RIVM report 310302001/2003

**Validation of the GC-MS method for the determination of chloramphenicol in bovine urine, meat and shrimp**
Method validation according to Commission Decision 2002/657/EC

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This investigation has been performed by order and for the account of MAP VGZ, within the framework of project 310302, Monitoring Veterinary Drugs.

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# Abstract

**Validation of the GC-MS method for the determination of chloramphenicol in bovine urine, meat and shrimp**

This report describes the validation of the quantification and the identification of an analytical method for the determination of low concentrations (0.1-1.0 µg/kg) of chloramphenicol in samples of urine, shrimps and meat. The validation study was based on the criteria described in Decision 2002/657/EC of the European Commission.
The analytical method consists of an enzymatic hydrolysis (urine) or enzymatic digestion (meat), followed by liquid-liquid extraction of chloramphenicol from the matrix with ethyl acetate. The extract is cleaned with Solid Phase Extraction (SPE), followed by LC fractionation. The SPE step can be omitted for shrimps. After derivatisation of the chloramphenicol, final separation and detection is performed with GC-MS with Negative Chemical Ionisation (NCI). Detection can also be carried out using Electron Impact (EI), which is a less sensitive technique. This method can be used for both screening and quantification. The limit of determination for all samples is approximately 0.05 µg/l or µg/kg. The detection capability for samples of urine is 0.3 µg/l. For shrimp samples, the detection capability is 0.1 µg/kg. If EI is used, the detection capability is 0.5 µg/l or µg/kg.

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# Samenvatting

Dit rapport beschrijft de validatie, de kwantificering en de wijze van identificatie van een analysemethode voor de bepaling van lage concentraties (0,1-1,0 µg/kg) chlooramphenicol in monsters urine, garnalen en spierweefsel (vlees) Deze validatie is gebaseerd op de criteria beschreven in de Beschikking van de Commissie 2002\657\EC. Na een eerste extractie, voorafgegaan door enzymatische hydrolyse (urine) of enzymatische digestie (spierweefsel), wordt chlooramphenicol geëxtraheerd vanuit de matrix met ethylacetaat. Het verkregen extract wordt vervolgens verder gezuiverd met vaste fase extractie (Solid Phase Extraction, SPE) en LC-fractionering. Bij het opwerken van monsters garnaal kan de zuiveringsstap over SPE worden overgeslagen.
Na derivatisering wordt het verkregen extract geanalyseerd met GC-MS.
Detectie kan plaatsvinden met negatieve chemische ionisatie (NCI), de meest gevoelige methode. Indien NCI niet beschikbaar is kunnen electron impact (EI) of positieve chemische ionisatie (PCI) als alternatief gebruikt worden.

De beschreven methode is zowel geschikt voor screening als bevestiging. De beslissingsgrens voor alle monsters bedraagt ongeveer 0,05 µg/l of 0,05 µg/kg. Het detectievermogen voor urinemonsters is 0,3 µg/l, voor garnalen is deze 0,1 µg/kg. Wanneer PCI of EI gebruikt worden is het detectievermogen 0,5 µg/l of 0,5 µg/kg.

# Summary

This report describes the validation, quantification and the identification of the method of analysis, at low concentrations (0.1 – 1.0 µg/kg) for chloramphenicol in samples of urine, shrimps and meat.
The validation is performed according to the criteria laid down in Commission Decision 2002/657/EC.
After a first extraction, preceded by enzymatic hydrolysis (urine) or enzymatic digestion (meat), chloramphenicol is extracted from matrix using ethylacetate. The extract is further purified using Solid Phase Extraction (SPE) and LC-fractionation.
For clean-up on shrimp samples the SPE-step can be omitted. After derivatisation final analysis is performed using GC-MS. Detection is performed using the Negative Chemical Ionisation mode (NCI), a very sensitive technique. However, if NCI is not possible, Electron Impact (EI) or Positive Chemical Ionisation (PCI) can be used as an alternative.
The method described in this report is suitable for both screening and confirmation.
The decision limit for all sample matrixes is approximately 0.05 µg/l or 0.05 µg/kg.
The detection capability for urine samples is 0.3 µg/l, for shrimp 0.1 µg/kg. When PCI of EI are used the detection capability is 0.5 µg/l or 0.5 µg/kg.

# 1. Introduction

Chloramphenicol {D (-)-threo-2.2-di-chloro-N-[b-hydroxy-a-(hydroxy-methyl)-p-nitro-phenyl-] –acetamide} (CAP) is an antibiotic with a broad spectrum of activity, frequently used in veterinary practice for therapeutic and prophylactic purposes. In humans CAP can cause serious health problems. In 1969 the FAO/WHO Expert Committee on Antibiotics has recommended a zero tolerance in meat products. [6].
The use for veterinary purposes is prohibited since no MRL can be established.

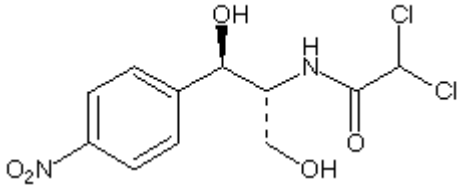


*Figure 1 : Structure of Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$)*
*CAS Registry number : [56-75-7] ; Molecular Weight = 323.13*

The existing Standard Operational Procedure (SOP) describing the detection and identification of residues of chloramphenicol (CAP) in biological matrices had to be updated because:

1. The purchase of a new GC-MS system with NCI gave the opportunity to analyse samples within the laboratory with significant improved sensititvity.
2. There is an increasing need for the analyses of samples of muscle tissue for the presence of low levels of CAP.
3. Since a few years the labeled internal standard CAP-d5 is available. The adventage of this internal standard over $^{37}Cl_2$-CAP is that the fragment-ions used for GC-MS confirmation give no interference with the fragment-ions of unlabeled CAP.
   So screening and quantification, can be performed in the same run as the confirmation analysis (identification).
4. New criteria for method validation (establishing performance characteristics) have been published (in house method validation) [1].

This report describes a validation study for the analysis of samples of urine, muscle tissue and shrimps. For both detection and qualitative confirmation the European Commission (EC) has set identification criteria which have to be fulfilled in order to prove the presence of an analyte with sufficient reliability. Method validation and confirmation of the identity is based on the latest international (EC) criteria [1]. According to these criteria it is mandatory that at least four ions are being 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% |

# 2. Materials and Methods

A detailed description of the analytical procedure is given in Appendix 2 (SOP ARO/060 rev. 3).

## 2.1 Sample pre-treatment and analytical procedure

The Keuringsdienst van Waren supplied for samples of urine muscle tissue and shrimps. Samples and chemicals are registrated in AROMIS. Prior to analysis samples were stored at –20°C. After hydrolysis of the urine samples and clean-up by liquid-liquid extraction (LLE) followed by solid phase extraction (SPE, C18), the purified extracts are injected on a HPLC system to collect the CAP-fraction. For shrimps the SPE step can be omitted. The CAP-fraction is derivatized and analyzed by GC-(NCI)-MS (Negative Chemical Ionisation-MS).

## 2.2 GC-MS-equipment

The GC-MS system used consisted of a Hewlett Packard gas chromatograph, type 6890, with an autosampler type 7673 and a Mass Selective Detector (MSD) type 5973N with MSDchem software from Agilent. Two different ionisation modes were used, Electron Impact (EI) and Negative- Positive Chemical Ionisation (NCI) with $CH_4$ as reactiongas. Separation of the extracts was achieved on a CPSil 5CB (25 m * 0.25 mm * 0.12 µm) column from Varian or on a ZB-1 7HG-G001-11 (30 m * 0.25 mm * 0.25 µm) column from Zebron. For the purpose of CAP analysis these columns are equivalent.

## 2.3 Derivatization and GC-MS

After extraction and extract clean up the purified extracts are derivatized. After derivatization to TMS-derivatives (Figure 2) samples, blanks and standards are evaporated and dissolved in iso-octane. Injection is performed in the splitless mode. After determination of the retention time in full scan mode, the following ions are monitored in the Selected Ion Mode:

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. Method validation

Prior to the actual validation full-scan spectra of standards of CAP and CAP-d5 in NCI, PCI and in EI mode (Figure 3-8) were recorded. After determination of the retention times, diagnostic ions (Selected Ion Monitoring) were selected. A chromatogram of a urine sample in the selected ion monitoring is presented in Figure 9. A chromatogram of the same urine sample, spiked with 0.3 µg/l of CAP, is presented in Figure 10.

## 3.1 Urine repeatability using NCI

This experiment consisted of analysis in 5-fold of samples of urine enriched with 0-0.5-1.0-2.0 and 5.0 ppb CAP. NCI was used for quantification.
After 96 hours the samples were re-analysed again by re-injection for determination of the storage lifetime of the derivatives.

