Clean-up and Concentration of *Chloramphenicol and Malachite green from Animal Products and Honey* for Residue Analysis

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Application Note AN0057 | www.LCTech.de/en
As of: July 2023, Version: 1.0

Content

1. Introduction

Chloramphenicol and other antibiotics are repeatedly found in aquatic foods such as crabs, shrimps, mussels from intensive aquaculture, but also in poultry (chicken, turkey) and other animal products. The use of these antibiotics is subject to strict regulations. The presence of these and other drugs used in veterinary medicine in food is prohibited and strictly controlled (EU 2019/1871, EU 2021/808). The so-called reference point of action (RPA) was previously set by the EU in 2019/1871 at 0.15 ppb for chloramphenicol and 0.5 ppb for malachite green. However, due to the now achievable lower limits of quantification, lower limits down to 0.1 and 0.05 ppb for chloramphenicol would also be possible and are under discussion. For the analysis of chloramphenicol and/or malachite green in animal and aquatic meat products, a complex extraction and clean-up is unavoidable. In order to prevent contamination, interference during the analysis or simply contamination of the analytical equipment by protein and fat components, selective extractions and clean-ups are usually used to separate interfering components from the analytes and to facilitate the determination. The application presented in this application note offers an ideal combination of a simple extraction approach combined with an effective clean-up and enrichment of the analytes (chloramphenicol and derivatives thereof, malachite and leucomalachite green), especially for meat products, fish and seafood, and thus represents a new, fast way of introducing samples to the analysis by means of a simple SPE method.

2. Product Highlight

The CrossTOX® column enables high matrix loading, efficient depletion of interfering substances (protein, fat components or sugars) that can influence the analysis.

Fast and easy extraction, high flow rates and high matrix capacity characterise the CrossTOX® column and offer an excellent possibility to detect even traces of chloramphenicol.

Only 1 extraction procedure, only 1 SPE column, only 1 sample concentration necessary - time and material are saved.

- Efficient non-dispersive clean-up column
- Loading capacity up to 0.5 g matrix (1000 ng chloramphenicol WFR >90 %)
- Suitability for aquatic and animal products (crabs, shrimps, scampi, mussels, fish and poultry, honey)
- Compatible with different extraction approaches:
  Methanol compatibility (up to 20 %), acetonitrile compatibility (up to 10 %)
  Lower loading volumes without loss of sensitivity
- Simple extraction process (methanol/water or acetonitrile/water)
- Loading, washing and eluting - selective clean-up
- Suitability for the analysis of chloramphenicol, malachite green by LC-MS/MS

3. Processing Protocol

20 grams of homogenised material are extracted using 100 mL methanol/water (80/20 (v/v)) for 3 - 5 minutes. The crude extract is clarified by filtration or by centrifugation at 3000 x g for 5 minutes. 2.5 mL sample is mixed with 17.5 mL deionised water (isotope-labelled standards are added before SPE clean-up) and transferred at a flow rate of max. 2 mL/min onto the CrossTOX® column. Peristaltic pumps or a vacuum manifold or robotic system (FREESTYLE QuEChERS) can be used for sample preparation.

The template vessel is rinsed with 2 mL deionised water and the rinsing solution is also loaded onto the CrossTOX® column. Afterwards, the column is dried by a short air flow and then 1 mL methanol is added. To increase the elution efficiency, the methanol should be allowed to soak into the column bed for 5 minutes to ensure complete elution. The eluate is then collected and can be concentrated for analysis. After concentration, it is redissolved by means of 50 - 150 µL of running medium and can be analysed in LC-MS/MS.
In case of heavy precipitation, it is recommended to use a syringe filtration before injection into the LC-MS/MS.

