1. INTRODUCTION

This method describes the analysis of chloramphenicol (CAP) in samples of bovine urine, meat and shrimps. After hydrolysis of the samples of urine's and clean up of the extracts by liquid liquid extraction and solid phase extraction (Sep-Pak C18), the purified extracts are injected on a HPLC system. For shrimps the SPE step can be omitted. The CAP-fraction is collected and derivatised and analysed by GC-(NCI)-MS (Negative Chemical Ionisation-MS). The method can be used for both screening and quantification. The decision limit for all samples is about 0,05 µg/L or µg/kg. The detection capability for samples of urine is 0,3 µg/L, for samples of shrimps the detection capability is 0,1 µg/kg. If GC-(PCI)-MS (positive chemical ionisation-MS) or GC-(EI)-MS (Electron Impact-MS) is used, the detection capability is 0,5 µg/L or µg/kg.

2. MATERIALS

Reference to a company and/or product is for purposes of identification and information only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environment (RIVM) to the exclusion of others which might also be suitable.

2.1. Chemicals and reagents

All chemicals, including standards and solutions, are of defined quality. Pure chemicals are of "Pro Analyse" quality or better, standards are checked for identity (GC-MS and/or FTIR) and purity (HPLC and/or TLC) and the shelf life and storage conditions of all prepared reagents and solutions are defined.
Milli-Q water is used.

2.1.1 Beta-glucuronidase/sulfatase (suc d'Helix Pomatia containing 100.000 units ß-glucuronidase and 100.000 units sulfatase per ml, Industr. Biol. France, 213473.
2.1.2 Acetic acid, Merck, 63.
2.1.3 Sodium acetate, Merck 6268.
2.1.4 Acetate buffer 2 mol/l, pH=5,2. Dissolve 25,2 g acetic acid (2.1.2) and 129,5 g sodium acetate (2.1.3) in 800 ml of water. Adjust the pH to 5.2±0.1 and add water to a final volume of 1000 ml.
2.1.5 Extrelut® with refills, Merck 115093.
2.1.6 Ethyl acetate, Merck 9623.
2.1.7 Sep-pak C18 cartridges, Waters 051910.
2.1.8 Ethanol, Merck 983.
2.1.9 Methanol, Merck 6007.
2.1.10 Methanol-water 1:9 (v/v) (= 10 vol%).
2.1.11 Methanol-water 9:11 (v/v) (= 45 vol%).
2.1.12 HPLC-eluens A: methanol-water 2:3 (v/v) (= 40 vol%). Filter eluens through a (Whatman GF/F) filter.
2.1.13 Derivatisation reagent: N,O-bis(trimethylsilyl) trifluoracetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), Alltech 18089.
2.1.14 Iso-octane, Merck 4718.
2.1.15 Subtilisin A, Sigma, P-5380.

2.1.16 Tris(hydroxymethyl)-amino-methane, Merck, 8382.
2.1.17 Tris buffer, 0,1 mol/l, pH 9,5. Dissolve 12,1 g of Tris(hydroxymethyl)-amino-methane in 800 ml of water. Adjust the pH at 9,5 ± 0.1 and add water to a final volume of 1000 ml.
2.1.18 Chloramphenicol-d5 internal standard, BGVV.
2.1.19 Chloramphenicol, Sigma C-9228.

Stock solutions of CAP containing 0,1 mg/ml were prepared in ethanol and stored at -20°C. Working solutions were prepared by sequential 10-fold dilutions of the 0,1 mg/ml solutions to a single series of appropriate standard solutions. These solutions were stored in the dark at approximately 4°C (range 1-10°C) for a maximum period of 12 months.

2.2. Apparatus

Standard laboratory glassware and equipment is used, with the addition of:

2.2.1. Centrifuge: HiCen 21 (Herolab) with A8.24 rotor.
2.2.2. Polypropylene centrifuge tubes 50 ml with caps.
2.2.3. pH-meter (Applikon).
2.2.4. Automatic pipettes (Gilson).
2.2.5. Ultrasonic waterbath (Bransonic).
2.2.6. Rotating apparatus (Heidolph).
2.2.7. Heating module for evaporating derivatisation reagents (Pierce).
2.2.8. Incubator (Salvis).
2.2.9. Rotavapor with waterbath at 40°C and vacuum pump (Büchi).
2.2.10. The HPLC-system consisted:
HPLC gradient-system (2 pumps 2150 and a controller 2252) (Pharmacia).
UV detector UV 2000 (Thermo Separations Products).
Autoinjector AS3000 (Thermo Separations Products).
Fraction collector (Foxy jr).
Workstation PC1000 for switching valves (Thermo Separations Products).
HPLC column Superspher 100 endcapped RP18 (5mm) 125 x 4 mm (Merck)
HPLC guard column Lichrospher 100 endcapped RP18 (5mm) 4 x 4 mm (Merck)
Flow rate : 0,7 ml/min
Column temperature : 40°C
Detection : 280 nm
Injection volume : 0,1 ml
HPLC Eluens pump A : methanol/water 2:3
HPLC Eluens pump B : methanol 100 %
The gradient conditions are:

