

# Detection of Low Level of Chloramphenicol in Milk and Honey with MIP SPE and LC-MS-MS

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## Introduction

**Molecularly imprinted polymers (MIPs)** are polymers that are prepared by polymerizing either preformed or self assembled monomer template complexes together with a cross-linking monomer. After removal of the template molecule, a polymer with binding sites for the template is obtained.



- MIPs exhibit selective target recognition – artificial receptors!
- Selectivity is predetermined for a particular analyte or group of analytes.



# Analysis of Chloramphenicol

- The analysis of chloramphenicol (CAP) is a challenging analysis for drug residues in food matrices.
- Chloramphenicol is an antibiotic that is effective against a wide range of gram positive and gram negative bacteria in both humans and animals, however potentially fatal side effects such as aplastic anemia are known in humans. The suspected carcinogenicity of CAP is also thought to be dosage independent. Due to this toxicity in humans, CAP is completely banned in food producing animals within EU and USA.
- The screening of samples for CAP is often done through immunoassays followed by confirmatory methods for quantitation. Confirmatory methods for CAP are based on mass spectrometry detection due to the sensitivity required (down to 0.3 µg/kg) and the additional benefit of analyte confirmation.

## Analysis of Chloramphenicol (contd.)

- Sample preparation can have a tremendous affect on the method performance due to differences in sample matrices and variability in cleanup efficiency by different sample preparation methods.
- Often, the presence of matrix effects in the samples requires preparation of different sets of standards for different matrices and sample sources.
- Here we compare three sample cleanup methods with regards to the chloramphenicol recovery and cleanup. These included liquid-liquid extraction (LLE), generic polymer SPE and molecularly imprinted polymer (MIP) SPE.

## Experimental

### Liquid-Liquid Extraction (LLE) Method for Milk and Honey<sup>1</sup>

- 1 mL of milk or 1 g of honey dissolved in 1 mL of water were transferred into a centrifuge tube.
- 5 mL acetonitrile was added and the samples were shaken for 10 sec. centrifuged at 4000 rpm for 5 min.
- 5 mL of chloroform was added to the supernatant and the samples were centrifuged at 1800 rpm for 3 min.
- The bottom organic layer was evaporated to dryness, reconstituted in LC-MS mobile phase and filtered prior to analysis.

# Polymeric SPE Method for Milk<sup>2</sup>

pretreatment: 5 mL portion of milk was weighted into 50 mL polypropylene tube. The milk was spiked with chloramphenicol at 8 ng/mL (total 40 ng). 15 mL trichloroacetic acid (10% in water) was added, vortexed and heated for 1 hr. at 65 °C. After cooling to room temperature the mixture was centrifuged at 3,000 rpm for 15 min., the supernatant filtered over glass wool, the filter rinsed with 10 mL of water. pH of the filtrate was adjusted with 0.1 mL sodium acetate (pH 5.0).

cartridge: 6 mL, 200 mg bed weight

condition: 3 mL methanol, 2 mL water and 2 mL 10 mM HCl

load: Milk extract loaded on cartridge

wash: 2 mL water, 2 mL water:methanol (95:5, v/v), 2 mL water:methanol (50:50, v/v)

elute: 2 x 1 mL methanol

post treatment: Samples evaporated to dryness and reconstituted in 0.4 mL water. Liquid-liquid extraction was performed by adding 0.6 mL of acetonitrile:dichloromethane (4:1). The solution was centrifuged at 7,000 rpm for 5 min. and the upper organic layer was transferred to a glass tube. The liquid-liquid extraction was performed twice more and all pooled organic fractions were evaporated to dryness under nitrogen at 60 °C. The residue was reconstituted into 0.200 mL of LC mobile phase and filtered through 0.2 µm nylon filter.