4. Analytics

Table 1: LC-MS/MS- analytical parameterisation

UPLC	Gradient
Column oven	38 °C
Separation column	Accucore Biphenyl 100 mm x 2.1 mm; 2.6 µm with precolumn
Flow rate, Solvent	0,4 mL/min; Solvent A: HPLC-water/methanol (98/2 (v/v), 5 mM ammoniumacetat, 1 % acetic acid) Solvent B: HPLC-wasser/methanol (2/98 (v/v), 5 mM ammoniumacetat, 1 % acetic acid)
0 - 2 min	95 % A; 5 % B
2 - 10 min	15 % A; 85 % B
10.1 - 18 min	5 % A; 95 % B
18.1 - 25	95 % A; 5 % B
Analytics	Heated ESI 3500V (+); 3500 V (-); Ion-transfer-tube 325 °C; evaporator 350 °C; collision gas 1,5 mTorr (argon)

Table 2: Precursors and productions for the analysis of the analytes by LC-MS/MS

m/z	Precursor	Product ions
Chloramphenicol M+H	323	**304.887** / 274.863 / 257.97 / 165.179 / 119.054
Chloramphenicol M-H	321	**257.077** / 193.893 / 176.167 / 152.149 / 121.13
D5-Chloramphenicol M-H	326	**262.077** / 199.893 / 181.167 / 157.149
Malachite green M+H	329	**207.99** / 241.190 / 313.000

5. Loading Capacities

The capacity of the CrossTOX® column for chloramphenicol was tested for concentrations of 50-1000 ng under experimental conditions to provide quantifiable results in case of massive exceedances of the limits (Diagram 1).

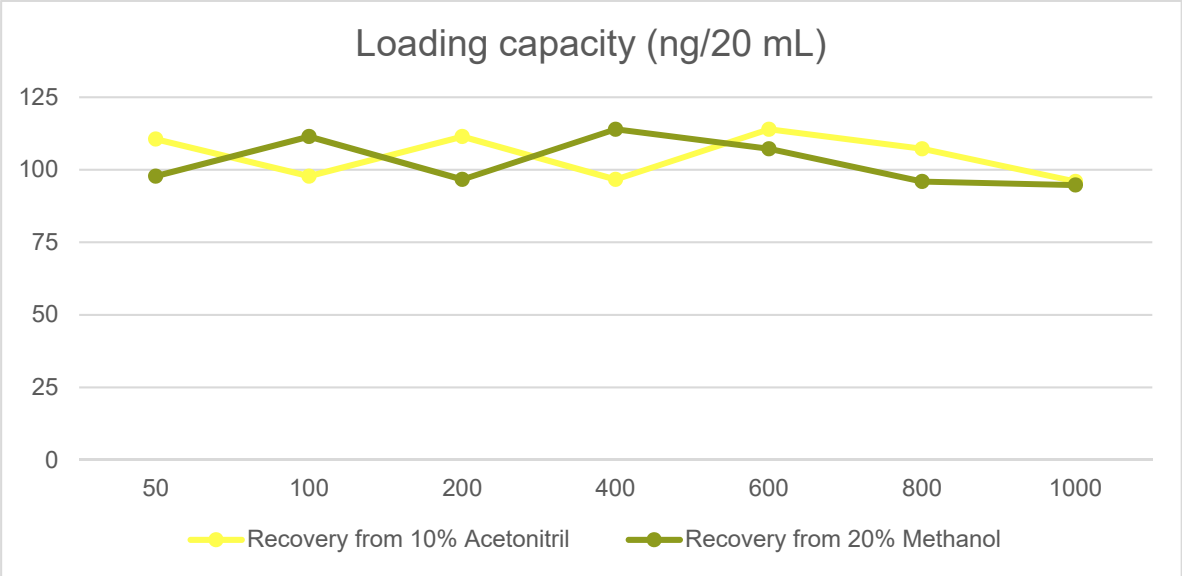

Diagram 1: Loading capacity of the CrossTOX® column by chloramphenicol at 10 % acetonitrile or 20 % methanol. Very good recoveries were consistently achieved when the solvent concentrations were maintained.

The loading capacity could be determined with up to 1000 ng under the conditions of sample processing with a recovery of more than 90 %. To determine whether the selectivity of the CrossTOX® column is suitable for effectively binding even the smallest traces of chloramphenicol to meet the minimum limits of quantification, concentrations of 0.01 to 50 ng chloramphenicol were tested in a matrix sample (crab sample) (diagram 2).