## 3.2 Validations of HPLC flushing protocol, comparison with EI

This experiment consisted of analysis of a 5-fold urine enriched with 0-0.25 and 0.50 ppb CAP. At the end of this experiment a series of samples containing 5 µg/l CAP and blank urine samples were analysed alternately. This experiment was used to check possible carry-over within the HPLC system. Detection was performed both with NCI and EI.

## 3.3 Extraction of CAP from muscle tissue and shrimps

This experiment consisted of analysis of shrimps and meat. Samples of meat were enriched with 0-0.25-0.50-0.50-0.75 and 1.0 µg/kg CAP each. Three different samples of shrimps, containing different amounts of CAP were enriched with 0-0.25-0.50 and 0.75 µg/kg CAP each. Meat samples were treated with β-glucuronidase/sulfatase in phosfate buffer. Shrimps were digested in Tris buffer containing Subtilisin. With this experiment the influence of the different extraction procedures on recovery was evaluated.

## 3.4 Comparising beta-glucuronidase versus Subtilisin

This experiment consisted of four different samples of shrimps (samples were found positive on previous analyses) and one sample of meat. The sample of meat was enriched with 0-0.25-0.50 and 0.75 µg/kg CAP. This experiment was performed in duplicate; half of the samples were digested with Subtilisin. The other half was treated with β-glucuronidase/sulfatase in phosfate buffer.

## 3.5 Comparising standards

From our colleagues of the LRVV [7] we received five random standards with unknown concentration of CAP, but enriched with 2.5 ng CAP-d5 as internal standard. The samples were derivatized and analysed at RIVM. The results were compared with the amounts of CAP declared by the LRVV.

## 3.6 Real life samples of urine

Two samples of urine obtained from TNO-Voeding (Zeist, The Netherlands) were analysed. Both urine samples were at RIVM also enriched with 0.2 µg/kg CAP each.

## 3.7 Different muscle tissues and shrimps

In this experiment other matrices were examined: meat of pigs, chicken and kidney of pigs. All samples were enriched with 0.2-0.3 and 0.4 ppb CAP. The series of samples was completed with 4 samples of shrimps found positive in earlier experiments. All samples were digested with Subtilisin. Analysis was performed in both NCI and PCI.

## 3.8 Evaluation extraction procedure for shrimps

In this experiment shrimps were analysed in three different procedures:
Procedure 1: extraction with water, followed by Extrelut$^R$ and LC-fractionation.
Procedure 2: digestion with Subtilisin followed by Extrelut$^R$ and LC-fractionation
Procedure 3: digestion followed by Extrelut$^R$, Seppak and LC-fractionation.
All samples were analysed with both NCI and PCI.

## 3.9 Robustness of the method

For checking the stability of the method of analysis, urine was analysed. This was performed in different series of samples of urine. In each series of urine, at least two random urine samples were enriched with 0.3 ppb CAP.
Various shrimps were analysed with various processing methods.

## 3.10 Proficiency study from CRL Berlin, BVL

The procedure described was also used during a CRL proficiency study for the analysis of CAP in pig muscle. Only EI was used.

# 4. Results and discussion

In Figure 2 (below) the molecular structure of CAP-di-TMS is shown. In Figures 3-8, full scan spectra of CAP, CAP-$D_5$ as TMS derivatives are shown in EI-mode and PCI-mode

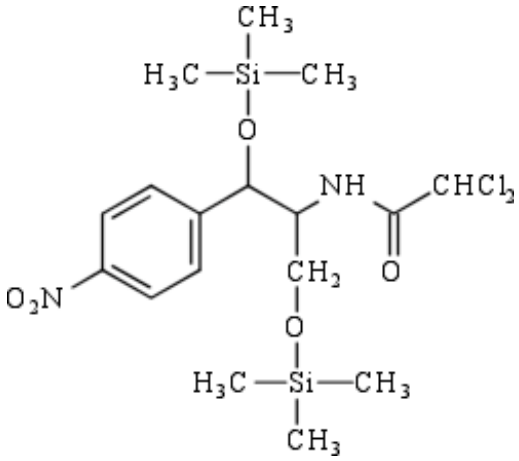

*Figure 2: Structure formula of CAP-di-TMS (Mw = 466).*

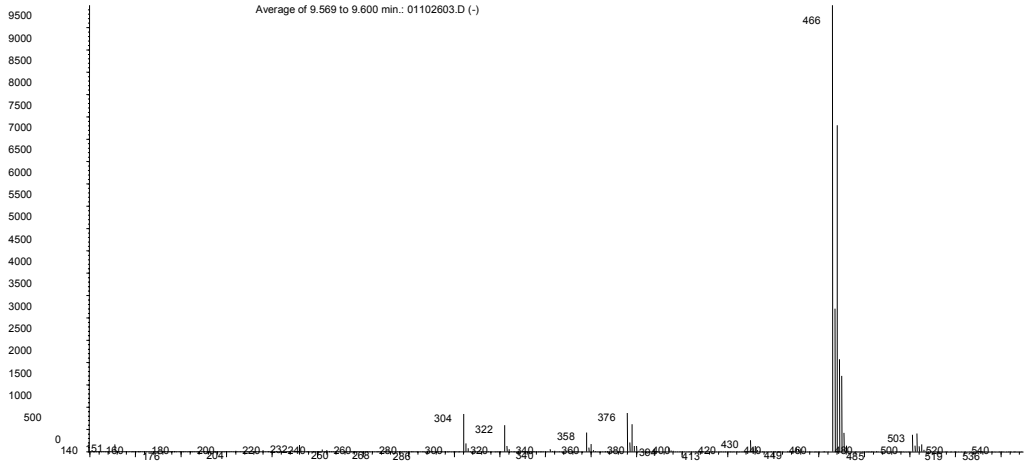


*Figure 3 : full-scan mass spectrum of CAP-di-TMS (NCI-mode)(20 ng injection).*

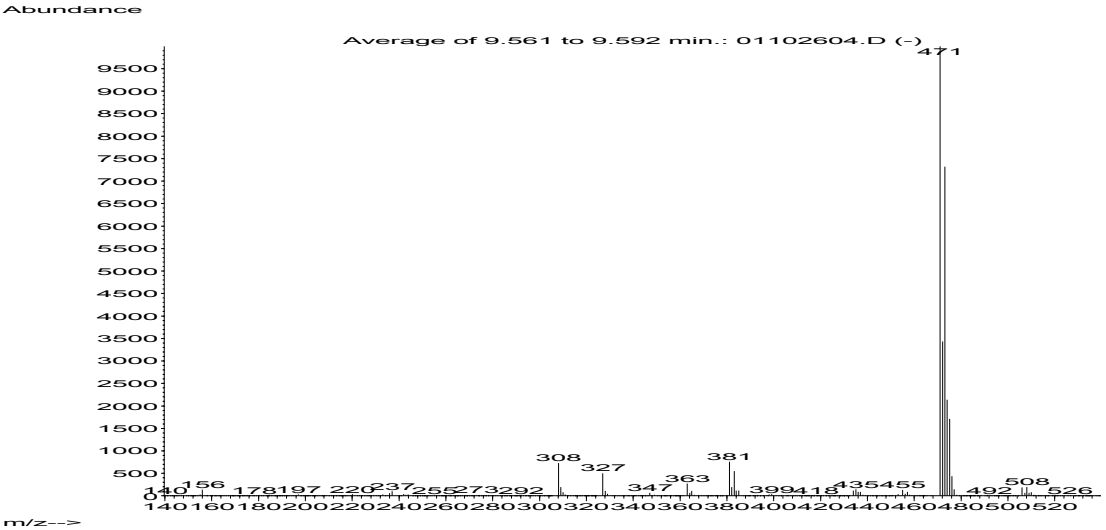


*Figure 4 : full-scan mass spectrum of CAP-d5-di-TMS (NCI-mode) (20 ng injection).*

