

SOLUTIONS BY



Detection of Chloramphenicol in Honey Using Automated Solid-Phase-Extraction and HPLC-MS/MS-Detection

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1. Introduction

In Germany, an average of 1 kilogram of honey per person is consumed every year. However, honey production in Germany only provides about 20 percent of this demand. Much to the dismay of the domestic beekeepers, the remaining demand, i.e. the majority, is covered by imports from abroad (predominantly Latin America and Asia). In 2002, the EU imposed a ban on imports of Chinese honey until 2004. The reason for this was the high contamination of honey with chloramphenicol – a banned antibiotic.

Chloramphenicol (CAP) has been used as a broad-spectrum antibiotic in animal and human medicine because of its very high bacteriostatic potency. However, in today's human medicine its use has been greatly reduced due to the severe side effects accompanying this drug.

In the EU, chloramphenicol has been banned for use in food-producing animals since 1994. In practical terms, this was achieved by including the ban in Annex IV (i.e. list of pharmacologically active substances for which maximum levels cannot be established) of the Regulation (EEC) 2377/90. The (EU) Regulation 37/2010 includes this prohibition, and hence replaces the above mentioned Annex to (EEC) 2377/90.

The ban is based on the suspicion of CAP causing aplastic anaemia in humans, as well as on the indication of possible reproductive toxicity.

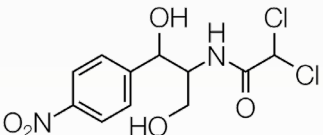
Over and over again, residues of antibiotics are found in honey, even though these have been banned in the EU. For example in South America, antibiotic use is still permitted, which means that they can get introduced to Germany through imports.



Figure 1: Honey

1.1 Analyte

Table 1:

Analyte	Molecular Formula	Molecular Weight [g/mol]	Structural Formula
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	323,14	

In the following, it is shown how honey samples can be fully automatically cleaned through solid phase extraction (SPE) with the FREESTYLE SPE robotic system ready for the measurement of chloramphenicol using LC-MS/MS. The results are compared with the manual preparation, which is currently mainly performed in the laboratory. Fully automated, sequential sample preparation enables a high sample throughput and liberates human resources for other tasks.

2. Method

2.1 Sample material and preparation

5 g of honey (blossom honey, Goldland (Aldi)) are weighed into a centrifuge tube (50 mL), and then 4 mL of water are added. The tube is shaken vigorously for 30 seconds. Thereafter, 1 mL standard solution ($c = 5 \text{ ng / mL}$ chloramphenicol and chloramphenicol-d5) is added, shaken vigorously for 30 seconds, and then 15 mL ethyl acetate are added and shaken once again vigorously for 30 seconds. The solution is then centrifuged at 3000 rpm for 10 min. Afterwards, 12 mL of the resultant supernatant is removed and evaporated under a nitrogen stream at 40 °C. The residue is dissolved in 10 mL of water.



Figure 2: Preparation of the sample

2.2 Chemicals, standard substances and consumables

- Water, double-distilled, Milli-Q by Millipore
- Acetonitrile ULC/MS, Biosolve B.V.
- Formic acid 99 % ULC/MS, Biosolve B.V.
- Ethyl acetate p.A., CHEMSOLUTE
- Standard substances and isotope labelled standards:
Chloramphenicol $\geq 98\%$ (HPLC), Sigma;
(Erythro) Chloramphenicol D5 100 $\mu\text{g}/\text{mL}$ in acetonitrile, LGC Standards
- SPE-cartridges CHROMABOND[®] HLB, 3 mL, 200 mg,
(REF 730924), MACHEREY-NAGEL
- HPLC separation column EC 150/2 NUCLEODUR[®] $\pi 2$, 5 μm (REF 760624.20),
MACHEREY-NAGEL

2.3 Devices

- 1290 Infinity II UHPLC, Agilent Technologies with QTRAP 5500, AB Sciex
- FREESTYLE SPE, LCTech GmbH (for configuration see 2.9)
- Concentrator with nitrogen
- Rotina 420R, Hettich Zentrifugen

2.4 Solid phase extraction, manual

Table 2: SPE steps in manual sample preparation

SPE steps	manual
Conditioning	3 mL methanol 5 mL ultrapure water
Loading	9 mL sample, 3 mL/min.
Washing	10 mL ultrapure water, 3 mL/min.
Drying	5 min. with vacuum
Elution	5 mL ethyl acetate/methanol (80:20, v/v)

2.5 Solid phase extraction – automated by LCTech

The FREESTYLE robotic system is a system for automated sample preparation. It consists of the basic building block FREESTYLE BASIC, which can be equipped with various modules, depending on the requirements of the user.