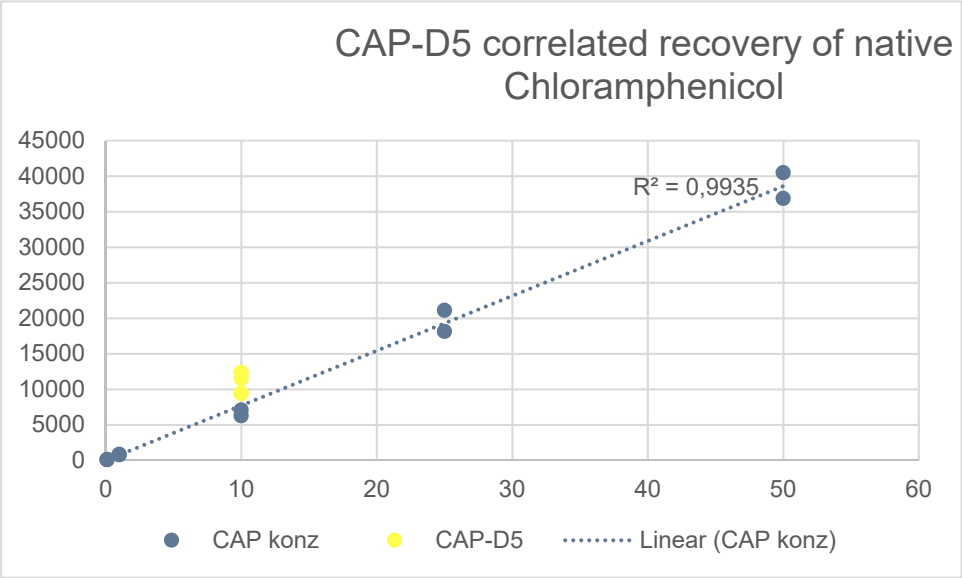

Diagram 2: Compensation of matrix effects by D5-labelled chloramphenicol, which was also cleaned up.

By using the D5-labelled chloramphenicol, matrix effects and sensitivity effects can be compensated. A correlation between the D5-labelled and the native chloramphenicol could be observed independent of the concentrations used (diagram 2).
A very good linearity between the applied concentration and the determined concentration could be achieved. A correlation with the internal standard was used to compensate for matrix effects. The recoveries and correlations meet the minimum legal requirements in terms of sensitivity, linearity and reproducibility. The data show that chloramphenicol can be reliably detected in foodstuffs even far below the legally required minimum limits of determination.

6. Recoveries and Chromatograms

6.1 Recoveries of Chloramphenicol in Different Matrices

Matrix depletion allows selective enrichment of the analytes without burdening the analytical equipment. The analysis of various animal foods showed excellent results in terms of recovery and measurement sensitivity. The suitability of the simple extraction and 1-step clean-up using the CrossTOX® column allows fast and efficient sample processing and the suitability of this method for a wide variety of animal matrices (Table 3).

Table 3: Recoveries of chloramphenicol from different animal matrices with different concentrations.

Matrix	Source	Recovery [%] at 0.5 µg/kg	Recovery [%] at 5 µg/kg
Litopenaeus vannamei	Indonesia	105	96
Litopenaeus vannamei	Ecuador	101	87
Pandalus borealis	Norway	102	99
Mytilus sp.	Indonesia	108	105
Oncorhynchus mykiss	Denmark	96	100
Gallus	Germany	101	99
Meleagris gallopavo	Germany	94	89

Table 3: Testing the clean-up and recovery of chloramphenicol from honey samples

Matrix	Spiking-Level [µg/kg]	Recovery [%]
Honey	0.1	90
Honey	0.5	96
Honey	1	82
Honey	5	98

The recoveries for honey samples meet the requirements of the European implementing regulation EU 2021/808. The sensitivities achieved can be lowered to 0.01 ppb by adjusting the recovery volumes from 150 µL to as low as 50 µL (Table 4).

