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QUANTITATIVE ANALYSIS OF CHLORAMPHENICOL IN MILK USING A SIMPLE EXTRACTION METHOD AND LC-MS/MS

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OVERVIEW

- A method for extraction of chloramphenicol in milk is presented
- Analysis is done by LC-MS/MS in negative electrospray
- Incurred milk samples were analysed
- Ion ratio was used for confirmation
- The estimated LOQ is 0.001 ppb
- The method is highly selective
- Comparison with a commercial lab are in good agreement

INTRODUCTION

Chloramphenicol (CAP) is a broad spectrum antibiotic used in human medicine only in extreme cases. It has toxic effects with no safe threshold level. It has been banned from use in food producing animals by the EU since 1994 with no MRL (Maximum Residue Level). CAP is sometimes used unlawfully to treat animal diseases or feed may be mislabeled or contaminated. CAP was recently found in Estonian milk samples. As Estonia is a member of EU, they are interested in exporting milk and mild powder to the rest of EU. They will then have to monitor CAP in milk and need a sensitive and selective method for analysis.

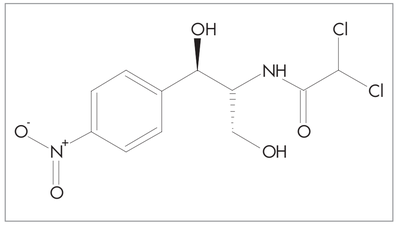
MATERIALS AND METHODS

Samples: They were incurred and blank milk samples from Estonian farms.

Chemicals: Chloramphenicol was from Sigma (C0378) and the internal standard chloramphenicol-d5 from Cambridge Isotopes Laboratories Inc. All other chemicals were of analytical grade.

Extraction:
2 ml of milk
↓
Add internal standard and freeze samples
↓
Remove fat while thawing the samples
↓
Add 100 µl of 1 M NaoH and 2 ml of EtOAc
↓
Shake gently and centrifuge at 3000 rpm for 5 minutes
↓
Remove organic phase and repeat extraction
↓
Evaporate to dryness under nitrogen
↓
Reconstitute in 150 µl mobile phase and analyse by LC-MS/MS

CHLORAMPHENICOL



LC CONDITIONS

HPLC: Waters 2695

Column: Waters Xterra MS C18 2.1x 50 mm, 3.5 µm

Mobile Phase: A:Water B: Acetonitrile

Gradient:

Time	% A	%B	Curve	Flow rate (ml/min)
0.00	95	5	1	0.3
1.00	95	5	1	0.3
3.00	5	95	6	0.3
4.00	5	95	1	0.3
4.10	95	5	6	0.3
7.00	95	5	1	0.3

Injection Volume: 10 µl

Temperature: Ambient

MASS SPECTROMETRY

Mass Spectrometer: Waters/Micromass Quattro Ultima Pt

Ionisation Mode: Negative electrospray

Instrument Settings:

Capillary:	1.00 kV
RF Lens 1:	40
Aperture:	0.2 V
RF Lens 2:	0.2
Source Temperature:	120o C
Desolvation Temperature:	300o C
LM 1 Resolution:	12.5
HM 1 Resolution:	12.5
Ion Energy 1:	0.5
Entrance:	-2
Exit:	0
LM 2 Resolution:	13.0
HM 2 Resolution:	13.0 Ion Energy 2: 1.0
Multiplier:	650 V
Collision Cell Pressure:	3.17 x 10-3

MRM TRANSITIONS MONITORED

Compound	*Transition*	*Dwell time (s)*	*Cone voltage (V)*	*Collision energy (eV)*
CAP d0	320.9>193.9	0.2	60	10
CAP d0	320.9>151.9	0.2	60	14
CAP d5 (IS)	326.0>157.0	0.2	60	14

RESULTS AND DISCUSSION

The product ion spectra for CAP-d0 and CAP-d5 (IS) are shown in figure 1. The transition 320.9>151.9 is used for quantification and the transition 320.9>193.9 is for confirmation using the ion ratio between the two transitions. The three transitions for a blank milk extract is shown in the chromatogram in figure 2 and the chromatogram for the lowest standard (0.01 ppb) in figure 3 showing the signal to noise (S/N) The S/N for the lowest standard (0.01 ppb) is 94:1. This gives an estimated limit of quantification (LOQ) about ten times lower, ie 0.001 ppb.