Equipped with the SPE module, the FREESTYLE handles all methods in the area of solid phase extraction. The fixed connection of the SPE column with the robot arm allows the columns to be moved to any position on the platform. In addition, it also caters for a controlled pressurisation of up to 4 bar, which is particularly important in applications where suspended matter can block the column. The FREESTYLE SPE is able to process all SPE standard column formats (1, 3, 6, 8, 15 mL) and LCTech glass columns (up to 15 mL) automatically.

The manual solid phase extraction can be easily transferred to the robotic system for automated sample preparation using the FREESTYLE SPE. The system's easy-to-use software provides a method editor that makes method development on the system fast and straightforward. With drag & drop, the general structure of the method can be defined in seconds. By moving bars, parameters such as volumes and speeds are specified.

The screenshot displays the FREESTYLE software interface for configuring Solid Phase Extraction (SPE) steps. The main window shows 'SPE Steps: Step number: 2' with ID: 530. The configuration includes:

- Load:** Transfer Type: 'Transfer Sample-Aliquot over sample loop', Input Vial Type: 'Type1@16', Sample Volume: 10,0 mL.
- Speeds:** Suction Speed: 5 mL/min, Dispensing Speed: 2 mL/min.
- Waiting times:** Waiting time after dosage: 0 s, Waiting Time after Step: 5 s.
- Dispense:** Radio buttons for 'into Waste' (selected) and 'into vial'.
- Calculated time:** 2,2 min for this step.
- Summary:** SPE Steps: 4, Total: 118.6 min.

At the bottom, there are two tables:

Input vials					
Step N°.	Owner	Count	Type	Basic type	ID
2	Load	1	Type1@16	LAD	530

Result vials				
Step N°.	Owner	Count	Type	Basic type
4	Drying	1	Type1@60	DRY

Figure 3: Screenshot of the FREESTYLE software showing the various SPE steps.

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FREESTYLE performs the following steps fully automatically:

Table 3: SPE steps in automated sample preparation

SPE steps	automated
Conditioning	3 mL methanol, 1 mL/min. 5 mL ultrapure water, 1 mL/min.
Loading	9 mL sample, 3 mL/min.
Washing	5 mL ultrapure water, 3 mL/min.
Drying	100 mL air with syringe, 100 mL/min.
Elution	5 mL ethyl acetate/methanol (80:20, v/v), 3 mL/min
Drying	20 mL air with syringe, 10 mL/min.

The exact parameter settings of the method on the FREESTYLE are shown in the following method report:








Step: Conditioning_MeOH		Basic type: Conditioning		Step: - ID: 623	
 Volume: 3 ml	Suction Speed: 20 ml/min Repetitions: 0 Waiting Time after Dosage: 0 sec.	Dispensing Speed: 1 ml/min Waiting Time after Step: 0 sec.	Port: 1 MeOH Dispense: into Waste		
Step: Conditioning_H2O		Basic type: Conditioning		Step: - ID: 622	
 Volume: 5 ml	Suction Speed: 20 ml/min Repetitions: 0 Waiting Time after Dosage: 0 sec.	Dispensing Speed: 1 ml/min Waiting Time after Step: 0 sec.	Port: 8 Water Dispense: into Waste		
Step: Loading		Basic type: Load - Transfer Sample-Aliquot over sample loop		Step: - ID: 610	
 Volume: 9 ml Vial Type: Type1@16	Suction Speed: 10 ml/min Waiting Time after Dosage: 0 sec.	Dispensing Speed: 3 ml/min Waiting Time after Step: 150 sec.	Dispense: into Waste		
without rinsing of vial					
Step: Washing_H2O		Basic type: Washing		Step: - ID: 624	
 Volume: 5 ml	Suction Speed: 10 ml/min Repetitions: 1 Waiting Time after Dosage: 0 sec.	Dispensing Speed: 3 ml/min Waiting Time after Step: 0 sec.	Port: 8 Water Dispense: into Waste		
Step: Drying		Basic type: Drying - Drying by defined air volume		Step: - ID: 625	
 Air volume: 100 ml	Suction Speed: 100 ml/min	Dispensing Speed: 100 ml/min	Dispense: into Waste		
Step: Elution_EE-MeOH_8:2		Basic type: Eluting		Step: - ID: 626	
 Volume: 5 ml	Suction Speed: 10 ml/min Repetitions: 0 Waiting Time after Dosage: 0 sec.	Dispensing Speed: 3 ml/min Waiting Time after Step: 0 sec.	Port: 11 EE-MeOH 8:2 Dispense: into vials Number of vials: 1 Vial Type: Type1@16		
Step: Drying		Basic type: Drying - Drying by defined air volume		Step: - ID: 627	
 Air volume: 20 ml	Suction Speed: 100 ml/min	Dispensing Speed: 100 ml/min	Dispense: stay on actual position		