A linearity of recovery with increasing chloramphenicol or malachite green concentrations, was observed up to a loading of 2000 ppb.

For aquatic and terrestrial meat products, sensitivities down to 0.05 ppb, could be achieved without adjustments/reduction of matrix loading.

6.2 Chromatography Chloramphenicol M-H

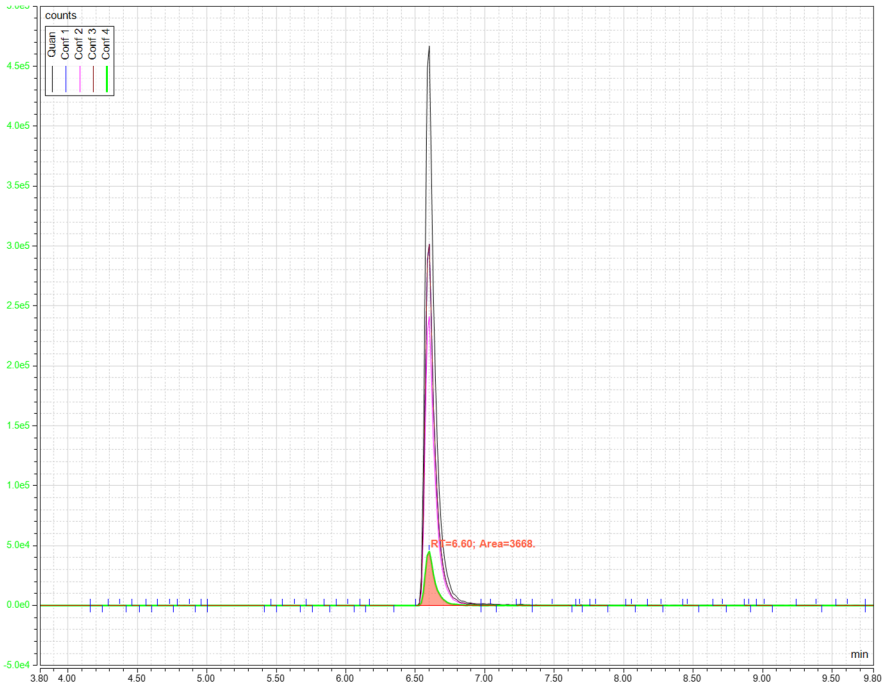

Figure 1: Chromatography of the productions of chloramphenicol under the parameters described in table 1 and 2.