# SupelMIP SPE Procedures

## Milk

pretreatment: Milk centrifuged for 15 min. at 3400 rpm. Collect layer between upper lipid layer and protein pellet

cartridge: 3 mL, 25 mg bed weight

condition: 1 mL methanol, 1 mL DI water

load: 1 mL milk samples spiked at 0.1 and 1.0 ng/mL

wash: 2 x 1 mL DI water, 1 mL 5% acetonitrile in 0.5% acetic acid, 2 x 1 mL DI water, 1 mL 20% acetonitrile in 1.0% ammonium hydroxide (aq)

Apply 0.7 bar vacuum for 5 min.

wash: 3 x 1 mL dichloromethane

Apply 0.7 bar vacuum for 5 min.

elution: 2 x 1 mL methanol:acetic acid:water (89:1:10, v/v/v)

Samples evaporated to dryness and reconstituted in LC mobile phase

## Honey

pretreatment: 1 g honey dissolved in 1 mL DI water at 45 °C

cartridge: 3 mL, 25 mg bed weight

condition: 1 mL methanol, 1 mL DI water

load: 1 mL honey samples spiked at 0.175 and 1.75 ng/g

wash: 2 x 1 mL DI water, 1 mL 5% acetonitrile in 0.5% acetic acid, 2 x 1 mL 1% ammonium hydroxide (aq)

Apply 0.7 bar vacuum for 5 min.

wash: 2 x 1 mL 2% acetic acid in dichloromethane

Apply 0.7 bar vacuum for 2 min.

elution: 2 x 1 mL dichloromethane:methanol (90:10, v/v)

Samples evaporated to dryness and reconstituted in LC mobile phase

# Ion Suppression Measurements

- Blank samples were cleaned by one of the sample preparation methods and were spiked with CAP directly before the analysis. These are the so called “matrix-matched samples.”
- The “matrix-matched” samples were compared to the standards prepared in buffer to determine if there is any ion suppression after sample preparation.



