Ultrasensitive Detection and Quantitation of Chloramphenicol using the new Elute UHPLC and EVOQ LC-TQ System

Quantitative Analysis of Chloramphenicol in Eggs using the EVOQ Elite TQ coupled to the Elute UHPLC.

A liquid chromatography tandem mass spectrometry (LC-MS/MS) method operated in multiple reaction monitoring (MRM) mode has been developed for detecting and confirming trace levels of chloramphenicol in egg matrix with minimal sample preparation. Three MRM transitions were used to ensure highly accurate peak assignment; the primary one for quantification and the other two for qualification. The limit of quantification for chloramphenicol in eggs was 0.02 ppb with both qualifying ions showing consistent ratios over the whole range. The calibration curve is linear from 0.02 ppb to 5.0 ppb with a correlation coefficient of $R^2 = 0.9967$.

Keywords: Elute UHPLC, EVOQ Elite, LC-TQ

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Introduction

Chloramphenicol is a broad spectrum antibiotic first employed in 1994. Although inexpensive, chloramphenicol is no longer recommended as a first line treatment in humans because of the potential for adverse effects and interactions. Chloramphenicol is commercialized for veterinary applications, but its use in food animals is restricted or prohibited in many countries because of the potential for adverse effects in humans eating food containing residues.

In 1986 the FDA banned the use of chloramphenicol in animals linked to food production; with the EU following in 1994.

The European Union Regulation (EC) 470/2009 states the zero tolerance position clearly. In detail the EU decision 2003/181/EC defines that a method for chloramphenicol detection must meet or exceed the Minimum Required Performance Level (MRPL) of 0.3 ppb.

Experimental

Sample Preparation

Chloramphenicol (98%) was purchased from Sigma-Aldrich (St. Louis, MO), and Chloramphenicol-d5 (100 µg/mL in acetonitrile) was purchased from Cambridge Isotope Lab (Andover, MA).

A phosphate extraction solution (PES) was prepared weighing 8.0 g of NaCl (137×10^{-3} mol/L), 0.20 g of KCl (2.7×10^{-3} mol/L), 1.15 g of $Na_2HPO4 \times 7H_2O$ (8×10^{-3} mol/L), 0.215 g of Na_2HPO4 (1×10^{-3} mol/L), 0.20 g KH_2PO4 (1×10^{-3} mol/L), and diluted with de-ioinized water. The pH was adjusted to 7.2 with hydrochloric acid 1 mol/L and the volume being made to 1000 mL with de-ionized water. The egg samples were prepared according to the method of Siqueira *et al*[1]. 4.0 mL PES and the internal standard was added to 1.0 g homogenized whole egg in a 15-mL centrifuge tube and ultrasonicated for 15 min. The slurry was centrifuged at 10.000 *g* for 10 min. The supernatant was transferred to a new centrifuge tube and mixed with 4.5 mL of Ethylacetate. After an additional 10 min centrifugation the top organic layer was taken and evaporated to dryness in an Eppendorf Vacuum Concentrator at 45 °C. The extract was reconstituted in 0.5 mL initial mobile phase (90:10 Water:MeOH).

Results and Discussion

Figure 1 shows the overlaid chromatograms for a positive sample for CAP alongside the egg matrix blank.

The Decision 2002/657/EC specifies several parameters to confirm detection of the compound of interest.

Chromatography (Bruker Elute UHPLC)

Column:	Restek Ultra AQ C18 150 x 2.1 mm, 3 µm	Gradient Conditions:	0.00 min 10% B 0.05 min 10% B 4.50 min 95% B 5.50 min 95% B 5.60 min 10% B 7.00 min 10% B
Injection Volume:	2 µL (µL pick up)		
Flow Rate:	0.3 mL/min		
Mobile Phase A:	1 mM Ammonium Acetate, 0.1% Acetic Acid in water		
Mobile Phase B:	90% Acetonitrile, 10% A		

Mass Spectrometry (EVOQ Elite™)