Figure 4: Parameter settings for the SPE method on the FREESTYLE SPE

2.6 Eluent exchange

- The eluate (from both the manual and automated clean-up) is transferred to a round cuvette (nano reaction vials, OD: 16 mm, 20 pcs, item no.: 91680).
- Rinse with 1 mL of ethyl acetate / methanol (80:20, v / v).
- The temperature on the thermoblock (Vario 4, item no.: 919300) is set to 40 °C.
- Under a gentle stream of nitrogen the eluate is concentrated, made up to 1 mL volume with water / acetonitrile (95: 5, v / v) and dissolved again.
- Then the eluate is injected.

2.7 Measurement with LC-MS/MS

The following measurement was performed on an Agilent 1290 Infinity II system together with an AB Sciex QTRAP 5500 MS detector.

The following configuration was used:

Table 4: Configuration of the LS-MS/MS with detector.

Column	EC 150/2 NUCLEODUR® π2, 5 μm
Eluent A	Water
Eluent B	Acetonitrile
Gradient	5-95 % B (in 7.5 min.) 9 5% B (1 min. hold) 95-5 % B (in 1 min.) 5 % B (5 min. hold)
Flow rate	0.3 mL/min
Injection volume	5 μL
Column temperature	35 °C
Detection	MS/MS, AB Sciex QTRAP 5500 Ion source: Turbo Spray (ESI) Scan type: MRM Polarity: negative Curtain gas: 35 psig Ion spray voltage: -4500 V Temperature: 450 °C Gas 1 (nebuliser): 45 psig Gas 2 (turbo gas): 45 psig CAD gas: 6 psig

2.8 Configuration FREESTYLE

1. FREESTYLE BASIC robotic system	P/N 12663-12
2. SPE module for FREESTYLE BASIC robotic system	P/N 12668
3. Rack for solvent delivery	P/N 13156
4. Column adapter for 3 mL SPE columns with jam strip	P/N 14612
5. Caps for 3 mL columns	P/N 14862
6. Rack for up to 18 SPE-columns	P/N 13946
7. Tray for 16 mL vials	P/N 11933
8. Frame for trays	P/N 11915
9. Adapter for SPE-columns	P/N 13382
10. Screw-thread bottle, 16 mL	P/N V0016
11. Screw cap, 16 mL	P/N V0016-SL
12. Seal for 16 mL vials	P/N V0016-D

2.9 Consumables by MACHEREY-NAGEL

1. CHROMABOND® HLB, 3 mL, 200 mg	P/N 730924
2. EC 150/2 NUCLEODUR® π2, 5 μm	P/N 760624.20
3. Nano reaction vials, AD 16 mm	P/N 91680

3. Results

3.1 Recovery rates

Table 5: Comparison of recovery rates achieved with automated and manual solid phase extraction.

Column	Chloramphenicol	Chloramphenicol (internal standard taken into account)	Chloramphenicol-d5
Automated SPE	86.8 ± 4.4 (n = 4)	90.8 ± 4.6 (n = 4)	95.6 ± 4.5 (n = 4)
Manual SPE	74.6 ± 2.7 (n = 3)	92.9 ± 3.4 (n = 3)	80.3 ± 4.7 (n = 3)