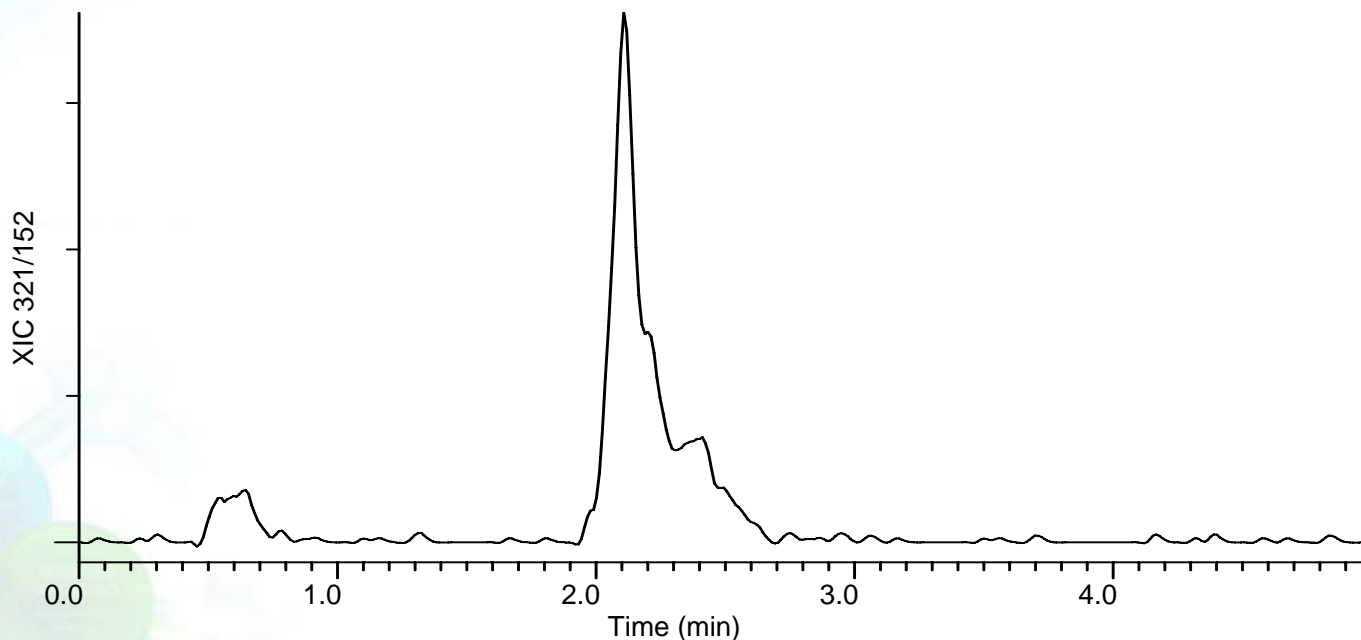
# LC-MS Conditions

column: Supelco Ascentis C18, 5 cm x 2.1 mm I.D., 3  $\mu\text{m}$  particles or  
Supelco Ascentis Express C18, 5 cm x 2.1 mm, 2.7  $\mu\text{m}$  particles  
mobile phase: 10 mM ammonium acetate (pH unadjusted) in water:acetonitrile 70:30  
flow rate: 0.2 mL/min.  
temp.: 35  $^{\circ}\text{C}$   
det.: MS/MS using Applied Biosystems 3200 Q-TRAP  
injection: 2.0  $\mu\text{L}$   
ion mode: negative  
MRM: 21.06/152.1  
ion source: Turbospray  
ionspray voltage: -2700 V  
source temp.: 475  $^{\circ}\text{C}$   
collision gas: 50 psi

