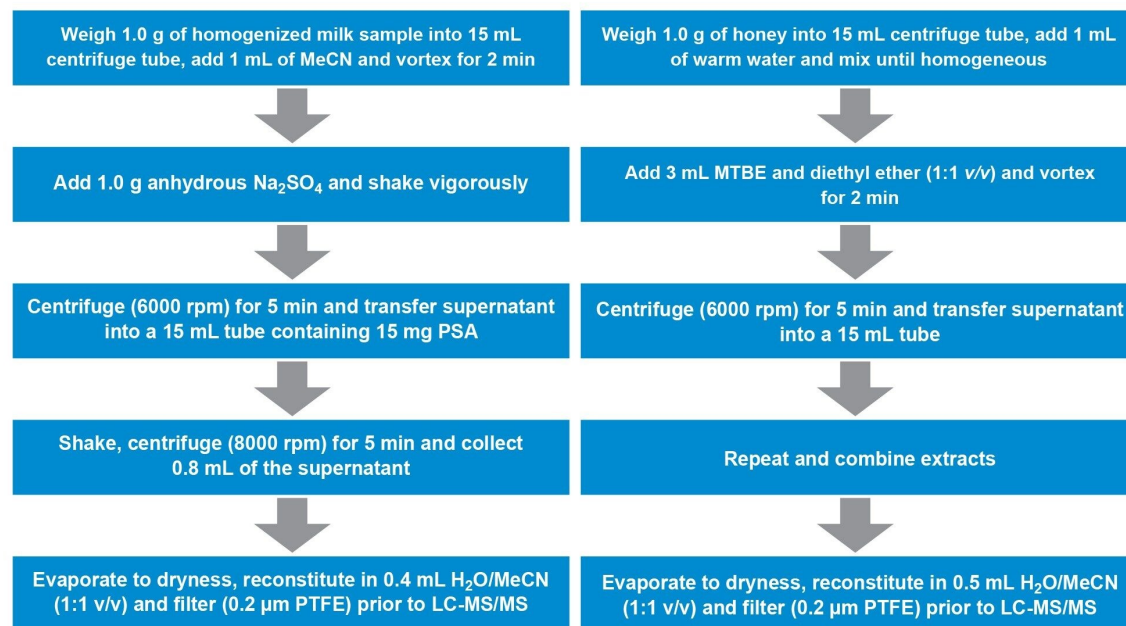


Figure 1. Overview of the details of sample extraction and clean up for the analysis of milk and honey.

Matrix-fortified standards, also known as procedural calibration standards, were prepared by spiking test portions of milk and honey with known amounts of CAP to provide standards over the range 0.025 to 0.50 μg/kg. Trueness and within-laboratory repeatability were determined from the analysis of six replicates of each commodity, prepared at three concentrations: 0.025, 0.050, and 0.100 μg/kg. No internal standards were used in this work.

LC Conditions

LC system:

ACQUITY H-Class UPLC with FTN SM

Vials: Total Recovery, with cap and pre-slit
PTFE/silicone septum, 1 mL volume (p/n: 186000385C)

Column(s): XBridge™ Premier BEH C₁₈ (2.5 μm, 2.1 x 100 mm)
(p/n: 186009828)

Column temperature: 40 °C

Sample temperature: 10 °C

Injection volume: 10 μL

Mobile phase A: Water

Mobile phase B: Methanol

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.45	90	10	Initial
0.2	0.45	90	10	6
3.0	0.45	2	98	6
3.2	0.45	90	10	6
6.0	0.45	90	10	6

MS Conditions

MS system:	Xevo TQ-S cronos
Ionization mode:	Electrospray (negative ion mode)
Capillary voltage:	-1.0 kV
Source temperature:	150 °C
Desolvation temperature:	500 °C
Desolvation gas flow:	900 L/hr
Cone gas flow:	0 L/hr
Cone voltage:	40 V

MRM Method (quantitative transition given in bold)

Compound	Retention time (min)	MRM	CE (eV)	Dwell time (s)
CAP	2.8	321>152	14	0.207
		321>257	8	0.207

The dwell times were set automatically using the auto dwell function to give a minimum of ten data points across each peak.

Data Management

MS software:	MassLynx v4.2
Informatics:	TargetLynx XS

Method Performance

Full validation following the requirements in (EU) 2021/808 requires the replicate spiked samples to be prepared and analyzed on three separate days by the same analyst.³ Here, the performance of the methods was evaluated by preparation and analysis of a single set of replicate spiked milk and honey samples. The following factors were assessed: sensitivity, calibration graph characteristics, trueness, and repeatability. Trueness means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value, in this case the concentration of the spiked sample. There was no need to evaluate absolute recovery as matrix-fortified calibration was used.

Results and Discussion

The XBridge Premier BEH C₁₈ Column provided sufficient retention and gaussian peak shape for CAP, which eluted at 2.8 min, with a total run time of six minutes.

