

THE SELECTIVE EXTRACTION OF CHLORAMPHENICOL FROM SHRIMP USING MOLECULARLY IMPRINTED POLYMERS (MIPs)

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Abstract

For the analysis and control of trace residues (pesticides, toxins, antibiotics, etc) in food and the environment, reliable and sensitive analytical methods are a necessity. However due to the complexity of the sample matrices in food and environmental monitoring, LC-MS/MS alone does not provide the sensitivity and accuracy often required by regulatory and food safety agencies. Common technique used to complement LC-MS/MS analysis is the pre-treatment of the sample by clean-up methods such as solid phase extraction (SPE), liquid-liquid extraction, supercritical fluid extraction, etc. This sample pre-treatment step is often the rate-limiting step within the analytical process, and can often take up to 10 times as long as the analysis itself. There is therefore a need to develop simple, robust and rapid extraction techniques. Solid-phase extraction is the most popular of these extraction techniques due to factors such as convenience, cost, time saving and simplicity. One rapidly developing method that obviates the need for multiple clean-up and extraction steps, thereby simplifying the pre-treatment procedure, is solid-phase extraction phases based on molecularly imprinted polymers (MIPs). The use of MIPs has proven to simplify sample clean-up, in addition to lower limits of detection and improved MS compatibility.

In this work, we evaluate the utility of molecular imprinted polymer SPE technology for the extraction of chloramphenicol from shrimp. Because selectivity is introduced during the development of the MIP phase itself, it allows for a binding site that is sterically and chemically complementary to the target analyte. For chloramphenicol, the SupelMIP SPE approach provided improved and significant increases in selectivity relative to a described conventional hydrophilic polymer SPE method. The SupelMIP Chloramphenicol method allowed high recoveries, above 90 %, low levels of interfering contaminants and very low limits of detection in the low ppt range.

Introduction

Chloramphenicol is a broad spectrum antibiotic that has recently been determined as a causative agent of aplastic anemia and possible carcinogen in humans. Because of these health concerns, the EU, US and Canada have banned the use of chloramphenicol in food-producing animals and livestock. Because the drug is still widely available in developing countries and no "safe" residue levels have been determined in food, public health concerns still arise. As of today, a "zero" tolerance level has been established for this antibiotic. It is therefore critical to develop a highly selective and sensitive analytical assay

to control and monitor chloramphenicol residues in difficult matrices such as food stuffs. This material has previously been described by Mohamed et al.¹ and Boyd et al.². In this work we discuss the selective use of SupelMIP SPE for the extraction and analysis of chloramphenicol from shrimp. This method is compared against a conventional hydrophilic polymer-based SPE method³.

Molecularly imprinted polymers (MIPs) are a class of highly cross-linked polymer-based molecular recognition elements engineered to bind one target compound or a class of structurally related target compounds with high selectivity. Selectivity is introduced during the MIP synthesis in which a template molecule, designed to mimic the analyte, guides the formation of specific cavities or imprints that are sterically and chemically complementary to the target analyte(s). The preparation of a MIP is generally described in figure 1. By careful design of the imprinting site, the binding cavities can be engineered to offer multiple interactions with the analyte of interest. Multiple non-covalent interaction points (ion-exchange, reversed phase and hydrogen bonding) between the MIP phase and analyte functional groups allow for stronger and more specific analyte retention than conventional separation materials. Improved selectivity is introduced through the use of harsh wash conditions during sample preparation methodology. As the selectivity of extraction is significantly improved, lower background is observed allowing analysts to achieve lower detection limits.

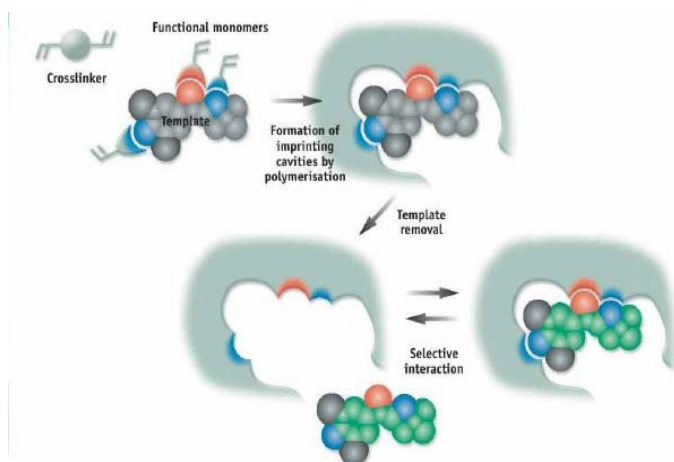


Figure 1, MIPs are prepared by first mixing a template molecule which may be the analyte of interest or a structural analogue of the analyte of interest with one or more functional monomers. The monomers form spontaneous complexes around the template. Upon complex formation, cross-linking monomers are then added with a suitable porogen to drive polymerization. An extensive wash procedure is used to remove the template from the polymer leaving imprints or binding sites that are sterically and chemically complementary to the template.