3.2 Chromatograms

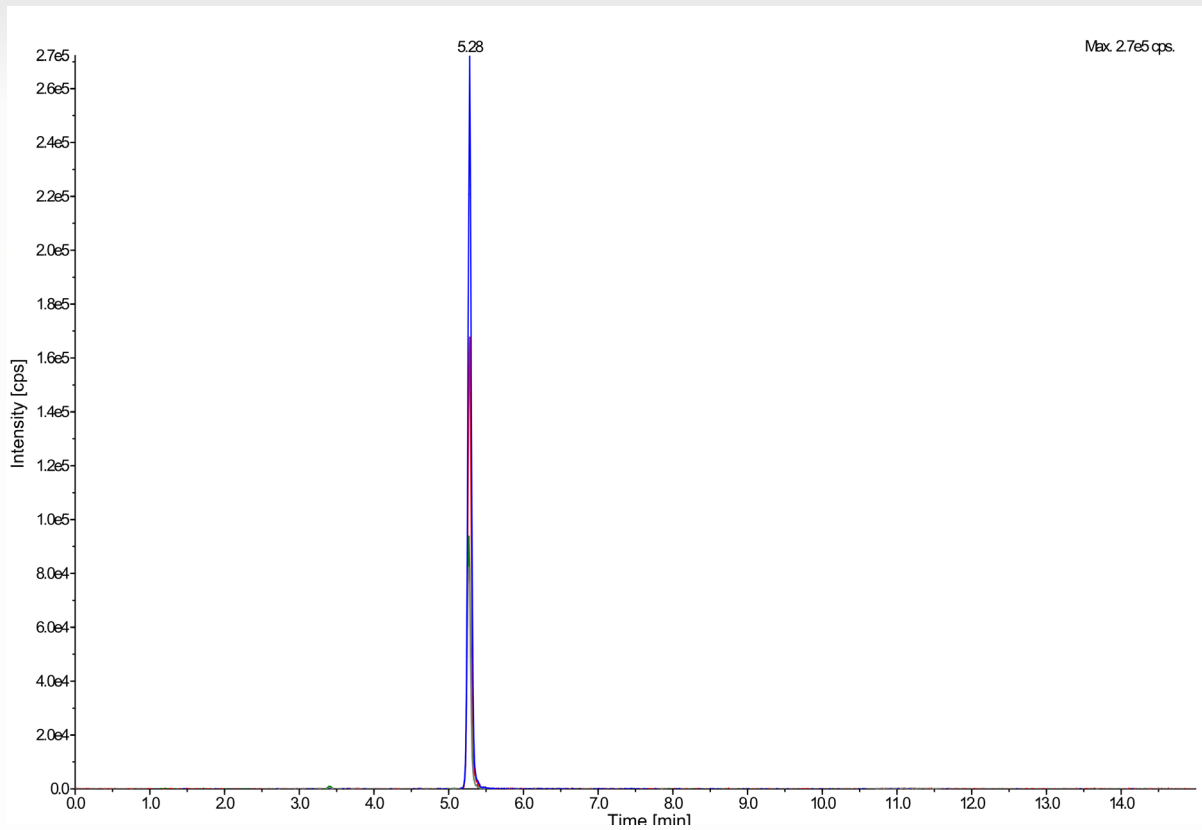


Figure 5: Chromatogram of a chloramphenicol standard solution, $c = 5 \text{ ng/mL}$.