Excellent sensitivity was demonstrated from the analysis of the matrix-fortified standards prepared from spiked samples of milk and honey. Figure 2 shows typical chromatograms for chloramphenicol from the analysis of the matrix-fortified standards at the lowest calibration level (LCL), 0.025 µg/kg, in milk and honey, which indicates that the methods are capable of being used for checking compliance with RPAs, after suitable validation. The response for chloramphenicol was linear over the range evaluated and graphs were created using 1/x weighting. The values for the coefficient of determination (R^2) were found to be >0.99 and residuals <10%.

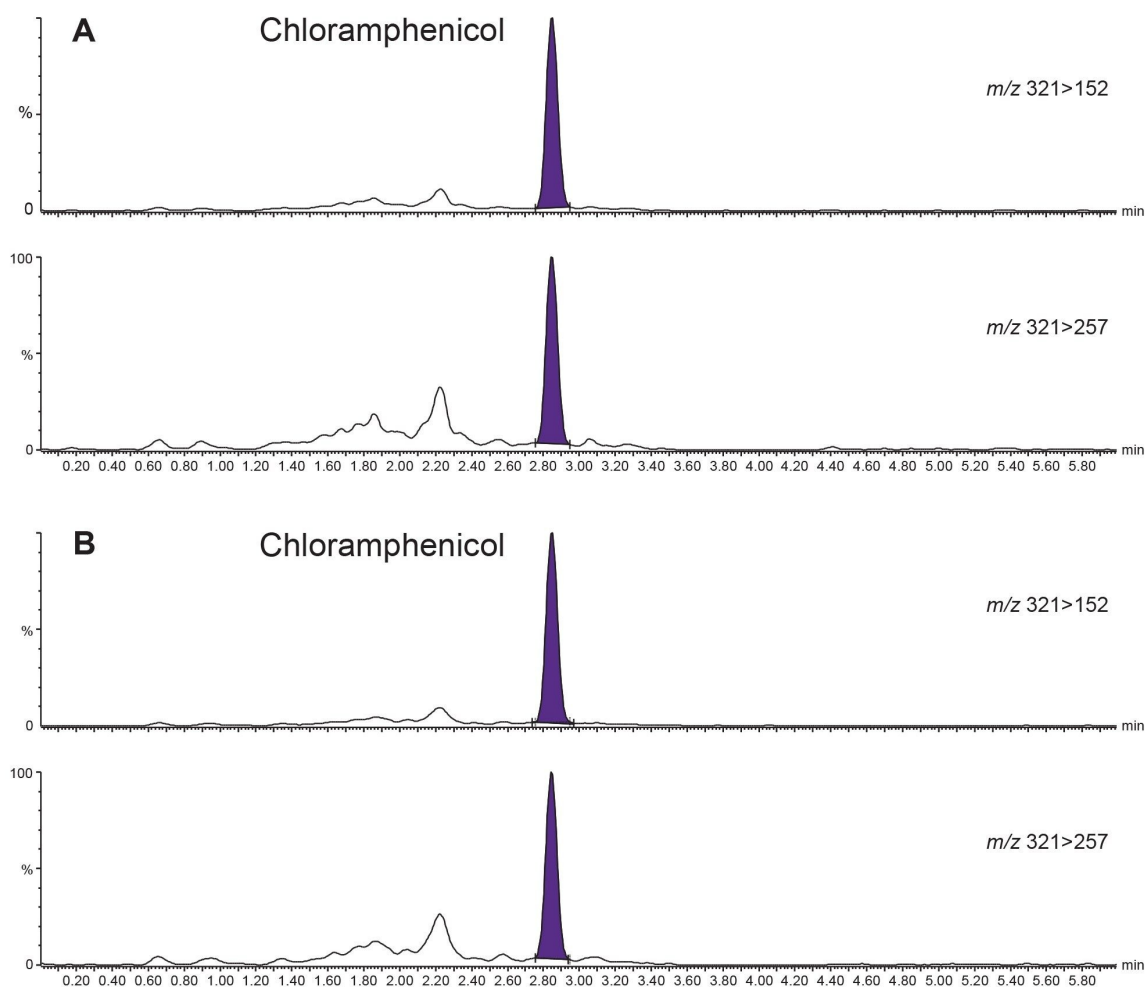


Figure 2. Chromatograms from the analysis of matrix-fortified standards at the LCL, 0.025 $\mu\text{g}/\text{kg}$, in milk (A) and honey (B).

The trueness, determined by measured recovery, was evaluated using the data from the analysis of the spiked samples. The mean measured recoveries for both methods, for each set of spikes at the three concentrations, were within the range 81 to 110% (-19 to +10% trueness). The values for repeatability of the methods were also satisfactory (2.7–9.7%RSD). The values for trueness and repeatability were within the criteria set out in Commission Implementing Regulation (EU) 2021/808, namely -50 to +20% and <20% respectively, without resorting to using an internal standard. Measured recoveries and repeatability (error bars) are shown in Figure 3.