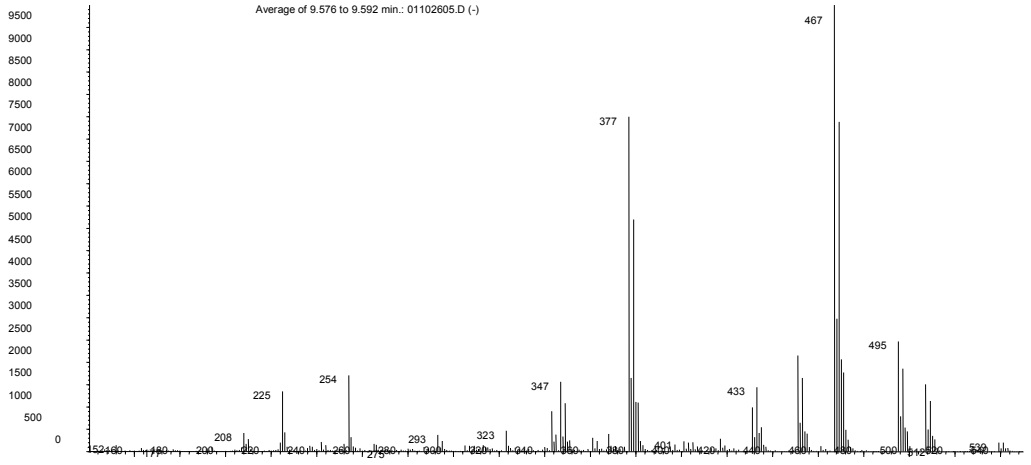


*Figure 5 : full-scan mass spectrum of CAP-di-TMS (PCI-mode)(20 ng injection).*

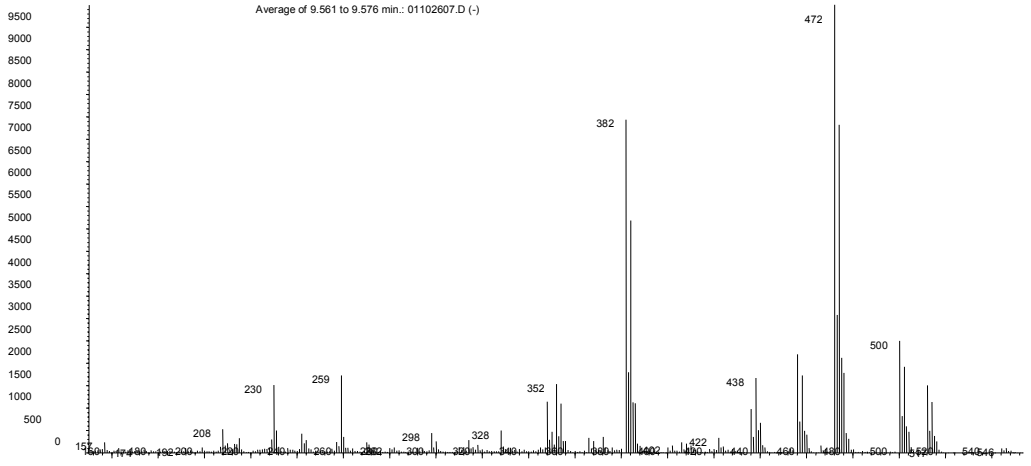


*Figure 6 : full-scan mass spectrum of CAP-d5-di-TMS (PCI-mode)(20 ng injection).*

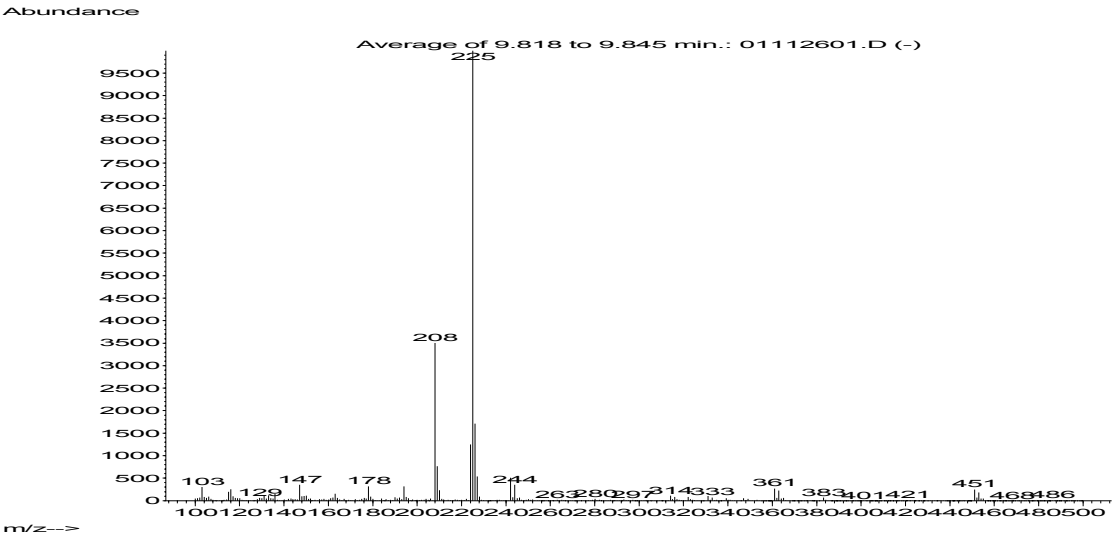


*Figure 7 : full-scan mass spectrum of CAP-di-TMS (EI-mode) )(20 ng injection).*

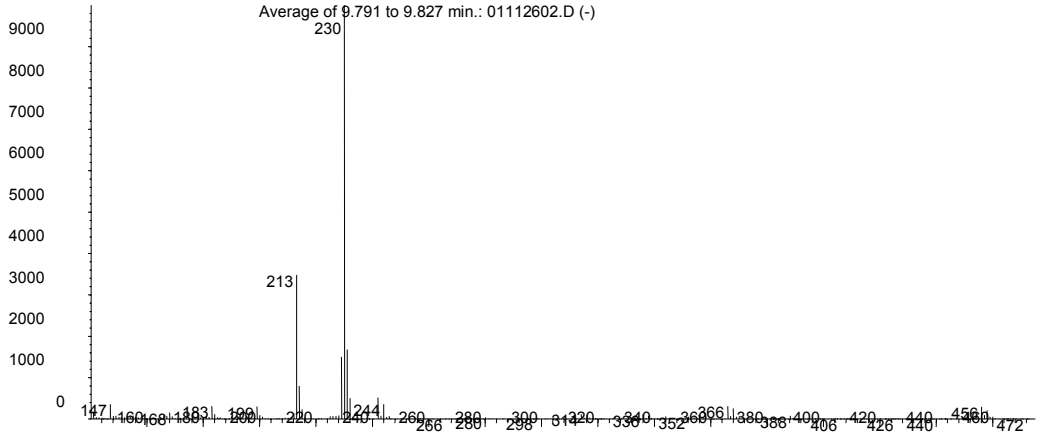


*Figure 8 : full-scan mass spectrum of CAP-d5-di-TMS (EI-mode) )(20 ng injection).*

## 4.1 Urine repeatability using NCI

Results of the validation experiment: urine enriched with 0-0.5-1.0-2.0 and 5.0 µg/l CAP, 5-fold analysis, are summarised in Table 1.

*Table 1: Repeatability of quantification and confirmation of CAP in samples of urine.*

| | µg/l CAP | | | | | average | S.D. |
|---|---|---|---|---|---|---|---|
| 0 µg/l urine | 0.00* | 0.10* | 0.31 | 0.27 | 0.48 | 0.23 | 0.19 |
| 0.5 µg/l urine | 0.48 | 0.48 | 0.43 | 0.44 | 0.43 | 0.45 | 0.03 |
| 1.0 µg/l urine | 1.00 | 1.04 | 1.00 | 0.98 | 0.97 | 1.00 | 0.03 |
| 2.0 µg/l urine | 1.98 | 2.11 | 2.19 | 2.11 | 2.06 | 2.09 | 0.08 |
| 5.0 µg/l urine | 5.08 | 5.05 | 4.69 | 4.76 | 4.52 | 4.82 | 0.24 |
| | * not confirmed | | All other | samples | confirmed | | |

Re-analysed after 96 hours stored at 4°C :

| | µg/l CAP | | | average | S.D. |
|---|---|---|---|---|---|
| 0 µg/l urine | 0.01* | 0.14* | 0.38 | 0.18 | 0.19 |
| 0.5 µg/l urine | 0.38 | 0.39 | 0.37 | 0.38 | 0.01 |
| 1.0 µg/l urine | 0.73 | 0.76 | 0.72 | 0.74 | 0.02 |
| 2.0 µg/l urine | 1.46 | 1.44 | 1.50 | 1.47 | 0.03 |
| 5.0 µg/l urine | 3.47 | 3.35 | 3.24 | 3.35 | 0.12 |
| | * not confirmed | | All other | samples | confirmed |

The positive results for the blank samples of urine most likely were caused by carry-over during HPLC. Amounts in the table are corrected for recovery of the internal standard. The average recovery during this experiment was 53%. Recovery is calculated by following formula:

$$\text{Average recovery (\%)} = \frac{\text{average of peakareas CAP-d5 in samples of urine}}{\text{average of peakareas CAP-d5 in standards}} \times 100\%$$

Based on this result the HPLC-protocol and washing procedures were modified, including extra blank runs. From the result it can be calculated that the repeatability is good, 7% or less variability (SD divided by average times 100%). The accuracy, as can be calculated from Table 1, ranges from 90% to 105%, equally acceptable. Prolonged storage of the derivatives is inadvisable causing significant under estimation at all levels.

## 4.2 Validation of HPLC flushing protocol, comparison with EI

This validation experiment consisted of analysis in six-fold of samples of urine enriched with 0-0.25 and 0.50 µg/l CAP. At the end of this experiment a series of samples containing 5 µg/l of CAP of blank samples of urine were analysed alternately. This last experiment was used as a check for carry-over of the HPLC system. Detection was performed with both NCI and EI.