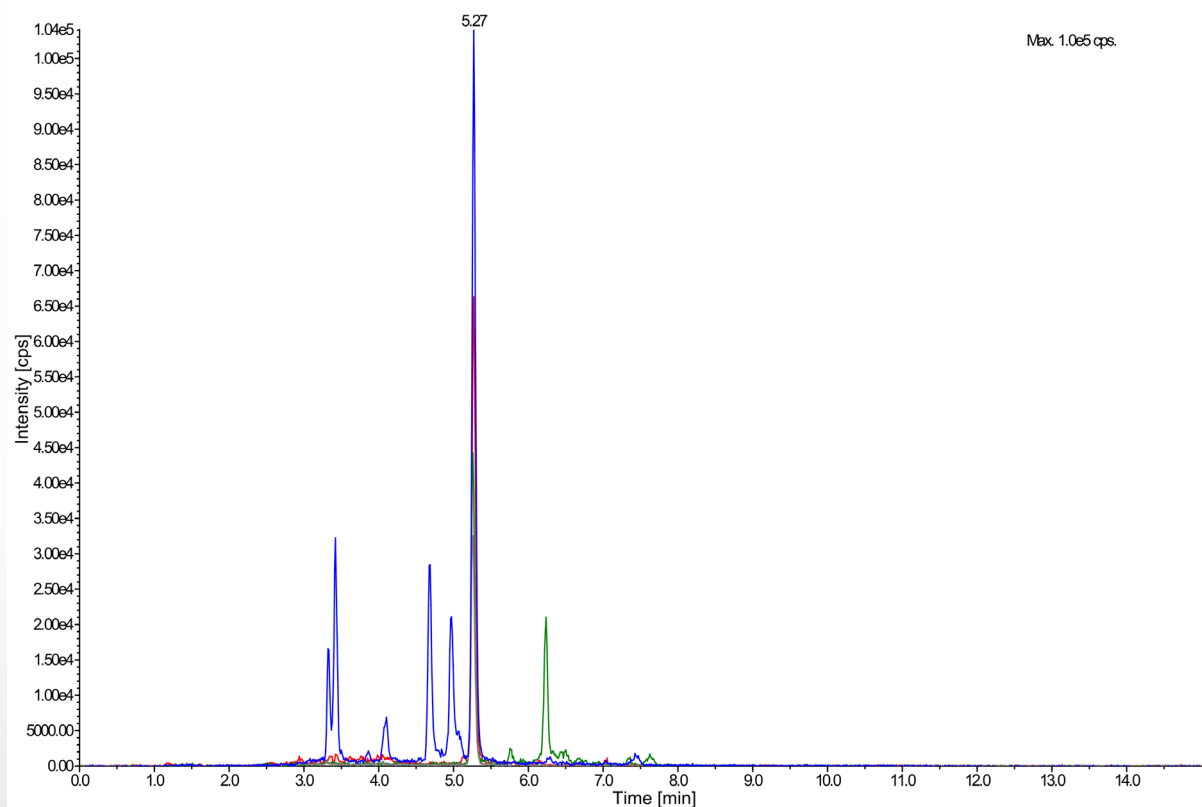


Figure 6: Chromatogram of a spiked honey sample, $1 \text{ }\mu\text{g/kg}$

Table 6: MRM transitions for chloramphenicol and for chloramphenicol-d5.

Analyte	[M-H] ⁻	Q1 (Quantifier)	Q2 (Qualifier)
Chloramphenicol	320.9	152.0	256.0
Chloramphenicol-d5	325.9	156.6	261.8