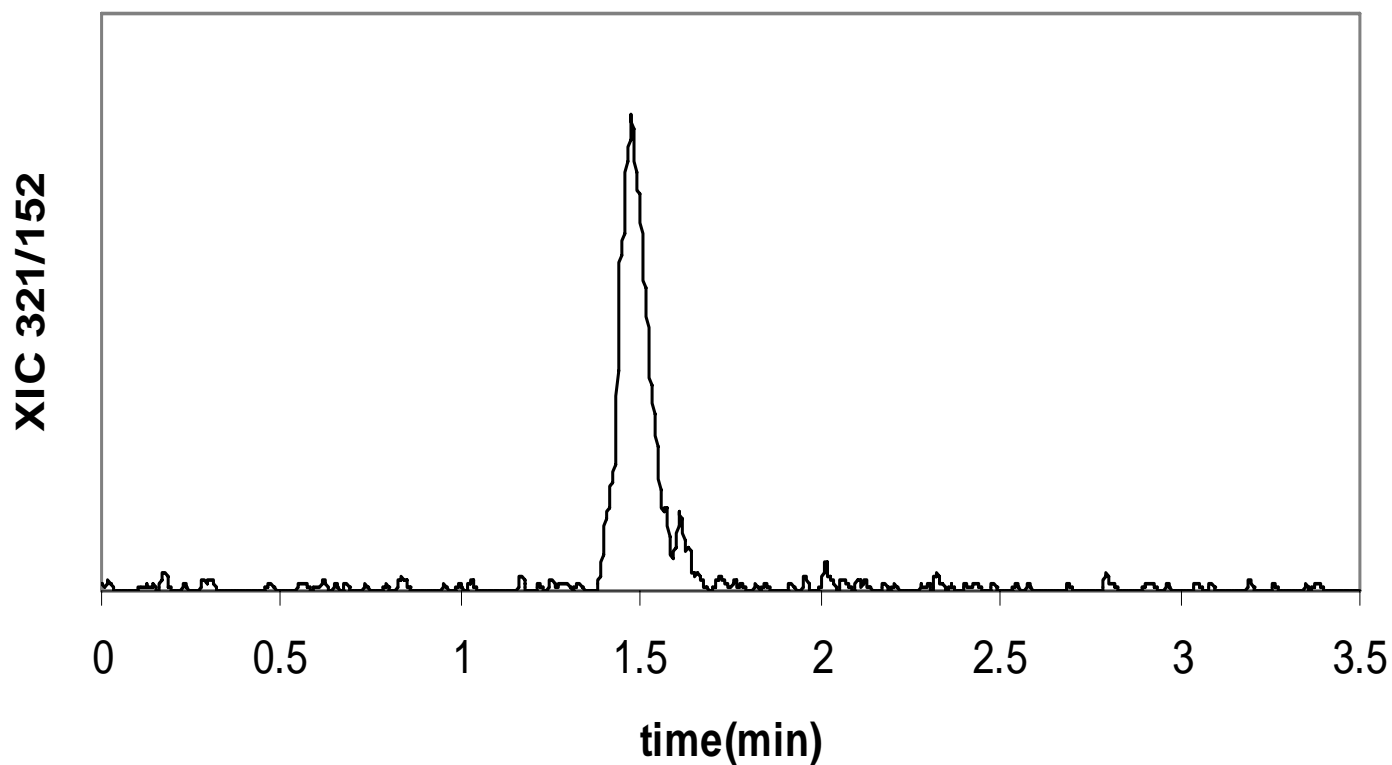


## Results

**Figure 1. Chromatogram of Chloramphenicol Extracted from Spiked Honey Sample by SupelMIP SPE at 0.1 ng/mL. HPLC Analysis done using Ascentis C18 Column.**



**Figure 2. Chloramphenicol (0.1 ng/mL) was Extracted from Milk Sample with SupelMIP. HPLC Analysis done using Ascentis Express C18 Column.**



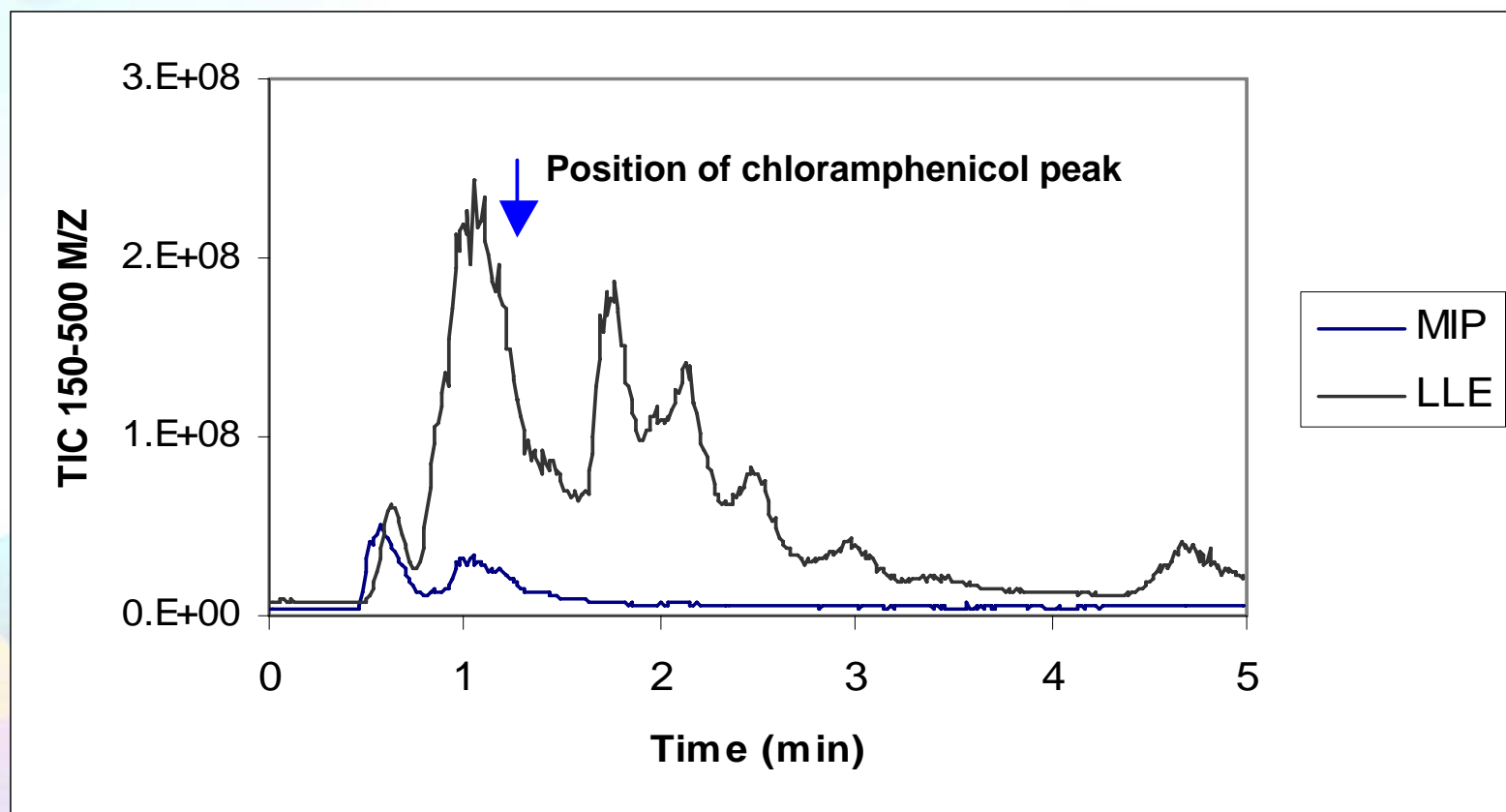
# Recoveries of Chloramphenicol at 0.1 ng/mL and 1.0 ng/mL from Milk and Honey