*Table 2: Repeatability at low levels, check for carry-over.*

| **NCI** | µg/l CAP | | | | | | average | S.D. |
|---|---|---|---|---|---|---|---|---|
| 0 µg/l urine | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.25 µg/l urine | 0.16 | 0.19 | 0.20 | 0.19 | 0.19 | 0.19 | 0.19 | 0.01 |
| 0.5 µg/l urine | 0.51 | 0.51 | 0.48 | 0.46 | 0.48 | 0.49 | 0.49 | 0.02 |
| 5.0 µg/l urine | 4.72 | 4.28 | 4.70 | | | | 4.57 | 0.25 |

The identity of CAP was confirmed according to EC criteria (1) in all samples > 0.15 µg/l.

Samples were re-analyzed with EI. When measured in screening mode (m/z 225 for CAP and m/z 230 for CAP-d5) the urine samples with 0.25 µg/l of CAP were positive. Only at the level of 5 µg/l CAP the identity could be confirmed. The urine samples, analysed directly after a high concentraction sample of 5 µg/l did not result in any CAP positive cases. Carry-over in the series was not found.

## 4.3 Extraction of CAP from muscle tissue and shrimps

This experiment consisted of analysis of samples of shrimps and muscle tissue (meat). Muscle tissue was enriched with 0-0.5-0.0-0.0-0.5 and 1.0 µg/kg of CAP. Three different samples of shrimps containing different amounts of CAP were enriched with 0-0.5-0.0 and 0.5 µg/kg of CAP. Meat samples were treated with β-glucuronidase/sulfatase in phosfate buffer. Samples were also digested in Tris buffer containing Subtilisin to investigate the influence on recovery.

*Table 3: Accuracy for muscle- and shrimp tissue.*

| Sample I.D | µg/kg of CAP | Corrected for level in sample | Accuracy |
|---|---|---|---|
| 99M1295 muscle tissue | 0 | | |
| 99M1295 + 0.25 µg/kg CAP | 0.25 | | 100% |
| 99M1295 + 0.50 µg/kg CAP | 0.47 | | 94% |
| 99M1295 + 0.75 µg/kg CAP | 0.72 | | 96% |
| 99M1295 + 1.0 µg/kg CAP | 0.92 | | 92% |
| 01M1431 shrimps | 0.10 | | |
| 01M1431 + 0.25 µg/kg CAP | 0.39 | 0.29 | 116% |
| 01M1689 shrimps | 1.49 | | |
| 01M1689 + 0.25 µg/kg CAP | 1.87 | 0.38 | 152% |
| 01M1689 + 0.50 µg/kg CAP | 1.99 | 0.50 | 100% |
| 01M1689 + 0.75 µg/kg CAP | 2.29 | 0.80 | 107% |
| 01M1698 shrimps | 0.11 | | |
| 01M1698 + 0.25 µg/kg CAP | 0.39 | 0.27 | 108% |
| 01M1698 + 0.50 µg/kg CAP | 0.65 | 0.54 | 108% |
| 01M1698 + 0.75 µg/kg CAP | 0.96 | 0.85 | 113% |

The average estimated absolute recovery for the samples of muscle tissue was 7%, for shrimps 50%. The estimation is based on the recovery of the internal standard (data not shown). For the samples with concentration > 0.09 µg/kg CAP EC criteria were fulfilled. In all cases the accuracy is good (92-152%), with no significant bias.
The estimated absolute recovery for muscle tissue, however, is relatively low.

## 4.4 Comparison of beta-glucuronidase versus Subtilisin

This experiment consisted of the analysis of four different samples of shrimps (samples were analysed before and found positive) and one sample of meat (00M2273).
The sample of meat was enriched with respectively 0-0.25-0.50 and 0.75 µg/kg CAP.
This experiment was performed in duplicate, the first half of the samples was digested.
The second half was treated with β-glucuronidase/sulfatase in phosfate buffer.
Data are summarised in table 4 and 5.

*Table 4: Comparison of beta-glucuronidase versus Subtilisin.*

| | µg/kg CAP | µg/kg CAP |
|---|---|---|
| shrimps | acetic buffer/ beta-glucuronidase/sulfatase | digestion Subtilisin |
| 01M1687 | 4.03 | 3.64 |
| 01M1689 | 1.40 | 1.57 |
| 01M1690 | 0.48 | 0.53 |
| 01M1700 | 0.0 | 0.0 |
| Estimated absolute recovery | 37% | 53% |

| | µg/kg CAP | µg/kg CAP |
|---|---|---|
| Meat | acetic buffer/ beta-glucuronidase/sulfatase | digestion Subtilisin |
| 00M2273 | 0.0 | 0.0 |
| + 0.25 µg/kg CAP | 0.21 | 0.23 |
| + 0.50 µg/kg CAP | 0.44 | 0.47 |
| + 0.75 µg/kg CAP | 0.77 | 0.75 |
| Estimated absolute recovery | 35% | 43% |

For the samples with a concentration > 0.0 µg/kg CAP the EC criteria were fulfilled. (See table 5). No significant differences in concentrations of CAP were observed for either muscle tissue or shrimps. Only the estimated absolute recovery after digestion with Subtilisin is somewhat higher. For both procedures the absolute recovery is significant higher than in the previous experiment.
It should be noted, however, that muscle tissue sample 00M2273 contained no residues of CAP.

*Table 5: Practical datasheet with ratio-calculation.*

| | | peakarea's | | | | | m/z 471 CAP-d5 | | ratio's (x 100%) | | | | ratio | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample ID | Rt CAP | m/z 376 | m/z 378 | m/z 450 | m/z 466 | m/z 468 | Rt | peakarea | 376/466 | 378/466 | 450/466 | 468/466 | 466/471 | |
| st. 20 ng CAP | 9,598 | 585647 | 406988 | 153040 | 4673252 | 3544075 | 9,598 | 774643 | 12,5 | 8,71 | 3,27 | 75,8 | 6,03 | |
| st. 15 ng CAP | 9,598 | 368785 | 258492 | 106529 | 2999438 | 2269975 | 9,598 | 644808 | 12,3 | 8,62 | 3,55 | 75,7 | 4,65 | |
| st. 10 ng CAP | 9,603 | 188782 | 130951 | 61054 | 1514169 | 1155781 | 9,597 | 488778 | 12,5 | 8,65 | 4,03 | 76,3 | 3,10 | |
| st. 7,5 ng CAP | 9,606 | 127967 | 90678 | 42407 | 1042420 | 789575 | 9,598 | 438631 | 12,3 | 8,70 | 4,07 | 75,7 | 2,38 | |
| st. 5.0 ng CAP | 9,609 | 74188 | 53129 | 26432 | 585741 | 442532 | 9,600 | 359897 | 12,7 | 9,07 | 4,51 | 75,6 | 1,63 | |
| st. 2,5 ng CAP | 9,617 | 32849 | 21992 | 11475 | 257347 | 189129 | 9,605 | 300314 | 12,8 | 8,55 | 4,46 | 73,5 | 0,86 | |
| st. 1,25 ng CAP | 9,614 | 17700 | 12944 | 6877 | 151514 | 113945 | 9,603 | 327833 | 11,7 | 8,54 | 4,54 | 75,2 | 0,46 | |
| st. 0,625 ng CAP | 9,618 | 8469 | 6094 | 3398 | 70531 | 51770 | 9,607 | 305254 | 12,0 | 8,64 | 4,82 | 73,4 | 0,23 | |
| st. 0,313 ng CAP | 9,620 | 4163 | 3194 | 1679 | 35443 | 26165 | 9,606 | 286911 | 11,7 | 9,01 | 4,74 | 73,8 | 0,12 | |
| 5 ng CAP-d5 | | | | | | | 9,609 | 278258 | | | | | 0,00 | |
| deriv.blanc | | | | | | | 9,587 | 790 | | | | | | CAP |
| HPLC blanc | | | | | | | 9,586 | 2859 | | | | | | µg/kg |
| 01M1687 shrimp | 9,599 | 174697 | 122141 | 59841 | 1428524 | 1058216 | 9,594 | 256893 | 12,2 | 8,55 | 4,19 | 74,1 | 5,56 | 3,64 |
| 01M1689 shrimp | 9,603 | 61820 | 44179 | 23869 | 500166 | 377729 | 9,594 | 205432 | 12,4 | 8,83 | 4,77 | 75,5 | 2,43 | 1,57 |
| 01M1690 shrimp | 9,607 | 21893 | 15627 | 9890 | 183283 | 138552 | 9,596 | 211630 | 11,9 | 8,53 | 5,40 | 75,6 | 0,87 | 0,53 |
| 01M1700 shrimp | 9,608 | 1145 | 939 | | 8973 | 7744 | 9,595 | 214663 | 12,8 | 10,5 | | 86,3 | 0,04 | 0,00 |
| 00M2273 meat | 9,587 | 873 | | | 4920 | 4432 | 9,575 | 183137 | 17,7 | | | 90,1 | 0,03 | 0,00 |
| 00M2273 meat + 0.25 µg/kg | 9,586 | 10870 | 6916 | 4705 | 87631 | 67704 | 9,574 | 209208 | 12,4 | 7,89 | 5,37 | 77,3 | 0,42 | 0,23 |
| 00M2273 meat + 0.50 µg/kg | 9,586 | 14374 | 10154 | 6380 | 118060 | 90295 | 9,575 | 152083 | 12,2 | 8,60 | 5,40 | 76,5 | 0,78 | 0,47 |
| 00M2273 meat + 0.75 µg/kg | 9,585 | 25597 | 18034 | 11284 | 212106 | 161117 | 9,575 | 176474 | 12,1 | 8,50 | 5,32 | 76,0 | 1,20 | 0,75 |
| 01M1687 shrimp | 9,586 | 168448 | 122981 | 56877 | 1401366 | 1053692 | 9,578 | 227800 | 12,0 | 8,78 | 4,06 | 75,2 | 6,15 | 4,03 |
| 01M1689 shrimp | 9,592 | 39932 | 28588 | 16163 | 332776 | 258603 | 9,584 | 152485 | 12,0 | 8,59 | 4,86 | 77,7 | 2,18 | 1,40 |
| 01M1690 shrimp | 9,596 | 13230 | 9528 | 5875 | 107332 | 82329 | 9,584 | 134254 | 12,3 | 8,88 | 5,47 | 76,7 | 0,80 | 0,48 |
| 01M1700 shrimp | 9,596 | | | | 5169 | 4053 | 9,585 | 108716 | | | | 78,4 | 0,05 | 0,00 |
| 00M2273 meat | 9,594 | | | | 1894 | 1402 | 9,583 | 126636 | | | | 74,0 | 0,01 | 0,00 |
| 00M2273 meat + 0.25 µg/kg | 9,591 | 7518 | 5863 | 3497 | 63068 | 47879 | 9,581 | 163142 | 11,9 | 9,30 | 5,54 | 75,9 | 0,39 | 0,21 |
| 00M2273 meat + 0.50 µg/kg | 9,588 | 13678 | 10835 | 6026 | 108942 | 82038 | 9,578 | 148607 | 12,6 | 9,95 | 5,53 | 75,3 | 0,73 | 0,44 |
| 00M2273 meat + 0.75 µg/kg | 9,592 | 20474 | 15086 | 8815 | 178485 | 130438 | 9,582 | 145220 | 11,5 | 8,45 | 4,94 | 73,1 | 1,23 | 0,77 |
| | | | | | | ratio o.k. | | | v | v | v | v | | |