VIP Heated-ESI :	Temp 380°C		Q1 Resolution = 1, Q3 Resolution = 2.5
Heated probe gas:	80 units	MRM scan time:	100 ms/MRM
Nebulizer gas:	60 units		CAP & d5-CAP MRM transitions
Cone gas temp:	350°C		m/z 320.9>152 (CE:12 V) Quantitation
Cone gas:	20 units		m/z 320.9>257 (CE: 8 V) Qualification
Spray voltage:	-4500v		m/z 320.9>194 (CE:10 V) Qualification
Active exhaust:	On		m/z 325.9>157 (CE:12 V) Internal Standard

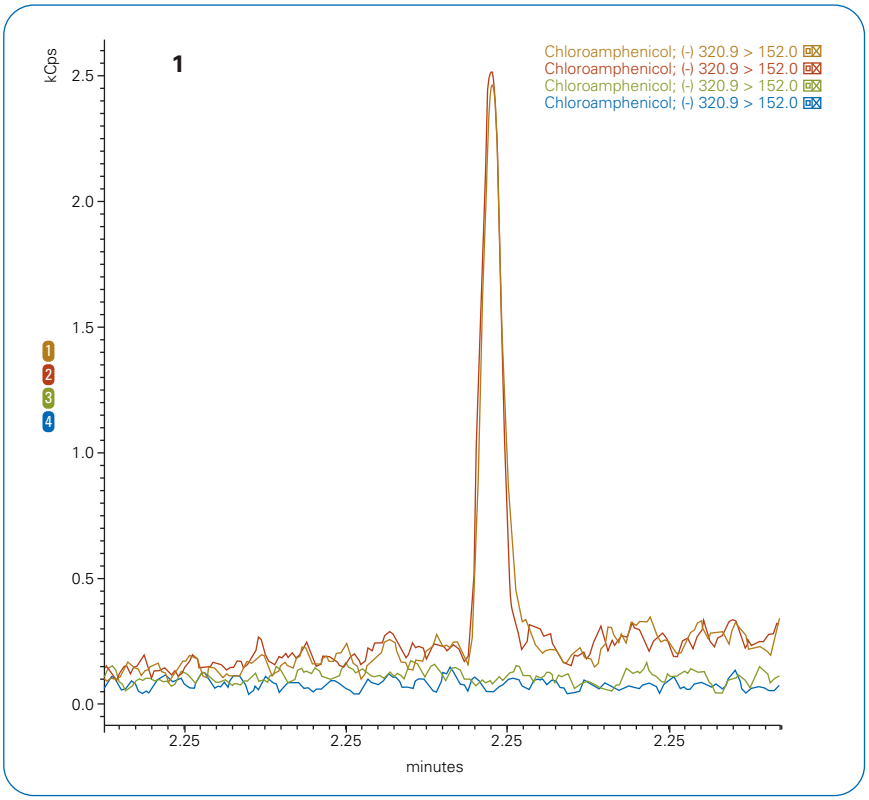

Fig. 1: Overlaid SRM chromatograms (m/z 320.9 m/z 152.0) (brown and red) for a positive egg sample and similar traces (blue and green) for a blank egg.