Extraction method	Milk		Honey	
	0.1 ng/mL	1 ng/mL	0.1 ng/mL	1 ng/mL
SupelMIP SPE	68%	94%	82%	79%
Polymeric SPE*	76%	-	-	-
Liquid-Liquid extraction	72%	94%	37%	50%

\*Only one concentration was done with polymeric SPE as the procedure takes one whole day to perform.

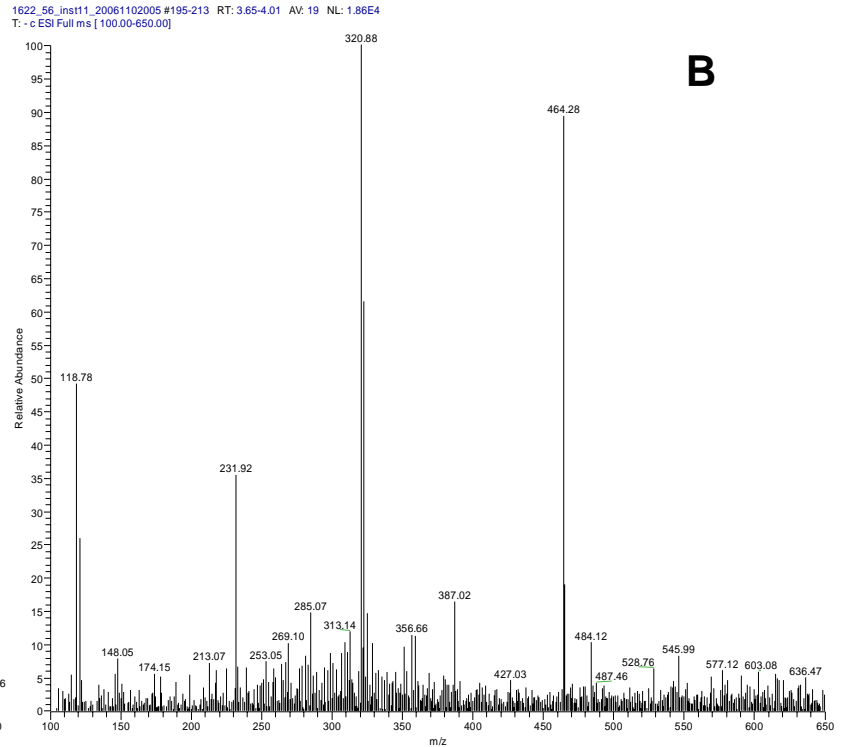
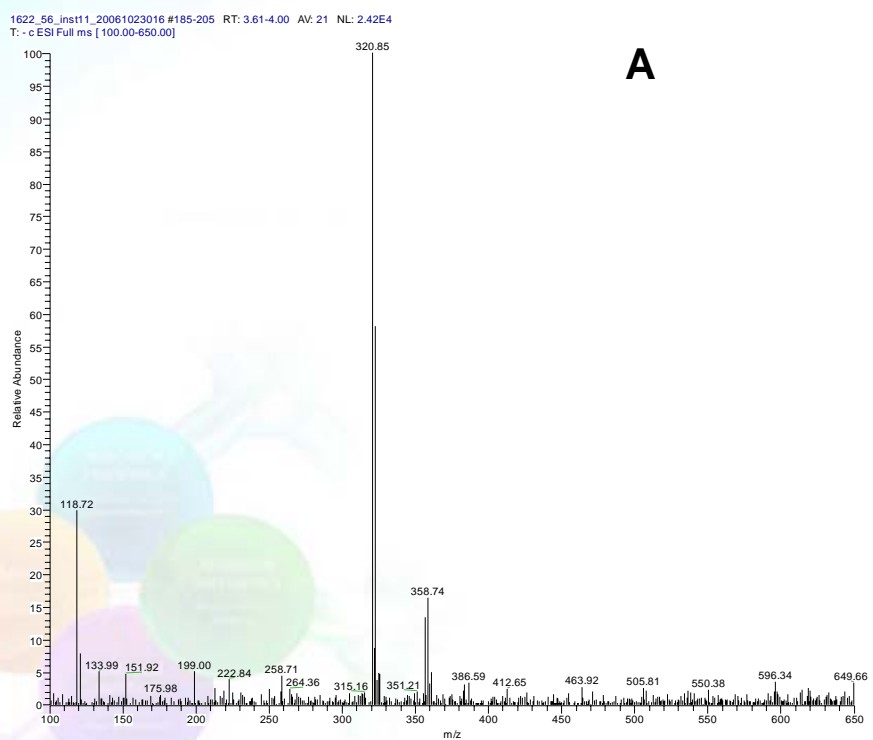
# Figure 3. Comparison of Extraction Background from Honey Sample between SupelMIP SPE and LLE