| standardline | |
|---|---|
| corr.coeff. | 0,999 |
| slope | 0,302 |
| intercept | 0,067 |

| ratio in standards | | | | |
|---|---|---|---|---|
| | v | v | v | v |
| average | 12,3 | 8,72 | 4,22 | 75,0 |
| maximal variation | 30% | 50% | 50% | 20% |
| minimum | 8,59 | 4,36 | 2,11 | 60,0 |
| maximum | 16,0 | 13,1 | 6,33 | 90,0 |

## 4.5 Comparison with standards from other sources

One additional approach for establishing possible bias is the comparison of the standards used with standards derived form independent sources. From the central laboratory of the RVV (LRVV) [7] we received five random standards with unknown concentration of CAP. These standards were enriched with 2.5 ng internal standard CAP-d5. The standards were derivatized and analysed at RIVM. The results were compared with the amounts of CAP declared by LRVV.

*Table 6 : Comparison of standards RIVM and LRVV.*

| | LRVV contents (ng) | RIVM result (ng) |
|---|---|---|
| standard LRVV 1 | 1.0 | 1.01 |
| standard LRVV 2 | 0.2 | 0.14 |
| standard LRVV 3 | 2.0 | 1.94 |
| standard LRVV 4 | 0.5 | 0.42 |
| standard LRVV 5 | 5.0 | 4.88 |

From these results it is concluded that standards used at RIVM and LRVV are comparable.

## 4.6 Analysis of control samples

Two samples that were found positive during routine control were analysed. The samples were also analysed enriched with 0.2 µg/l CAP at RIVM.

*Table 7: Results of positive samples from regulatory control programmes.*

| Urine sample ID | µg/l CAP | Corrected for level in sample | Accuracy |
|---|---|---|---|
| 01M1980 | 0.20 | | |
| 01M1981 | 0.11 | | |
| 01M1980 + 0.2 µg/l CAP | 0.42 | 0.22 | 110% |
| 01M1981 + 0.2 µg/l CAP | 0.32 | 0.21 | 105% |

In both samples the identity of CAP was confirmed.

## 4.7 Muscle tissues from other species and shrimps

In this experiment other matrices were examined: muscle tissue of pigs and chicken and kidney of pigs. All samples were enriched with 0.2-0.3 and 0.4 µg/kg CAP.
The experiment was completed with 4 samples of shrimps found positive in earlier experiments. All the samples were digested with Subtilisin and analysed in both NCI and PCI mode.

*Table 8 : Comparising of different matrices.*

| | **NCI results** µg/kg CAP | **PCI results** µg/kg CAP | |
|---|---|---|---|
| 01M1687 shrimps | 3.7 | 4.1 | |
| 01M1689 shrimps | 1.43 | 1.54 | etimated |
| 01M1690 shrimps | 0.56 | 0.59 | absolute |
| 01M1700 shrimps | 0.0 | 0.0 | recovery : 36% |
| 01M2134 muscle tissue : pigs | 0.0 | 0.0 | |
| 01M2134 + 0.2 µg/kg CAP | 0.20 | 0.17 | estimated |
| 01M2134 + 0.3 µg/kg CAP | 0.31 | 0.28 | absolute |
| 01M2134 + 0.4 µg/kg CAP | 0.41 | 0.42 | recovery : 15% |
| 01M2133 chicken | 0.0 | 0.0 | |
| 01M2133 + 0.2 µg/kg CAP | 0.21 | 0.19 | estimated |
| 01M2133 + 0.3 µg/kg CAP | 0.32 | 0.31 | absolute |
| 01M2133 + 0.4 µg/kg CAP | 0.41 | 0.43 | recovery : 18 % |
| 01M2135 kidney | 0.0 | 0.0 | |
| 01M2135 + 0.2 µg/kg CAP | 0.21 | 0.26 | estimated |
| 01M2135 + 0.3 µg/kg CAP | 0.31 | 0.35 | absolute |
| 01M2135 + 0.4 µg/kg CAP | 0.40 | 0.41 | recovery : 23 % |

NCI : samples > 0.0 µg/kg CAP confirmed according EU-criteria.
PCI : samples > 0.5 µg/kg CAP confirmed according EU-criteria.

From this experiment it is concluded that both NCI and PCI can be used for screening and quantification at low levels. Confirmation is only possible when NCI is used.

## 4.8 Evaluation extraction procedure for shrimps

In this experiment shrimps were analysed in three different procedures:
Procedure 1: extraction with water, followed by Extrelut® and LC-fractionation.
Procedure 2: digestion with Subtilisin followed by Extrelut® and LC-fractionation
Procedure 3: digestion followed by Extrelut®, Seppak and LC-fractionation.
All samples were analysed with both NCI and PCI.

*Table 9 : Comparison of extraction procedures and ionisation modes.*

| shrimps | **NCI** Procedure 1 µg/kg CAP | **NCI** Procedure 2 µg/kg CAP | **NCI** Procedure 3 µg/kg CAP | **PCI** Procedure 1 µg/kg CAP | **PCI** Procedure 2 µg/kg CAP | **PCI** Procedure 3 µg/kg CAP |
|---|---|---|---|---|---|---|
| 01M1690 | 0.51 * | 0.53 * | 0.51 * | 0.57 * | 0.62 * | 0.56 * |
| 01M1782 | 0.08 * | 0.09 * | 0.05 * | 0.10 | 0.08 | 0.05 |
| 01M2163 | 1.32 * | 1.19 * | 1.25 * | 1.41 * | 1.25 * | 1.33 * |
| 01M2164 | 0.07 * | 0.06 * | 0.05 * | 0.06 | 0.06 | 0.05 |
| 01M2165 | 0.04 * | 0.03 * | 0.04 * | 0.05 | 0.04 | 0.0 |
| 01M2166 | 1.23 * | 1.19 * | 1.49 * | 1.41 * | 1.38 * | 1.59 * |
| 01M2167 | 0.85 * | 0.87 * | 1.24 * | 0.94 * | 0.99 * | 1.36 * |
| 01M2168 | 1.11 * | 1.09 * | 1.07 * | 1.24 * | 1.19 * | 1.17 * |
| recovery (average) | 27% | 75% | 47% | 29% | 74% | 61% |

* confirmed according EU-criteria

NCI: confirmed according EU-criteria > 0.02 ppb.
PCI: confirmed according EU-criteria > 0.10 ppb.