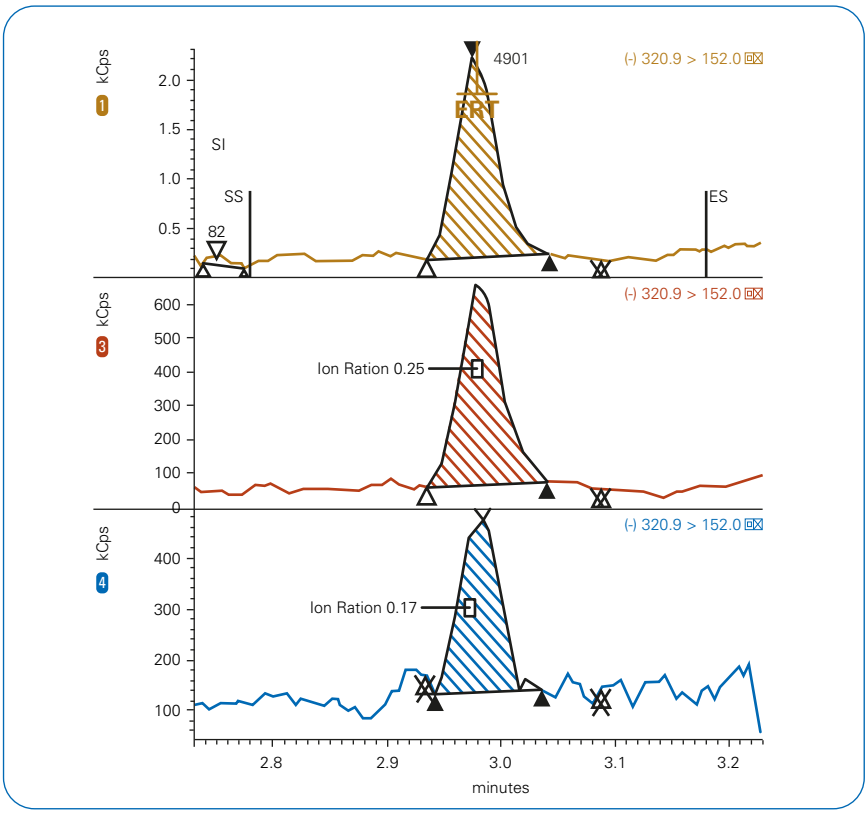

Fig. 2: Sample Chromatogram for an egg sample containing 0.115 ppb of chloramphenicol. The qualifying trace (brown) on top and below qualifier 1 with 0.25 times and qualifier 2 (blue) with 0.17 times the area.

Since chloramphenicol is a forbidden Group A substance, a minimum of 4 identification points (IP) is required. This can be fulfilled by a minimum of 2 MRMs, with 1.0 IP assigned for the precursor ion and 1.5 IPs assigned for each MRM product ion. The ion ratio for each MRM must match those of the certified reference material within specified tolerances. For qualification MRMs with ion ratios between 0.20 and 0.50 of the most intense transition are used with the maximum permitted tolerance of ±25%. In the current analysis, the ion ratios were found consistently <15% RSD for the concentration levels from 0.02 ppb to 5 ppb levels, as shown in an example of the real sample chromatogram in Figure 2, shown as stacked chromatograms for each of the three MRMs for CAP, along with their ion ratios. The chromatogram represents one typical dataset from the ten replicates of an egg sample being calculated with 0.115 ppb of chloramphenicol. The signal-to-noise ratio (PP) is well above 20 for the lowest abundant qualifier 2 and better than 70 for the quantifier trace.

An 8-level calibration curve from 0.020 to 5.0 ppb was generated with each level analyzed in ten-fold replicates. Repeatability of randomly chosen levels is presented in Table 1. Excellent linearity was obtained with R^2=0.9967 (no weighting) as displayed in Fig. 3.

The basis for good reproducibility in LC-MS/MS experiments is closely linked to the quality and resolution of the chromatographic separation prior to MS/MS analysis. The excellent chromatographic peak resolution provided by the Elute UHPLC system is shown in Fig. 4 a) with peak widths between 2.1-2.4 s. Whilst Figure 4b shows the resulting excellent reproducibility of 5 typical chromatograms at the 0.2 ppb level.