Method

In this study, an extraction method using SupelMIP Chloramphenicol SPE phase was compared against a recommended method using a conventional hydrophilic polymer SPE phase³. Table 1 describes the two extraction protocols, including sample pre-treatment. Resulting extracts were analyzed via LC-MS. For details see appendix 1.

Table 1. Sample pretreatment methods and SPE procedures for extraction of chloramphenicol from shrimp, SupelMIP CAP resp. Hydrophilic polymer metod.

<p>SupelMIP procedure <i>Sample pre-treatment:</i> Ethyl acetate extraction: 5 g of peeled shrimps were homogenized and 50 mL EtOAc added with internal standard d5-CAP (150 ng/kg). Vortex for 2 minutes. Filter the supernatant through a filter (5.5 μm). Evaporated to dryness and redissolve the residue in 10 mL of DI water.</p> <p>SupelMIP SPE procedure (Appendix 1) Column conditioning 1 ml MeOH 1 ml H₂O Sample loading Load 2 ml sample on the column Wash 2 x 1 ml H₂O 1 ml 5 % AcN in 0.5 % AcH 2 x 1 ml 1 % ammonia 1 ml 20 % AcN in 1 % ammonia Vacuum dry for 10 min. 3 x 1 ml DCM Vacuum dry for 2 min. Analyte elution 2 x 1 ml 10 % MeOH in DCM The samples were evaporated at 45 °C for 40 min and reconstituted in 150 μl 30 % AcN in 10 mM NH₄Ac.</p>	<p>Hydrophilic polymer procedure³ <i>Sample pre-treatment:</i> 5 g of peeled shrimps were homogenized and internal standard were added (d5-CAP, 150 ng/kg). extracted with LLEx2 using 10 + 5 ml of ethylacetate were Vortex 2 minutes for each extraction. The ethyl acetate was then evaporated to dryness at 50 °C. The residues were solved in 1 ml 50:50 methanol/water and diluted with 20 ml water, shaken (1 min.) and centrifuged (10 min. 1500 rpm).</p> <p>Hydrophilic polymer SPE procedure: Column conditioning 1ml Methanol 1mL DI Water Sample loading Load 21 mL sample on the column. Wash 1 mL 30 % methanol. Analyte elution 1 mL 80 % methanol The samples were evaporated at 45 °C for 40 min and reconstituted in 150 μl 30 % AcN in 10 mM NH₄Ac.</p>
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Results and discussion

Upon sample extraction using the two procedures described in Table 1, resulting extracts were analyzed via LC-MS/MS. Recovery was determined for each protocol against a calibration curve (data not shown) using external standards. An average chloramphenicol recovery of 96.8 ± 5.2 % (n=6) was obtained using the SupelMIP method and 75.6 ± 11.7 % (n=6) for the hydrophilic polymer SPE method. An average chloramphenicol absolute recovery of 75.5 ± 6.9 % (n=6) was obtained using the SupelMIP method and 25.5 ± 35.6 % (n=6) for the hydrophilic polymer method. A summary of the data is presented in Table 2. Comparing the extracts from the two methods a pronounced difference in selectivity was determined. The SupelMIP showed higher recovery and significantly higher precision compared to the hydrophilic polymer method. Figure 2, shows chromatograms (MRM 321/152) of a spiked shrimp sample extract (100 ng/kg). The intensity was significantly higher for the chloramphenicol peak when analysing the extract from the SupelMIP method than from the hydrophilic polymer. The signal/noise ratio for the SupelMIP was double that of the hydrophilic polymer SPE method.

Table 2. Recoveries in extracts using the SupelMIP SPE Chloramphenicol method respectively the hydrophilic polymer method. The SupelMIP shows high recoveries and precision.