In terms of estimated accuracy there are no significant differences between the different procedures. However, digestion with Subtilisin significantly improves the absolute recovery.

## 4.9 Ruggedness

For checking the ruggedness of the method of analysis, samples of urine were analysed. This was performed during seven experiments. In each series of samples, at least two random samples were enriched with 0.3 µg/l CAP. (Table 10).
Various samples of shrimps were analysed, extraction with water. Water was chosen for reasons of time-efficiency in spite of the significant lower absolute recovery that is obtainable. The results are summarised in Table 11-13.

*Table 10: Analysis (NCI) of samples of urine.*

| datafile MSD (.D) | ARO sample ID | CAP µg/l | result |
|---|---|---|---|
| 01111310 | 2001M1750 | | negative |
| 01111311 | 2001M1751 | | negative |
| 01111312 | 2001M1752 | | negative |
| 01111313 | 2001M1753 | | negative |
| 01111314 | 2001M1754 | | negative |
| 01111315 | 2001M1755 | | negative |
| 01111316 | 2001M1756 | | negative |
| 01111317 | 2001M1757 | | negative |
| 01111318 | 2001M1758 | | negative |
| 01111319 | 2001M1759 | | negative |
| 01111320 | 2001M1989 | | negative |
| 01111321 | 2001M1990 | | negative |
| 01111322 | 2001M1991 | | negative |
| 01111323 | 2001M1992 | | negative |
| 01111324 | 2001M1753 + 0.3 µg/l CAP | 0.24 | **confirmed** |
| 01111325 | 2001M1991 + 0.3 µg/l CAP | 0.23 | **confirmed** |
| 01111520 | 2001M1993 | | negative |
| 01111521 | 2001M1994 | | negative |
| 01111522 | 2001M1995 | | negative |
| 01111523 | 2001M2019 | | negative |
| 01111524 | 2001M2020 | | negative |
| 01111525 | 2001M2021 | | negative |
| 01111526 | 2001M2025 | | negative |
| 01111527 | 2001M2026 | | negative |
| 01111528 | 2001M2029 | | negative |
| 01111529 | 2001M2032 | | negative |
| 01111530 | 2001M2035 | | negative |
| 01111531 | 2001M2038 | | negative |
| 01111532 | 2001M2047 | | negative |
| 01111533 | 2001M2050 | | negative |
| 01111534 | 2001M2020 + 0.3 µg/l CAP | 0.29 | **confirmed** |
| 01111535 | 2001M2029 + 0.3 µg/l CAP | 0.31 | **confirmed** |
| 01112111 | 2001M2055 | | negative |
| 01112112 | 2001M2064 | | negative |
| 01112113 | 2001M2069 | | negative |
| 01112114 | 2001M2072 | | negative |
| 01112115 | 2001M2075 | | negative |
| 01112116 | 2001M2078 | | negative |
| 01112117 | 2001M2081 | | negative |
| 01112118 | 2001M2084 | | negative |
| 01112119 | 2001M2085 | | negative |
| 01112120 | **2001M2086** | **0.08** | **confirmed** |
| 01112121 | 2001M2087 | | negative |
| 01112122 | 2001M2088 | | negative |
| 01112123 | 2001M2089 | | negative |

| datafile MSD (.D) | ARO sample ID | CAP µg/l | result |
|---|---|---|---|
| 01112124 | 2001M2090 | | negative |
| 01112125 | 2001M2081 + 0.3 µg/l CAP | 0.33 | **confirmed** |
| 01112126 | 2001M2088 + 0.3 µg/l CAP | 0.30 | **confirmed** |
| 01120619 | 2001M2092 | | negative |
| 01120620 | 2001M2095 | 0.04 | **not confirmed** |
| 01120621 | 2001M2098 | | negative |
| 01120622 | 2001M2101 | | negative |
| 01120623 | 2001M2202 | | negative |
| 01120624 | 2001M2203 | | negative |
| 01120625 | 2001M2204 | | negative |
| 01120626 | 2001M2205 | | negative |
| 01120627 | 2001M2206 | | negative |
| 01120628 | 2001M2207 | | negative |
| 01120629 | 2001M2101 + 0.3 µg/l CAP | 0.30 | **not confirmed** |
| 01121210 | 2001M2208 | | negative |
| 01121211 | 2001M2209 | | negative |
| 01121212 | 2001M2210 | | negative |
| 01121213 | 2001M2211 | | negative |
| 01121214 | 2001M2212 | | negative |
| 01121215 | 2001M2216 | | negative |
| 01121216 | 2001M2218 | | negative |
| 01121217 | 2001M2220 | | negative |
| 01121218 | 2001M2222 | | negative |
| 01121219 | 2001M2224 | | negative |
| 01121220 | 2001M2226 | | negative |
| 01121221 | 2001M2228 | | negative |
| 01121222 | 2001M2230 | | negative |
| 01121223 | 2001M2232 | | negative |
| 01121224 | 2001M2228 + 0.3 µg/l CAP | 0.25 | **confirmed** |
| 01121225 | 2001M2232 + 0.3 µg/l CAP | 0.26 | **confirmed** |
| 01121310 | 2001M2234 | | negative |
| 01121311 | 2001M2236 | | negative |
| 01121312 | 2001M2241 | | negative |
| 01121313 | 2001M2243 | | negative |
| 01121314 | 2001M2247 | | negative |
| 01121315 | 2001M2248 | | negative |
| 01121316 | 2001M2253 | | negative |
| 01121317 | 2001M2254 | | negative |
| 01121318 | 2001M2257 | | negative |
| 01121319 | 2001M2261 | | negative |
| 01121320 | 2001M2264 | | negative |
| 01121321 | 2001M2268 | | negative |
| 01121322 | 2001M2269 | | negative |
| 01121323 | 2001M2274 | | negative |
| 01121324 | 2001M2248 + 0.3 µg/l CAP | 0.27 | **confirmed** |
| 01121325 | 2001M2254 + 0.3 µg/l CAP | 0.26 | **not confirmed** |

| datafile MSD (.D) | ARO sample ID | CAP µg/l | result |
|---|---|---|---|
| 01121810 | 2001M2275 | | negative |
| 01121811 | 2001M2278 | | negative |
| 01121812 | 2001M2281 | | negative |
| 01121813 | 2001M2286 | | negative |
| 01121814 | 2001M2289 | | negative |
| 01121815 | 2001M2289 + 0.3 µg/l CAP | 0.26 | **confirmed** |
| 01121816 | 2001M2291 | | negative |
| 01121817 | 2001M2294 | | negative |
| 01121818 | 2001M2587 | | negative |
| 01121819 | 2001M2588 | | negative |
| 01121820 | 2001M2589 | | negative |
| 01121821 | 2001M2590 | | negative |
| 01121822 | 2001M2591 + 0.3 µg/l CAP | 0.26 | **confirmed** |
| 01121823 | 2001M2591 | | negative |
| 01121824 | 2001M2592 | | negative |
| 01121825 | 2001M2593 | | negative |
| 01121826 | 2001M2594 | | negative |
| 01121827 | 2001M2595 | | negative |
| 01121828 | 2001M2596 | | negative |
| 01121829 | 2001M2596 + 0.3 µg/l CAP | 0.26 | **confirmed** |

Summarized results:

- In all samples the internal standard CAP-d5 was detected (0.5 µg/l CAP-d5).
- In total 14 spiked samples with 0.3 µg/l CAP were analysed, 12 samples could be confirmed according EU-criteria. Average result: 0.27 µg/l CAP (SD = 0.03).
- Urine sample 2001M2101 could not be confirmed, 4 ions were detected, but only one ratio was within the range according the criteria.
- Urine sample 2001M2254 could not be confirmed, 5 ions were detected, but only two ratios were within the range according the criteria.
- One sample of urine (2001M2086) was found positive: 0.08 µg/l CAP.
- One sample of urine (2001M2095) was suspect: 0.04 µg/l CAP, the ratios however were not within the EU-criteria.