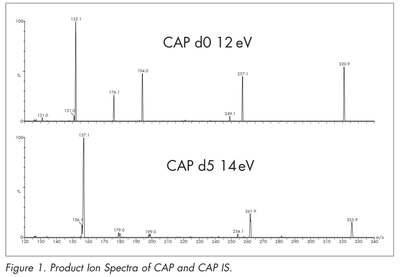


Figure 1. Product Ion Spectra of CAP and CAP IS.

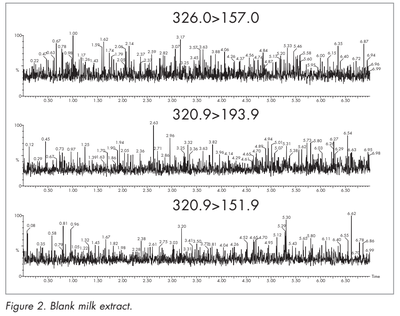


Figure 2. Blank milk extract.

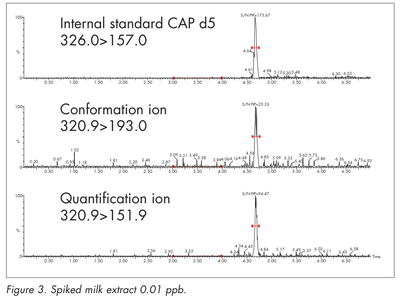


Figure 3. Spiked milk extract 0.01 ppb.

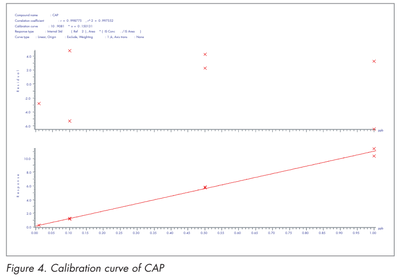


Figure 4. Calibration curve of CAP

The milk samples were either blank, spiked or incurred samples from Estonian milk farms. They had earlier, in September 2002, been analysed by a commercial German laboratory. They were all run in duplicates. The calibration curve is linear as shown in figure 4. It is not covering a vary wide dynamic range but as there is no MRL for CAP, this is enough.

To confirm the presence of CAP in the milk samples the second transition was used as a conformation ion. For the samples the ratio between the ions should be the same as between standards. Figure 5 shows a standard with the expected ion ratio. The expected ion ratio is 2.0 A chromatogram from an incurred sample is shown in figure 6 together with the calculated ion ratio.

The results from the milk sample analysis are shown in table 1. The ion ratios are very close to the expected ones and the comparison with reference lab analysis shows good agreement.

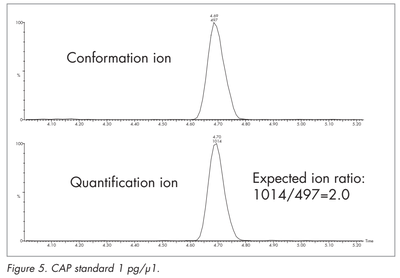


Figure 5. CAP standard 1 pg/µ1.

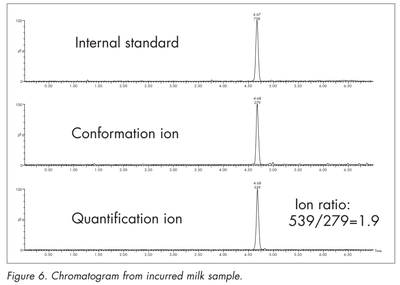


Figure 6. Chromatogram from incurred milk sample.

Sample	Type	Conc.(ppb)	Ion ratio	Ref lab
4968	Blank	< 0.01	NA	< 0.01
5187	Blank	< 0.01	NA	< 0.01
5158	Incurred	0.03	1.9	0.03
5170	Incurred	0.05	1.9	0.06
5175	Incurred	0.06	1.9	0.08
5169	Incurred	0.20	1.8	0.22

Results are the mean of two analysis.
NA means not applicable.
Ref. Lab are the results from the German laboratory.

Table 1. Results from milk sample analysis.

CONCLUSIONS

- A simple extraction method for CAP in milk is presented.
- The use of a deuterated internal standard is very important.
- The method gives an estimated LOQ of 0.001 ppb which is more than sufficient.
- The method is very selective showing no interferences in blanks, spiked standards or incurred samples.
- The ion ratio between the two product ions is used for conformation showing good agreement with the expected value.
- Comparison of the CAP analyses in incurred milk samples with a commercial lab show good agreement

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