0.0 minute to	8.0 minute	A = 100 %	and	B = 0 %
8.1 minute to	10.0 minute	A = 0 %	and	B = 100 %
10.1 minute to	10.2 minute	A = 100 %	and	B = 0 %

The time (2,5 min.) during which the fraction is collected is calculated as follows:
start fraction : Rt from the peak + 0,5 minutes – 1,0 minutes.
end fraction : Rt from the peak + 0,5 minutes + 1,5 minutes
(0,5 minutes describes the transfer time between the detector and the collecting tube).

2.2.11. GC-MS equipment:

Gas chromatograph (Hewlett Packard, type 6890).
GC-column, fused silica CpSil5CB 25 m x 0,25 mm ID, film thickness 0,12 µm (Varian). Or equivalent column.
Automatic injector (Hewlett Packard, type 7673A).
Mass selective detector (Agilent, type 5973N).
For NCi and PCI : CH_4 as reaction gas.
Computer and printer.

The following conditions are used during GC-MS analysis:
Injectionport: splitless 250°C.
Temperature program oven: 80°C (1 minute); rate 20°C/minute to 325°C.
Constant flow 1,1 ml/min helium.
Temperature transferline: 280°C.
Solventdelay of MS: 9.0 min.
NCI : ions for screening and confirmation: m/z 376-378-450-466-468-471.
Ions used for quantification: m/z 466 (CAP) and 471 (CAP-d5).
PCI : ions for screening and confirmation: m/z 254-377-467-469-472.
Ions used for quantification: m/z 467 (CAP) and 472 (CAP-d5).
EI : ions for screening and confirmation: m/z 208-225-230-361-451.
Ions used for quantification: m/z 225 (CAP) and 230 (CAP-d5).

3. ANALYTICAL PROCEDURE

Samples are stored in the dark at –20°C.
Each series should contain at least one positive (spiked 0,5 µg/l) and one blank control sample.

3.1 Sample preparation (urine).

3.1.1 Pipet 5 ml of urine in a 20 ml vial and adjust the pH of the sample to 5,2 with acetic acid (2.1.2).
3.1.2 Add 1,0 ml of 2,0 mol/l acetate buffer (2.1.4).
3.1.3 Add 2,5 ng of internal standard CAP-d5 (2.1.18).
3.1.4 Add 0,05 ml β-glucuronidase/sulfatase (2.1.1) and incubate during the night at 37°C or for 2 hours at 50°C.
3.1.5 Add water to a final volume of 20 ml.
3.1.6 Apply the sample to an Extrelut^R column (2.1.5) and equilibrate 15-20 minutes.
3.1.7 Continue the analysis at 3.4.

3.2. Sample preparation (meat).

3.2.1 Weigh 5 g of minced meat into a polypropylene centrifuge tube (2.2.2).
3.2.2 Add 2,5 ng of internal standard CAP-d5 (2.1.18).
3.2.3 Add 20 ml Tris buffer (2.1.17) containing 0,005 g subtilizin A (2.1.15).
3.2.4 Shake by placing the tube on a Vortex for a minute.
3.2.5 Incubate during 2 hours at 55°C, shake every 20 minutes.

3.2.6 Centrifuge the tube for 30 minutes at 18,000 rpm (2.2.1) and decantate the supernatant over a funnel within a plug of glasswool on an Extrelut^{R} column (2.1.5) and equilibrate 15-20 minutes.
3.2.7 Continue the analysis at 3.4.