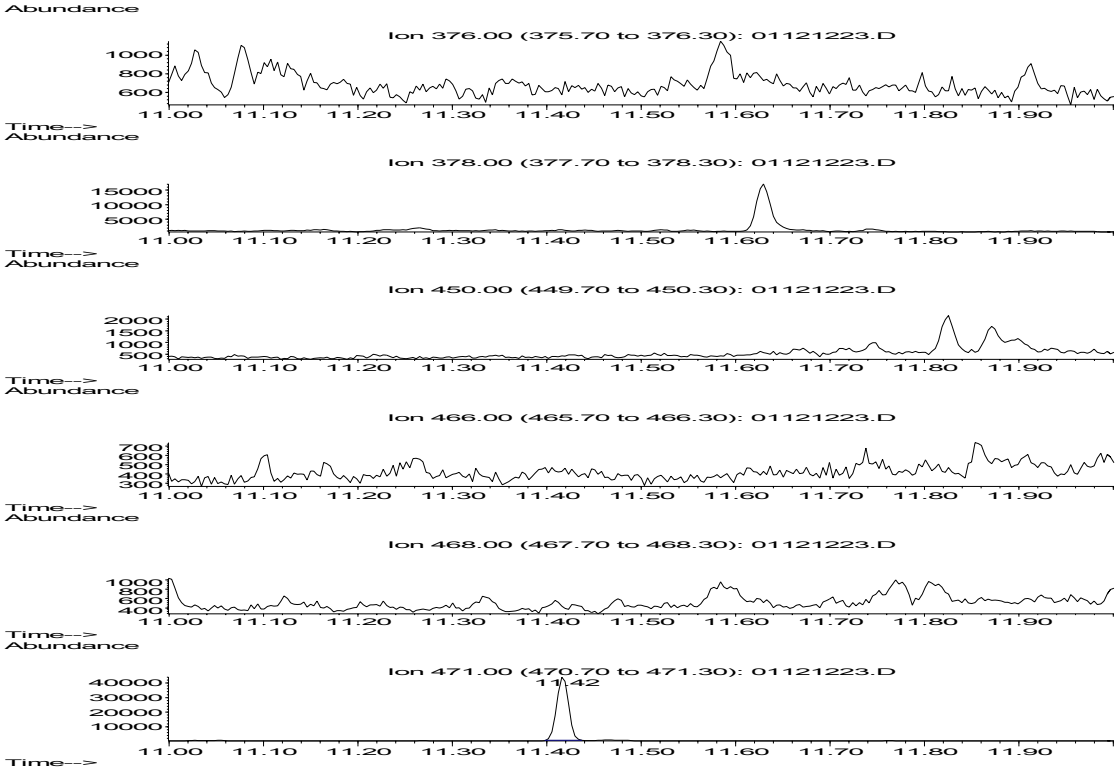


*Figure 9 : GC-(NCI)-MS-chromatogram of a sample of urine 2001M2232 (negative) spiked with 0.5 µg/l CAP-d5 ($r_t$=11.42 min).*

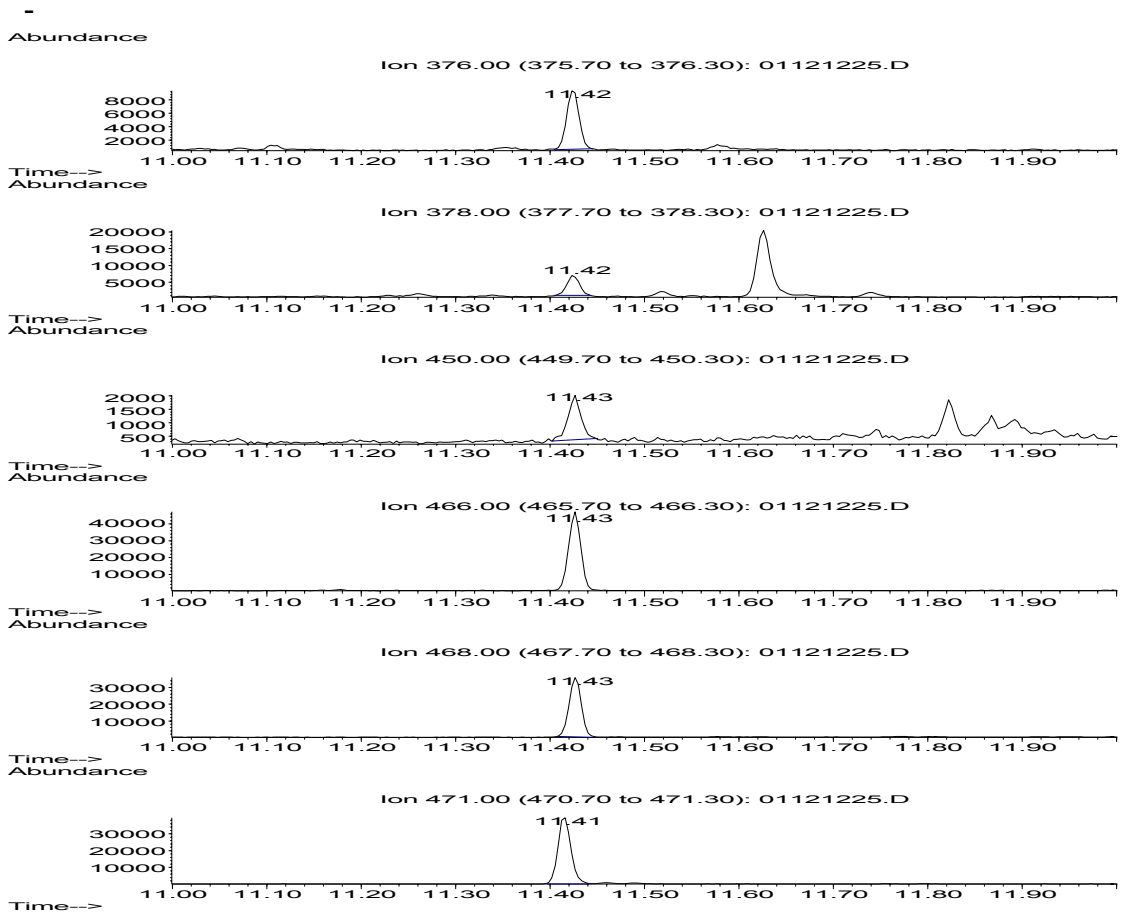


*Figure 10 : GC-(NCI)-MS-chromatogram of a sample of urine (2001M2232) spiked with 0.3 µg/l CAP and 0.5 µg/l CAP-d5 ($r_t$=11.42 min).*

*Table 11 : Analysis (NCI) of samples of shrimps (extraction with water).*

| Shrimps ARO sample ID (A11825/45) | Result µg/kg CAP | Ratios confirmed according EC-criteria |
|---|---|---|
| 01M1430 (Netherlands) | 0 | no |
| 01M1430 + 2 µg/kg CAP | 1.9 | yes |
| 01M1429 (Norway) | 0.5 | yes |
| 01M1431 (Iceland) | 0.2 | yes |
| 01M1431 + 1 µg/kg CAP | 1.0 | yes |
| 01M1430 | 0.1 | yes |
| 01M1411 | 0.4 | yes |
| 01M1412 | 1.8 | yes |
| 01M1413 | 1.1 | yes |
| 01M1414 | 0.1 | yes |
| 01M1415 | 2.3 | yes |
| 01M1416 | 0.5 | yes |
| 01M1417 | 0.2 | yes |
| 01M1418 | 0.1 | yes |
| 01M1419 | 0.5 | yes |
| 01M1420 | 6.2 | yes |

The average estimated absolute recovery was: 23%.

*Table 12: Analysis (NCI) of samples of shrimps (extraction with water).*

| shrimps ARO sample ID (A11825/46) | Result µg/kg CAP | Ratio confirmed according EC-criteria |
|---|---|---|
| 01M1429/01M1430 | 0.15 | yes |
| 01M1429/01M1430 | 0.15 | yes |
| 01M1431 (Iceand) | 0.13 | yes |
| 01M1430 (NL) | 0.00 | no |
| 01M1431 + 0,5 ppb | 0.60 | yes |
| 01M1429 + 0,5 ppb | 0.70 | yes |
| 01M1430 + 0,5 ppb | 0.47 | yes |
| 01M1430 (NL) | 0.00 | no |
| 01M1430 (NL) | 0.00 | no |
| 01M1421 | 0.49 | yes |
| 01M1422 | 0.07 | no |
| 01M1423 | 0.36 | yes |
| 01M1424 | 0.34 | yes |
| 01M1425 | 0.29 | yes |
| 01M1426 | 0.76 | yes |
| 01M1427 | 0.73 | yes |

The average estimated absolute recovery was: 20%.

*Table 13 : Analysis (NCI) of samples of shrimps (extraction with water).*

| shrimps ARO sample ID (A11825/52) | Result µg/kg CAP | Ratio confirmed according EC-criteria |
|---|---|---|
| 01M1422 | 0.07 | yes |
| 01M1422 + 0.5 µg/kg CAP | 0.46 | yes |
| 01M1422 | 0.06 | no |

The average estimated absolute recovery was: 15%.

From Table 12 it can be concluded that concentrations of CAP in shrimps > 0.2 µg/kg can be analysed accurately and confirmed according the latest EU-criteria.

## 4.10 Proficiency study from CRL (BVL in Berlin, Germany)

The results of a CRL proficiency study for the analysis of CAP in pig muscle are summarised below. Analysis was performed in EI mode.