	SupelMIP SPE Chloramphenicol method	Hydrophilic polymer method
Absolute recovery (%)	75.5 ± 6.9	25.5 ± 35.6
Recovery (%)	96.8 ± 5.2	75.6 ± 11.7

Significantly cleaner mass spectra are also observed for the SupelMIP SPE extract relative to the conventional hydrophilic polymer extract. In Figure 3, Q1 SIM chromatograms are shown (321 amu) of the shrimp extracts spiked with 200 ng/kg and cleaned-up using the SupelMIP CAP and a hydrophilic polymer. The SupelMIP chromatogram shows a chloramphenicol peak with a very low amount of interfering contaminants. Limit of detection using the SupelMIP SPE Chloramphenicol was determined to be 7 ng/kg.

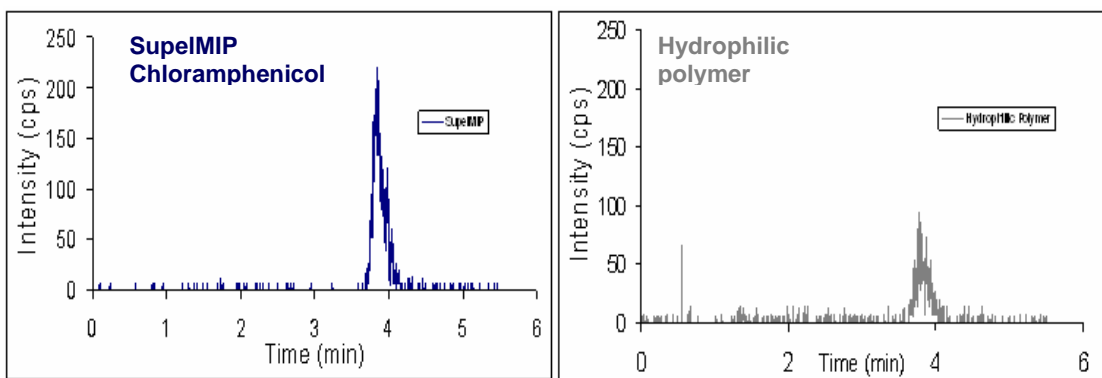


Figure 2. Chromatogram, MRM 321/152. Comparison of shrimp sample extracts spiked with 100 ng/kg CAP cleaned-up using SupelMIP material resp. a hydrophilic polymer. CAP Retention time 3.8 min. The intensity is significantly higher for the chloramphenicol peak in the SupelMIP chromatogram than in the chromatogram of the extract from the hydrophilic polymer.

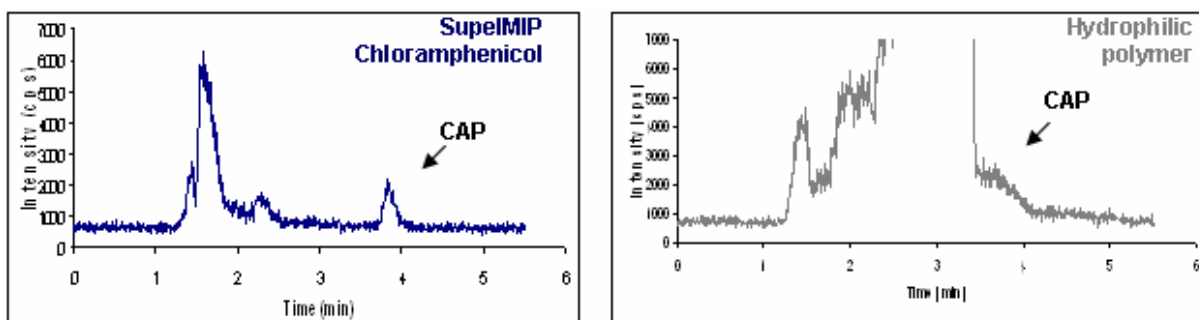


Figure 3. Chromatogram, Q1 SIM 321 amu, of the Shrimp extracts spiked with 200 ng/kg and clean-up using the SupelMIP CAP resp. a hydrophilic polymer. CAP retention time 3.8 min. The SupelMIP chromatogram shows a cleanly resolved chloramphenicol peak with a very low amount of interferences.

Conclusion

In this report, we discussed the utility of molecularly imprinted polymer SPE technology for the extraction of chloramphenicol from shrimp. As selectivity is introduced during the development of the MIP phase itself, it allows for a

binding site that is sterically and chemically complementary to the target analyte(s). The multiple interactions that take place between the imprinted binding site and analyte(s) of interest offer strong interactions enabling the use of harsh wash conditions during the SPE process. For chloramphenicol in shrimp, the SupelMIP SPE approach provided a significant increase in selectivity relative to the described conventional hydrophilic polymer SPE method. The method was robust with a high precision (5 %). The recoveries were determined to be above 90 %. The extracts were cleaner and contained lowered amounts of interferences, allowing lowered detection limits (LOD 7 ng/kg shrimp). This method is particularly advantageous where trace detection limits and routine analysis are required.