Comparison of cleanup by SupelMIP SPE vs Liquid-Liquid Extraction



# Figure 4. Mass Spectrum from full Ion Chromatogram for MIP-cleaned (A) and Generic Polymer-Cleaned (B) Milk Samples that were Spiked with Chloramphenicol.

## Comparison of sample cleanup by SupelMIP SPE vs Generic Polymer SPE



# Discussion

## Recoveries

- All sample preparation methods performed well for milk samples. At low level of CAP (0.1 ng/mL) the recovery values obtained were acceptable and range 68-76%.
- Honey samples cleaned with LLE showed lower recovery values than samples cleaned with SupelMIP SPE. This was primarily attributed to the presence of ion suppression effects with the LLE method.

## Discussion (contd.)

### **Sample cleanliness**

- Sample cleanliness can have a tremendous affect on the analytical results. Low or no matrix interferences after the sample preparation step can result in improvement in the sensitivity of the assay.
- SupelMIP SPE showed the best sample cleanup by the evaluation of total sample background (through TIC MS scan and UV-Vis). The selectivity of the MIP is introduced through the polymer synthesis, and contributes to the sample cleanliness.

### **Ion suppression effect**

- Lower recovery values for honey samples prepared with liquid-liquid extraction were attributed to the presence of ion suppression effects. These effects are present when the sample contains less volatile co-extracted matrix components. For these samples, matrix-matched standards are necessary in order to perform the quantitative analysis.

## Summary

- Molecularly imprinted polymer (MIP) SPE performed better for sample cleanup comparing to generic polymer SPE and liquid-liquid extraction.
- At trace levels the sensitivity of the method can be increased by using a more selective sample preparation method, such as MIP.
- When using SupelMIP SPE for cleanup, no matrix effects are observed in the sample and a standard prepared in buffer solution can be used for the calibration curve.
- Comparing to conventional SPE cleanup procedure, SupelMIP SPE procedure took much less time. Significant time savings can result.

## Acknowledgements

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- We thank Brian Boyd, Anna-Karin Wilborg and other scientists from MIP Technologies AB (Sweden) for the development of chloramphenicol MIPs and SPE extraction procedures.

## References

1. Ronning, H.T.; Einarsen, K.; Asp, T.N.; *J. Chromat A*, 1118 (2006) 226-233.
2. Guy, P.A.; Royer, D.; Mottier, P.; Gremaud, E.; Perisset, A.; Stadler, R.H.; *J.Chromat. A* 1054 (2004) 365-371.