*Table 14: ARO-results proficiency study from CRL (EI).*

| | sample ARO-ID | results CAP (µg/kg) | | CAP confirmed (EU-criteria) | | |
|---|---|---|---|---|---|---|
| | | experiment 1 | experiment 2 | experiment 1 | experiment 2 | |
| | 00M0815 + 3 µg/kg CAP | 2.4 | 3.4 | no | yes | |
| | 00M0815 + 2 µg/kg CAP | 1.7 | 2.3 | no | no | |
| | 00M0815 + 1 µg/kg CAP | 1.0 | 1.2 | no | no | Assigned CRL value |
| CRL - code | 00M0815 | neg | neg | no | no | |
| SJB084 | 01M0636 | neg | neg | no | no | neg |
| SJB299 | 01M0638 | 0.3 | 0.3 | no | no | neg |
| SJB428 | 01M0640 | 2.5 | 2.9 | yes | no | 2.1 |
| SJB955 | 01M0643 | 2.5 | 2.7 | yes | yes | 2.1 |
| SJB152 | 01M0637 | 5.1 | 5.5 | yes | yes | 4.9 |
| SJB840 | 01M0642 | 5.3 | 5.8 | yes | yes | 4.9 |
| SJB309 | 01M0639 | 6.4 | 7.7 | yes | yes | 6.5 |
| SJB784 | 01M0641 | 6.5 | 7.3 | yes | yes | 6.5 |

# 5. Conclusions

The method described in ARO-SOP 060, rev. 3, can be used for the screening and confirmation of low levels of CAP in samples of bovine urine, muscle tissue (meat) from different species and shrimps. The use of NCI allows screening and confirmation of low levels, 0.1 µg/l or µg/kg in different matrices.

The validation status is currently in agreement with the criteria described in Commission Decision 2002/657/EC.

Validation experiments show that the method is rugged with a good (better than 10%) repeatability. The method is already used in national surveillance programs and performs well.

The pre-validation study demonstrated that Subtilisin digestion of shrimp tissues significantly improves the absolute recovery of the analyte. However, accuracy and repeatability are not influences by the choice of extraction procedure. Based on time-efficiency it was decided to use water extraction as procedure of choice (“fit for purpose”) during subsequent validation studies.

# References


1 Official Journal of the European Communities (2002): L221, 8-36. Commission Decision No. 2002/657/EC of 12 August 2002 implementing Council Directive 96/23 concerning the performance of analytical methods and the interpretation of results.

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4 L.A. van Ginkel, H.J. van Rossum, P.W. Zoontjes, H. van Blitterswijk, G. Ellen, E. van der Heeft, A.P.J.M. de Jong and G. Zomer. Development and validation of a gaschromatic-mass spectrometric procedure for the identification and quantification of residues of chloramphenicol. Analytical Chimica Acta, 237 (1990) 61-69.

5 E. van der Heeft, A.P.J.M. de Jong, L.A. van Ginkel, H.J.van Rossum and G. Zomer. Confirmation and quantification of chloramphenicol in cow's urine, muscle and eggs by Electron Capture Negative Ion chemical Ionization Gas Chromatography/Mass Spectrometry. Biological Mass spectrometry, vol 20, 763-770 (1991).

6 World Health Organization 12$^{th}$ report of the joint FAO/WHO Expert Committee of Food Additives. WHO Techn.Report series no.430, FAO Nutrition Meeting reports series no.45 FAO/WHO, Geneva (1969).

7 J. Böhmer, LRVV (Laboratory meat inspection, Wageningen, the Netherlands). Letter LRVV/jp/010796, standards CAP for comparising.

## Appendix 1 Mailing list

| | |
|---|---|
| 1 - 8 | Voedsel en Waren Autoriteit / Keuringsdienst van Waren |
| 9 | VWA/Keuringsdienst van Waren Noord |
| 10 | VWA/Keuringsdienst van Waren Oost |
| 11 | VWA/Keuringsdienst van Waren Zuid |
| 12 | VWA/Keuringsdienst van Waren Noordwest |
| 13 | VWA/Keuringsdienst van Waren Zuidwest |
| 14 | Directeur-Generaal Volksgezondheid |
| 15 | Voorzitter van de Gezondheidsraad |
| 16 | VWA/Directie RVV |
| 17 | Directie RIKILT |
| 18 | Hoofd LRVV |
| 19 | Algemene Inspectie Dienst |
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| 32 | Bureau Rapportenregistratie |
| 33 | Bibliotheek RIVM |
| 34-38 | Bureau Rapportenbeheer |
| 39-43 | Reserve |

## Appendix 2 Standard Operational Procedure (SOP)

| | | | |
|---|---|---|---|
| | | S.O.P. | : ARO/060 |
| **TITLE:** | **Determination of chloramphenicol in bovine urine, meat and shrimps by GC-MS** | Page | : 1 of 5 |
| | | Revision | : 3 |
| | | Date | : 05-02-04 |

### 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 and subsequent 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 derivatized and analyzed 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.

The method was validated according to the criteria layed down in Commission Decision 2002/567 as described in RIVM report 310302 001.

### 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 for Public Health and the 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.

2.1.1 Beta-glucuronidase/sulfatase (suc d'Helix Pomatia containing 100.000 units ß-glucuronidase and 100.000 units sulfatase per ml, Brunschwig Chemie (Amsterdam, the Netherlands).
2.1.2 Acetic acid, Merck (Amsterdam, the Netherlands).
2.1.3 Sodiumacetate, Merck (Amsterdam, the Netherlands).
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$^{R}$ with refills, Merck (Amsterdam, the Netherlands).
2.1.6 Ethyl acetate, Merck (Amsterdam, the Netherlands).
2.1.7 Sep-pak C18 cartridges (Waters-Millipore, Etten-Leur, the Netherlands).

2.1.8 Ethanol, Merck (Amsterdam, the Netherlands).
2.1.9 Methanol, Merck (Amsterdam, the Netherlands).
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 Derivatization reagent: N,O-bis(trimethylsilyl) trifluoracetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), (Pierce).
2.1.14 Iso-octane (Merck).
2.1.15 Subtilisin A (Sigma, P-5380).
2.1.16 Tris buffer, 0.1 mol/l, pH 9.5. Dissolve 12.1 g of Tris(hydroxymethyl)-amino-methane (Merck, 8382) in 800 ml of water. Adjust the pH at 9.5 ± 0.1 and add water to a final volume of 1000 ml.
2.1.17 Chloramphenicol-d5 internal standard (BGVV).
2.1.18 Chloramphenicol (Sigma).

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 serie 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

For operating instructions and maintenance status files see ARO management information system Cardbox.
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 (Beun de Ronde).
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 derivatization reagents (Pierce).
2.2.8 Incubator (Salvis).
2.2.9 Rotavapor with waterbath at 40°C and vacuum pump (Buchi).
2.2.10 The HPLC-system consisted:
HPLC gradient-system (2 pumps 2150 and a controler 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.
Temp.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 standaard CAP-d5 (2.1.17).
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 standaard CAP-d5 (2.1.17).
3.2.3 Add 20 ml Tris buffer (2.1.16) 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 standaard CAP-d5 (2.1.17).
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).
3.4.5 Add 5 ml of water to the vial and dissolve further.
3.4.6 Wash a sep-pak C18 column (2.1.7) with 2 ml of methanol.
3.4.7 Wash the column with 5 ml of water.
3.4.8 Transfer the sample to the column.
3.4.9 Wash the vial with 5 ml of water and transfer the water to the column.
3.4.10 Wash the vial with 5 ml of methanol/water 1/9 (2.1.10) and transfer the methanol/water to the column.
3.4.11 Elute the column with 5 ml of methanol/water 9/11 (2.1.11).
3.4.12 Evaporate the solvent and dissolve the extract in 0.12 ml of HPLC eluens A.
3.4.13 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).
3.4.14 Inject 3 times a blanc and collect the third blanc fraction (HPLC-blanc).
3.4.15 Inject 0.10 ml of the sample on the HPLC-system and collect the CAP fraction.

3.4.16 Evaporate the solvent, dissolve the extract in 0.3 ml of ethanol and transfer it into a derivatization-vial.
3.4.17 Pipet at least five different amounts (range for example : 15- 0 ng) of CAP-standards into derivatization-vials and add to each vial internal standard of 2.5 ng CAP-d5.
3.4.18 Evaporate (2.2.7) the vials with the standards, the samples, the HPLC-blank and a derivatization-blank.
3.4.19 Add 0.05 ml of derivatization reagent (2.1.13) to each vial and incubate during 1 hour at 60°C.
3.4.20 Evaporate (2.2.7) the solvent and dissolve the extract in 0.025 ml of iso-octane (2.1.14).
3.4.21 Transfer the solvent into injection-vails 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 (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 the in-house validation study are described in detail in report 310302 001.