References

1. Mohamed R, Richoz-Payot J, Gremaud E, Mottier P, Yilmaz E, Tabet JC and Guy P, 2007. Advantages of molecularly imprinted polymers LC-ESI-MS/MS for the selective extraction and quantification of Chloramphenicol in milk-based matrices. Comparison between a classical sample preparation, *Anal.Chem.* (2007); 79(24); 9557-9565.
2. Boyd, B, Björk H, Billing J, Shimelis O, Axelsson S, Leonora M, Yilmaz E, Development of an improved method for trace analysis of chloramphenicol using molecularly imprinted polymers, *Journal of Chromatography A* (2007), 1174
3. Young, M.S., Jenkins, K.M. 2004. SPE based methods for LC/MS determination of chloramphenicol and related compounds in shrimp. www.waters.com

Appendix 1.

Details of the SupelMIP SPE Chloramphenicol method

Sample pretreatment

Method 1. Ethyl acetate extraction: Homogenize 5 g of peeled shrimps and add 50 mL EtOAc with internal standard d5-CAP (150 ng/kg). Vortex for 2 minutes. Filter the supernatant through a filter (5.5 µm). Evaporated to dryness and redissolve the residue in 10 ml of DI water.

Condition cartridge

Note: recommended flow rate ~0.5 mL/min.

- ◆ 1 mL methanol
- ◆ 1 mL DI water

Do not allow the polymer to dry between conditioning and sample application steps. If the MIP goes dry, repeat the complete conditioning.

Load

Note: recommended flow rate ~0.5 mL/min.

Apply 2 mL sample to the cartridge.

Larger volumes are not recommended.

Wash

Perform the washings steps in the described order. The maximum flow rate during the wash steps should not be greater than 0.5 mL/min.

- ◆ 2 x 1 mL DI water
- ◆ 1 mL 5 % acetonitrile / 95% acetic acid (0.5%v/v aq.)
- ◆ 2 x 1 ml 1 % (v/v) ammonia (aq.)
- ◆ 1 mL 20 % acetonitrile / 80% ammonia (1%v/v aq.) *

Apply gentle vacuum, around 0.1 bar (75 mm Hg), between each wash step.

◆ Apply full vacuum through cartridge to remove residual solvent from cartridge.

Use strong vacuum at ~0.5-0.7 bar for 10 min. to ensure a dry column before proceeding to next step.

◆ 3 x 1 mL dichloromethane

Apply gentle vacuum, around 0.1 bar (75 mm Hg), between each wash step.

◆ Apply full vacuum through cartridge to remove residual solvent from cartridge.

Use strong vacuum at ~0.5-0.7 bar for 2 min. to ensure a dry column before elution.

Analyte elution

Note: recommended flow rate ~0.2 mL/min.

Elute chloramphenicol with 2 x 1 mL 10 % methanol:90 % dichloromethane (v/v) **

Apply a gentle vacuum between each fraction. Use 2 x 1 mL and NOT 1 x 2 mL elution solvent.

Evaporate to dryness (45°C for 40 min.) and reconstitute in 150 µL in LC mobile phase (30 % Acetonitrile / 70% 10 mM Ammonium Acetate prior to analysis.

If the flow rate is greater than 0.2 mL/min you may increase the number of elution aliquots to recover more analyte, e.g. 3 x 1 mL.

Analytical procedure

Column:	Ascentis C18 (100mm X 2.1mm, 3µm)
Mobile phase:	10 mM ammonium acetate (pH 6.6):acetonitrile (70:30)
Flow rate:	0.2 mL/min.
Temperature:	22 °C
Run time:	Isocratic 5 mins.
Injection Volume:	20 µL
Detection:	MS/MS, MRM transitions
MRM transitions:	Quantification (321.00/152.00) Identification (321.00/257.00) I.S. (326.00/157.1)
Ion mode:	ESI Negative
Ion source:	Turbospray
Ion spray voltage:	-2000 V
source temperature:	500 °C
ion source gas 1:	70 psi
ion source gas 2:	40 psi
Curtain gas:	10 psi
DF:	200 V
Dwell time:	150 msec

Transitions	Q1/Q3	DP	EP	CEP	CE	CXP
Chloramphenicol	321/152	-35	-10	-12	-28	-2
d5-Chloramphenicol	326/157.1	-35	-10	-12	-26	-4