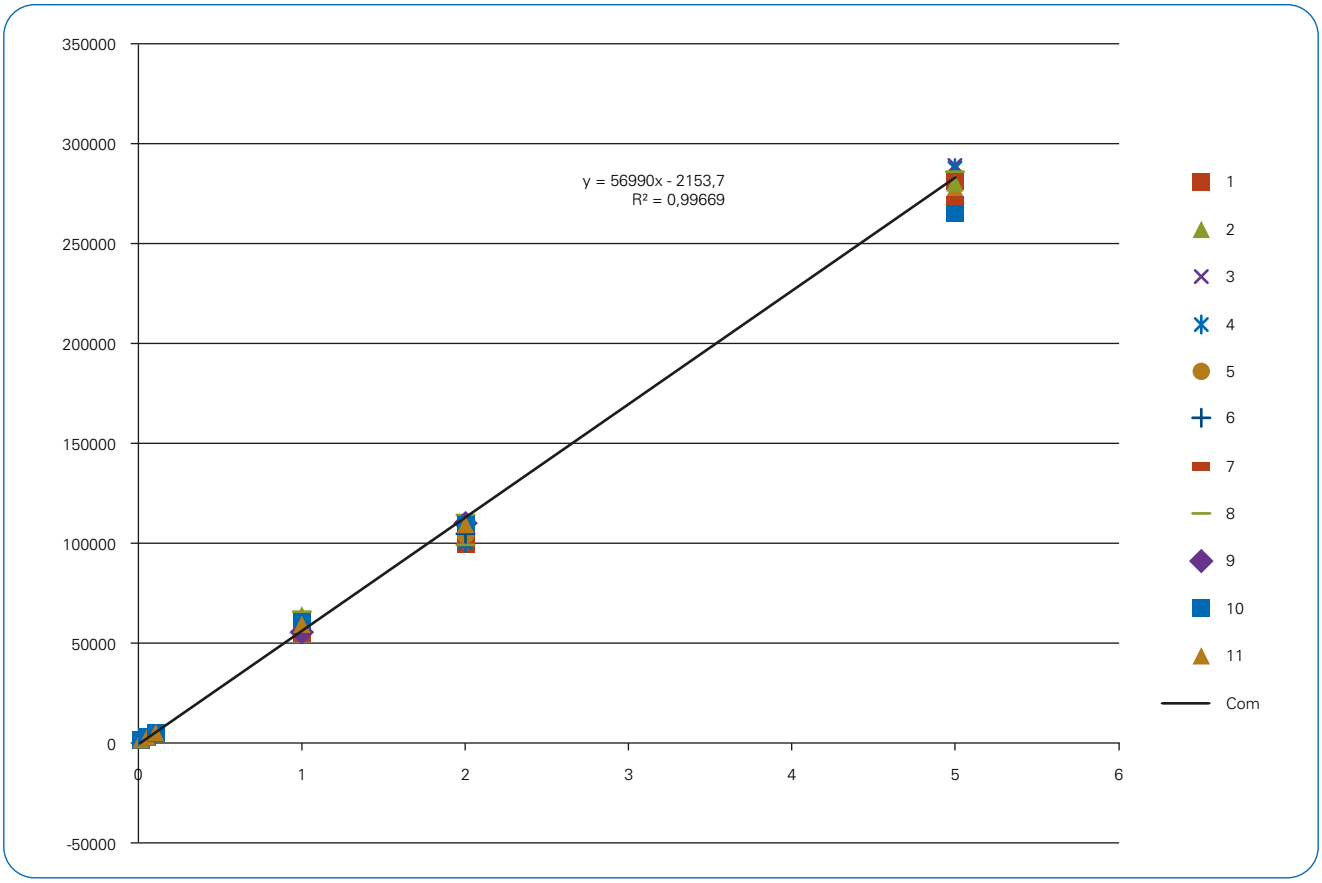

Fig. 3: Ten repetitions (1 – 11) of the calibration curves have been measured on different days. In black the averaged curve is shown, see Table 1 for further details.

Table 1: Individual results for the ten-fold measurement of calibration curves on different days showing a reproducibility well below RSD 7%.

Conc. (pg/mL)	0.02	0.05	0.10	1.0	2.0	5.0
Repetition						
1	1003	2295	4565	56954	99021	280362
2	1070	2563	4465	56382	102547	279124
3	1165	2275	4522	57694	105748	286648
4	1037	2436	4707	58307	99024	287172
5	1010	2296	4854	59051	102357	267403
6	1023	2210	4615	62471	103554	271560
7	1042	2633	4877	64338	105748	272736
8	978	2300	4964	64729	110585	285016
9	1098	2342	4833	55440	109251	270967
10	1053	2181	4839	60724	105848	266956
11	1048	2353	4724	59609	107825	276398
SD	53,7	147,2	172,7	3308,7	3801,7	8210
RSD	5.1%	6.3%	3.7%	5.6%	3.6%	2.9%