3.3. Sample preparation (shrimps).

3.3.1 Weigh 5 g of minced shrimps into a polypropylene centrifuge tube (2.2.2).
3.3.2 Add 2,5 ng of internal standard CAP-d5 (2.1.18).
3.3.3 Add 20 ml of water.
3.3.4 Shake by placing the tube on a Vortex for a minute followed by placing for 1 hour on a rotating apparatus (2.2.6).
3.3.5 Place the tubes in an ultrasonic waterbath (2.2.5) for 30 minutes.
3.3.6 Centrifuge the tube for 30 minutes at 18.000 rpm (2.2.1) and decantate the supernatant on an Extrelut^{R} column (2.1.5) and equilibrate 15-20 minutes.
3.3.7 Elute the CAP from the column with 60 ml of ethylacetate (2.1.6) into a 100 ml flask.
3.3.8 Evaporate the solvent till < 5 ml on a rotavapor at 40°C (2.2.9).
3.3.9 Transfer the solvent into a tube and evaporate.
3.3.10 Dissolve the dry extract in 0,12 ml of HPLC eluens A.
3.3.11 Continue at point 3.3.14.

3.4. Sample clean-up for urine and meat.

3.4.1 Elute the CAP from the column with 60 ml of ethylacetate (2.1.6) into a 100 ml flask.
3.4.2 Evaporate the solvent on a rotavapor (2.2.9) at 40°C till < 5 ml.
3.4.3 Transfer the solvent into a 20 ml vial and evaporate.
3.4.4 Dissolve the extract in 0,2 ml of methanol (2.1.9).
3.3.6 Add 5 ml of water to the vial and dissolve further.
3.3.7 Wash a sep-pak C18 column (2.1.7) with 2 ml of methanol.
3.3.8 Wash the column with 5 ml of water.
3.3.9 Transfer the sample to the column.
3.3.10 Wash the vial with 5 ml of water and transfer the water to the column.
3.3.11 Wash the vial with 5 ml of methanol/water 1/9 (2.1.10) and transfer the methanol/water to the column.
3.3.12 Elute the column with 5 ml of methanol/water 9/11 (2.1.11).
3.3.12 Evaporate the solvent and dissolve the extract in 0,12 ml of HPLC eluens A.
3.3.13 Inject 3 times 25 ng of CAP on the HPLC system and assign the retention time of CAP. Calculate the fraction collecting time (see 2.2.10).
3.3.14 Inject 3 times a blank and collect the third blank fraction (HPLC-blank).
3.3.14 Inject 0,10 ml of the sample on the HPLC-system and collect the CAP fraction.
3.3.15 Evaporate the solvent, dissolve the extract in 0,3 ml of ethanol and transfer it into a derivatisation-vial.
3.3.16 Pipet at least five different amounts (range for example : 15- 0 ng) of CAP-standards into derivatisation-vials and add to each vial internal standard of 2,5 ng CAP-d5.
3.3.17 Evaporate (2.2.7) the vials with the standards, the samples, the HPLC-blank and a derivatisation-blank.
3.3.18 Add 0,05 ml of derivatisation reagent (2.1.13) to each vial and incubate during 1 hour at 60°C.
3.3.19 Evaporate (2.2.7) the solvent and dissolve the extract in 0,025 ml of iso-octane (2.1.14).

3.3.20 Transfer the solvent into injection-vials and inject 0,002 ml splitless on the GC-MS.

4. INTERPRETATION AND CALCULATION.

Quantitative results are obtained by construction a calibration curve. The peakarea of the selected ion of CAP (NCI : m/z 466) and the peakarea of the selected ion of the internal standard of CAP-d5 (NCI : m/z 471) are calculated and the ratio is the response variable. A calibration curve is constructed by the ratio between the response variable versus the concentration of the standards. Unknown samples are calculated by interpolation.

Quantification is only valid if:

- the retention time of the standard and the unknown peak differs no more than 0,05 minutes.
- the internal standard CAP-d5 elutes before the analyte CAP.
- the difference in retention time between CAP and CAP-d5 in the standards may not significant differ from this retentiontime-difference in the suspected sample.
- the maximum of the signal originating from the analyte exceeds the noise + 3 S.D.
- the coefficient of correlation of the calibrationcurve is better than 0,98.
- calibration curves are calculated using least squares linear regression analysis.

For identification according to the EC-criteria (Sanco 1805/2000 REV 1) it is mandatory that at least 4 ions are monitored. Each ion monitored (response) should fulfil the criterion that the maximum exceeds the average noise + 3 S.D. If this criterion is fulfilled the 3 different ratios are calculated. The same ratios are calculated for the standard analyte, preferably at the corresponding concentration. For positive identification the responses obtained for the unknown sample should be:

Relative intensity (% of base peak)	relative range of the response for EI	relative range of the response for NCI and PCI
> 50%	± 10%	± 20%
> 20% <50%	± 10%	± 25%
> 10%-20%	± 20%	± 30%
≤ 10%	± 50%	± 50%

5. VALIDATION.

The results of a bulk in-house validation study are described in detail in the RIVM report 573005016