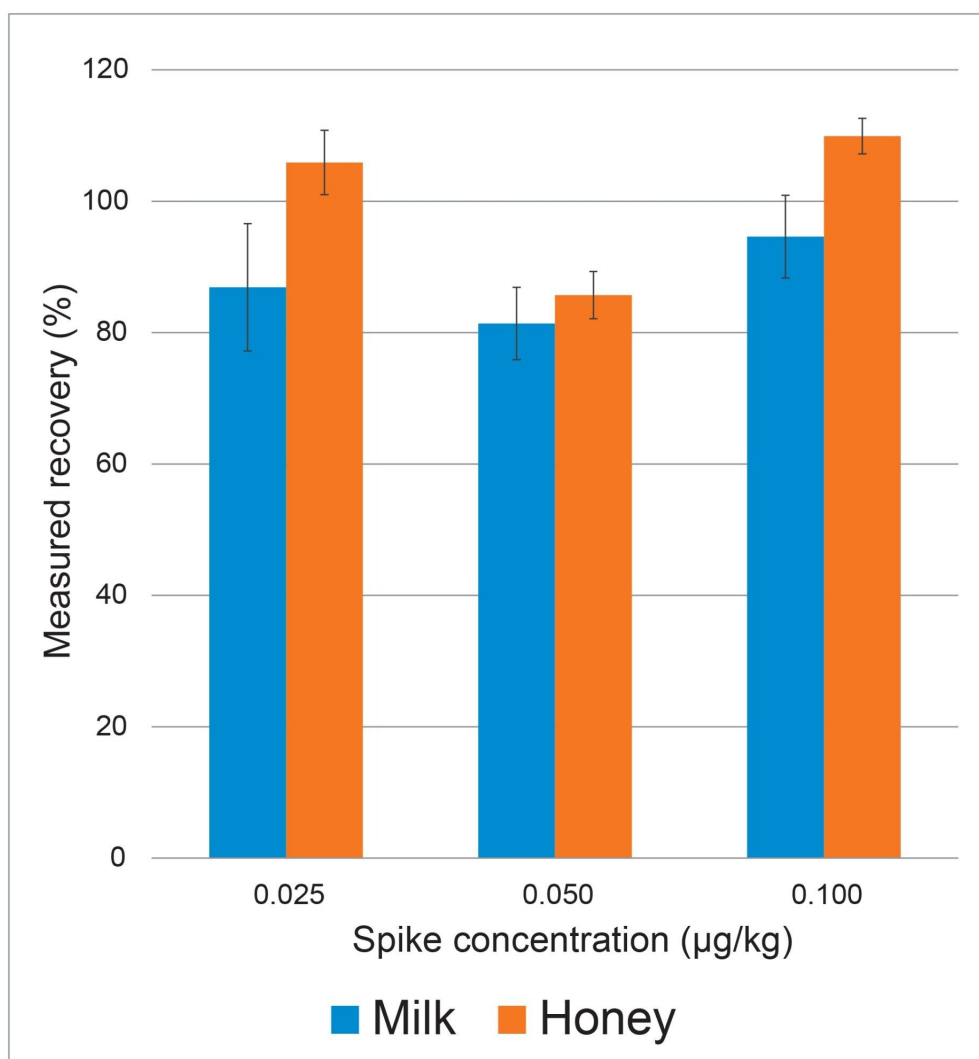


Figure 3. Summary of the values for measured recovery and repeatability (error bars) from the analysis of milk and honey, spiked at 0.025, 0.050, and 0.100 µg/kg.

In addition, the data from the analysis of the replicate spikes was assessed with respect to meeting required identification criteria. The two transitions for each analyte, enough to meet the required four identification points for banned substances, gave peaks with ion ratios and retention times within the recommended tolerances, when compared with the standards.

Conclusion

This application note describes two sensitive and accurate methods for the determination of CAP in milk and honey using LC-MS/MS (Xevo TQ-S cronos). The method allowed for reliable quantification down to concentrations below the revised RPA (0.15 µg/kg) and performance was successfully verified. The method exhibited high sensitivity for both milk and honey (LCL 0.025 µg/kg). The results from analysis of the spikes were within the required tolerances for trueness and repeatability, respectively. The procedures can be applied to the analysis of milk and honey, after suitable validation, to check compliance with the RPA for CAP at 0.15 µg/kg.

References

1. COMMISSION REGULATION (EU) 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC.
2. Renata Jandova, Sara Stead. Xevo TQ-S cronos for the Analysis of Banned Veterinary Drug Residues: Determination of Nitrofurans Metabolites and Chloramphenicol in Chicken Muscle at Regulatory Limits. Waters Application Note, [720007233](#). 2021.
3. COMMISSION IMPLEMENTING REGULATION (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC.

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