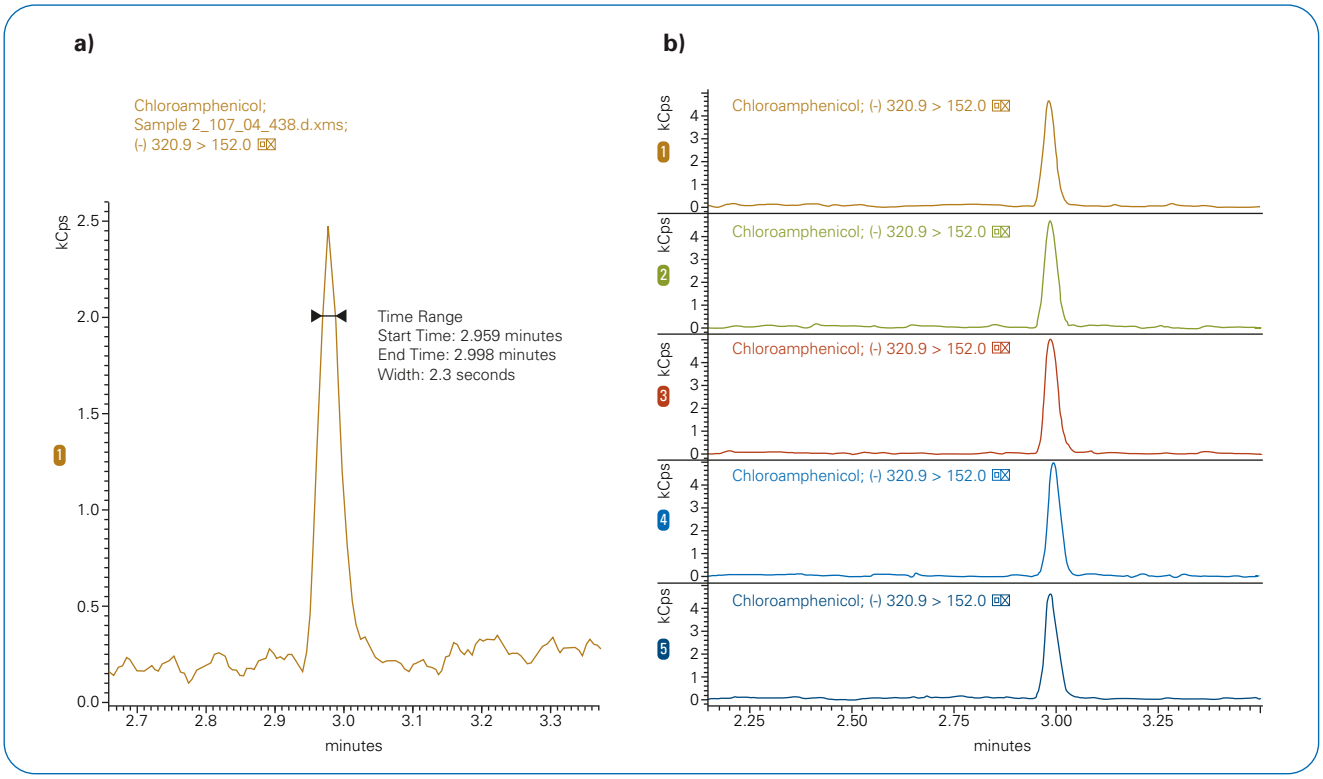

Fig. 4: As shown in a) the chromatogrpahic peak width at half max is constantly within the 2.1 – 2.4 seconds range.These sharp and focused peaks are key for the excellent reproducibility of the area under the curve as shown in b) by 5 typical extracted ion chromatograms at the 0.2 ppb concentration level.

Conclusion

A simple and fast LC-MS/MS method has been developed for analyzing trace levels of CAP in egg. The EVOQ Elite™ coupled to the Elute UHPLC is able to identify and quantify concentrations as low as 0.02 ppb CAP in a matrix sample as egg. Even this difficult matrix containing incl. emulsifiers does not affect chromatographic reproducibility of the Elute UHPLC system as demonstrated in Fig. 4.

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References

[1] S.R.R. Siqueira et. al, Journal of Separation Science 2009, 32, 4012-19

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