6.3 Chromatography D5-Chloramphenicol M-H

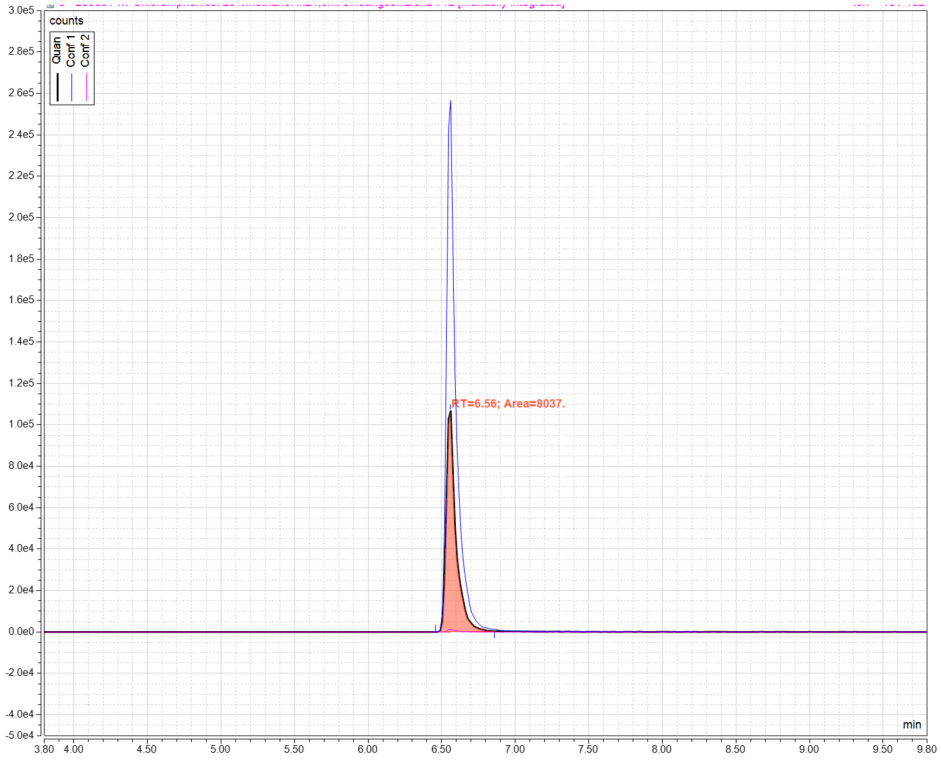

Figure 2: LC-MS/MS chromatography of the D5-labelled chloramphenicol and the corresponding productions when using the analytical parameters mentioned in Table 1 and 2.

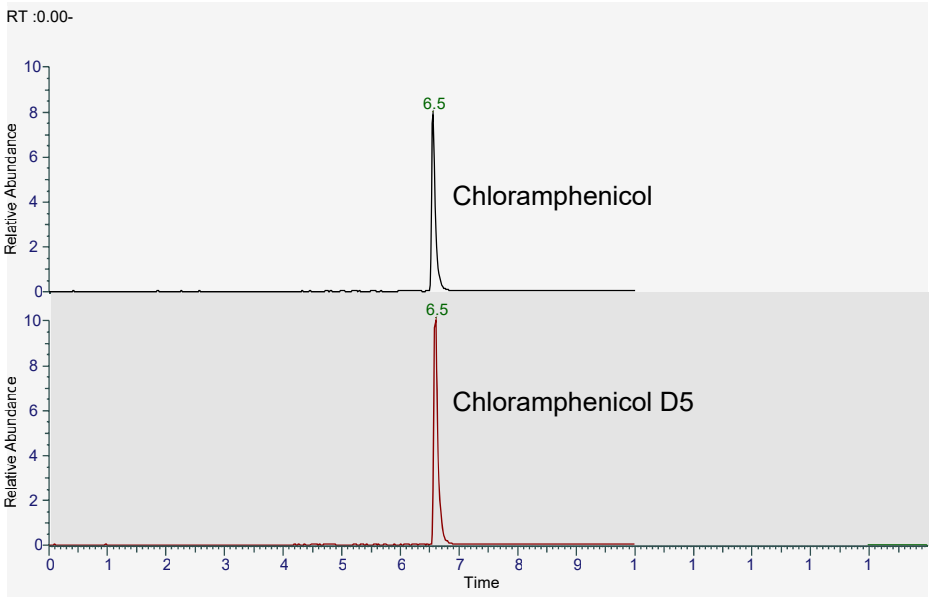

Figure 3: Simultaneous analysis of chloramphenicol, D5-chloarmphenicol in the SRM mode of the LC-MS/MS with corresponding retention times.

7. Conclusion

Due to its non-dispersive clean-up properties, the CrossTOX® column is an ideal column for the enrichment of antibiotics such as chloramphenicol or even malachite green, which are often used in aquaculture and factory farming to prevent infections in livestock. This means that these analytes can be reliably determined even far below the legal limits. Due to the irreversible binding of fats to the column material, substances such as chloramphenicol and malachite green can be selectively eluted and transferred to the analysis. The high matrix capacity and matrix compatibility predestine the CrossTOX® column for sample preparation in food and feed control.

With a loading capacity in the range of 0 - 1000 ng chloramphenicol at 20 mL sample volume and 20 % methanol content, recoveries were determined linearly with over 90 %. With this application, a two-step clean-up using 2 SPE columns and the associated solvent exchange can be avoided. Due to the excellent clean-up properties of the CrossTOX® column, matrix components that negatively influence the analytics or the measurement sensitivity are massively reduced, which leads to a faster, better and reliable analysis of meat, fish and honey samples for chloramphenicol or malachite green.

Any Questions?
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