3.3 Calibration curves

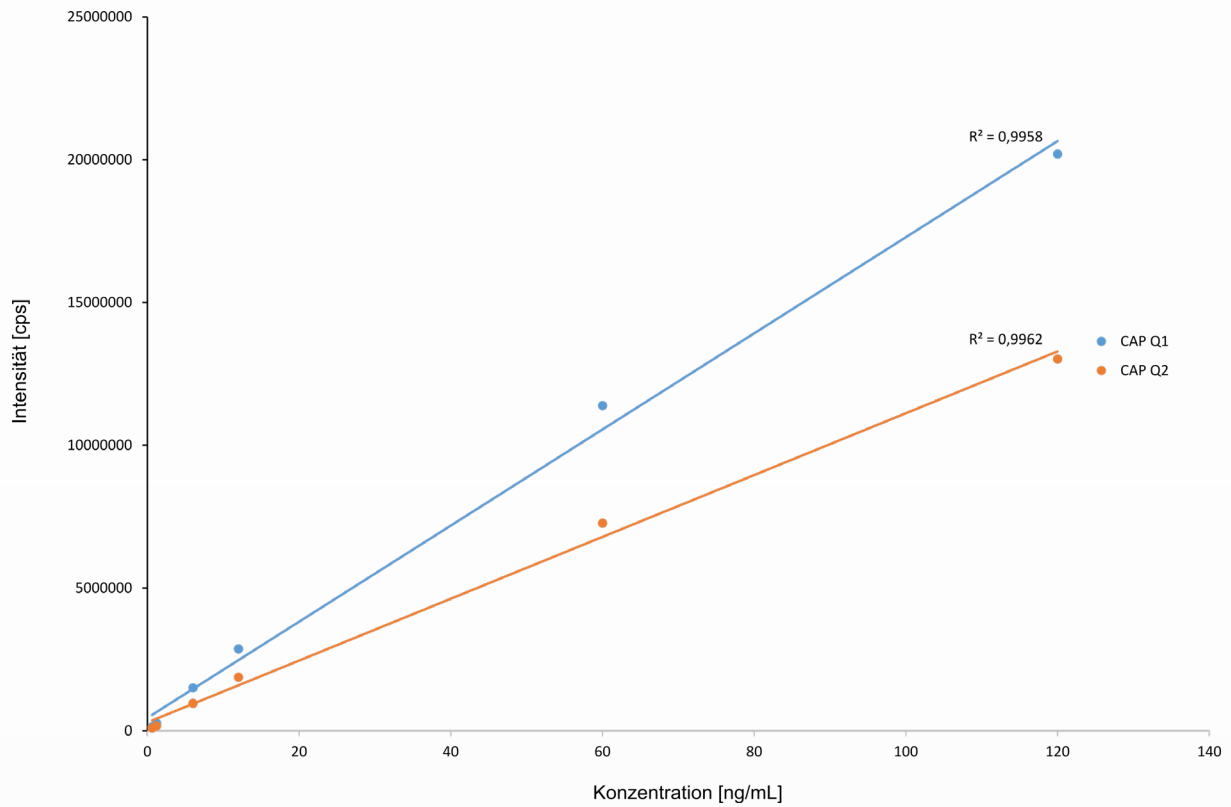


Figure 7: Calibration curves of chloramphenicol, concentration range 0.5-100 ng/mL.

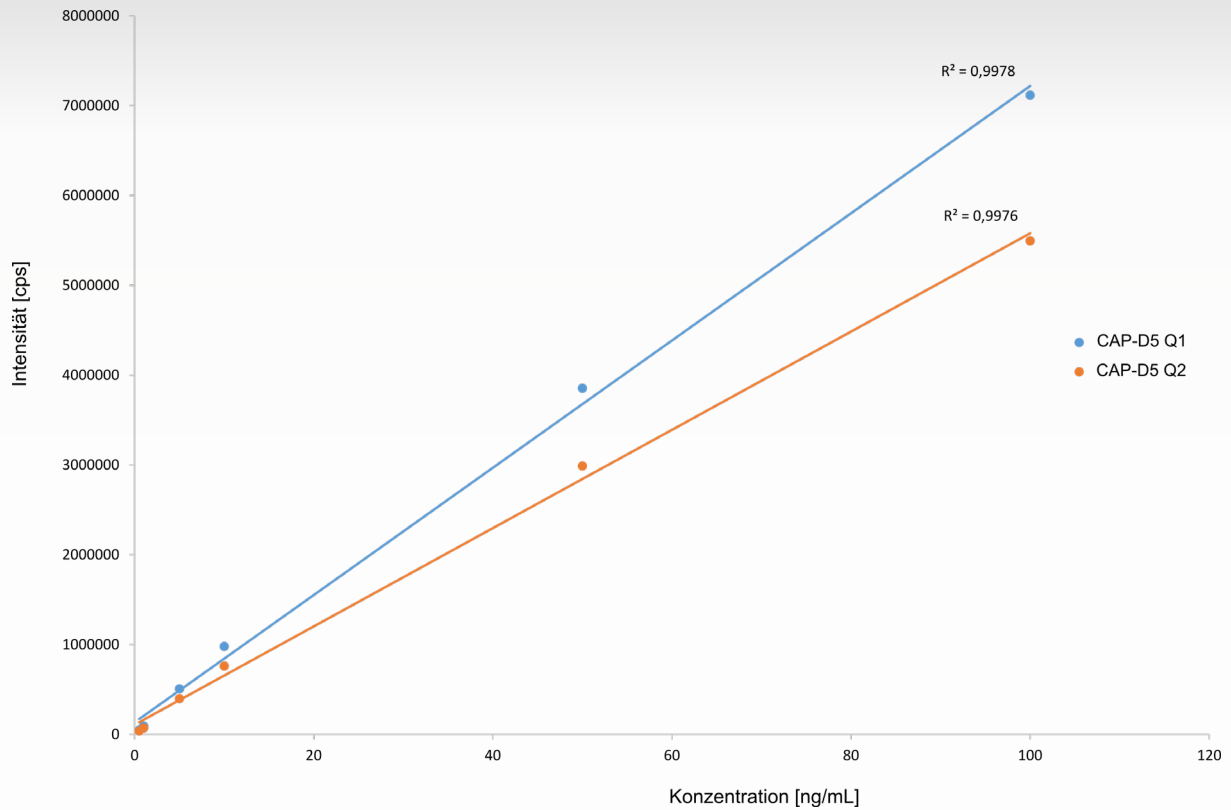


Figure 8: Calibration curves of chloramphenicol-d5, concentration range 0.6-120 ng/mL.

3.4 Summary

From these results, it is evident that solid phase extraction of chloramphenicol from the honey matrix using CHROMABOND® HLB leads to excellent recovery rates. As can be seen from Table 5, automated processing results in even higher recovery rates than manual processing with a good reproducibility (< 5 %) for both methods.

The chromatograms show that the peak for chloramphenicol clearly separates from the matrix interferences and thus can be easily identified and quantified.

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