Project number: 71.520.01
Project: Proficiency study for the analysis of chloramphenicol in porcine muscle

Project manager: Drs. J.A. van Rhijn

Report 2001. 024 July 2001

Proficiency study for the analysis of chloramphenicol in porcine muscle

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ABSTRACT

In the framework of the proficiency test for the analysis of chloramphenicol (CAP) in pig muscle, organised by AFSSA Fougères in its position as Community Reference Laboratory (CRL), eight muscle samples were analysed for the presence of chloramphenicol. Because the quantitative analysis was carried out using LC-MS/MS, operating in multiple reaction monitoring (MRM) mode recording the transition of the precursor ion into two product ions, confirmatory information was attained. The LC-MS/MS method was therefore used for quantification as well as for confirmation of the identity of the analyte present. No separate screening was carried out.
The results indicated the presence of chloramphenicol in seven out of eight samples. These results could be confirmed according to EU criteria.

SAMENVATTING

Proficiency study voor de analyse van chloramphenicol in varkensvlees

Rapport 2001.024

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5 bijlagen, 2 referenties

In het kader van de proficiency study voor de analyse van chloramphenicol in varkensvlees, georganiseerd door AFSSA in haar bevoegdheid als Communautair Referentie Laboratorium (CRL), zijn acht vleesmonsters geanalyseerd op de aanwezigheid van chloramphenicol. De kwantitatieve analyse is uitgevoerd met behulp van LC-MS/MS. Aangezien de MS is gebruikt in multiple reaction monitoring (MRM) mode, waarbij de overgang van het precursor ion naar twee product ionen wordt gemeten, wordt tevens informatie over de identiteit van het analiet verkregen. De LC-MS/MS methode is dus gebruikt voor zowel de kwantitatieve analyse als de bevestiging van de identiteit van het analiet. Er is geen afzonderlijke screening aan de LC-MS/MS metingen voorafgegaan.
De resultaten wezen uit dat chloramphenicol aanwezig is in zeven van de acht monsters. Deze resultaten konden worden bevestigd volgens EU criteria.

1 INTRODUCTION

In the framework of the proficiency test for the analysis of chloramphenicol (CAP) in pig muscle, organised by AFSSA Fougères in its position as Community Reference Laboratory (CRL), eight muscle samples were analysed for the presence of CAP.
CAP is a banned substance. The analytical approach for banned substances, in contrast to registered ones, has to focus on detecting and identifying the analyte at a level as low as possible.
The muscles were analysed using LC-MS/MS. Therefore the quantitative analysis and the confirmatory analysis are combined in a single method. No separate screening preceded the LC-MS/MS analysis.
This report describes the analytical procedures for the quantification and confirmation and the results of the proficiency study are reported.

2 EXPERIMENTAL

2.1 Materials

All solvents and reagents used were of analytical grade or better. All chemicals, including the Extrelut-3® columns, were obtained from Merck (Darmstadt, Germany). The chloramphenicol reference standard (batchno. 60740) was provided together with the samples by AFSSA (Fougères, France) and was obtained from Sigma (St. Louis, MO, USA). [$^{37}Cl_2$]-Chloramphenicol was used as internal standard and was obtained from RIVM (Bilthoven, The Netherlands).
A Waters (Milford, MA, USA) Symmetry C18 column (L=15 cm, ID=3.0 mm) was used to establish the separation.
Porcine muscle samples were received in a frozen condition from AFSSA and were coded: SJN-140, SJN-400, SJN-454, SJN-466, SJN-505, SJN-602, SJN-661 and SJN-935.

2.2 Sample preparation

Blank porcine muscle for the preparation of QC samples, was thoroughly minced. An aliquot of 5 g of the blank muscle or the provided muscle samples was taken and transferred to a stomacher bag.
[$^{37}Cl_2$]-Chloramphenicol was used as internal standard and was added to all samples and QC samples at a level of 5 µg/kg. Chloramphenicol was added to the blank muscle to prepare the QC samples. The samples were thoroughly homogenised. After 30 min, 10 ml water was added to the samples in the stomacher bag and the muscle homogenate was extracted in a stomacher apparatus during 3 minutes. The content of the stomacher bag was transferred to a centrifuge tube and was centrifuged for ten minutes (3500 g, 15°C). Three ml of the supernatant was transferred to an Extrelut-3® extraction column. After 30 minutes of equilibrating, chloramphenicol was eluted with 15 ml dichloromethane. The dichloromethane was evaporated to dryness under nitrogen at 30 °C. The residue was dissolved in 0,5 ml water. An aliquot of 50 µl was injected in the LC-system without further purification.

2.3 LC-MS/MS analysis

Separation was established on a Waters Symmetry C18 (L=15 cm, ID=3.0) column using a gradient in methanol (Table 1).

Table 1. Gradient used for LC-MS/MS analysis

Time (min)	Water (%)	Methanol (%)	Flow rate (ml/min)
0	100	0	0.4
15	40	60	0.4
20	40	60	0.4
21	100	0	0.4

The column was connected to a Micromass (Manchester, UK) Quattro Ultima triple quadrupole mass spectrometer equipped with an Atmospheric Pressure chemical Ionisation (APcI) interface operating in the negative ion mode.
The mass spectrometer was operated in multiple reaction monitoring (MRM) mode selecting the parent ion and recording two product ions characteristic for the analyte and the internal standard (Table 2).

Table 2. Instrumental settings and diagnostic ions, the most abundant ion is underlined

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Collision energy (eV)
CAP	322	192	0.25	15
		<u>151</u>	0.25	15
[$^{37}Cl_2$]-CAP	326	192	0.25	15
		<u>151</u>	0.25	15

The sum of both diagnostic ions is used for quantitative determination and their ratio is calculated for identification. [$^{37}Cl_2$]-CAP was used as an internal standard for the quantification of CAP by means of isotopic dilution.

3 RESULTS AND DISCUSSION

3.1 Quantitative analysis

For quantitative analysis, $^{37}Cl_2$ isotope labelled CAP is used as the internal standard. This substance, however, differs only four amu from the native CAP molecule. A CAP molecule contains two chloride atoms. Consequently overlap in the isotope pattern occurs and may give rise to "cross-talk" signals in the ion traces of the internal standard arising from high concentrations of the native compound.
CAP has a mono-isotopic molecular weight (MW) of 322. The natural abundance of the ^{37}Cl isotope is 24 %. Therefore, theoretically, about 10 % of CAP is present as the $^{37}Cl_2$ isotope (MW=326) [1] (Annex I).
Consequently, in practice, a signal is observed in the ion traces of the internal standard, corresponding to about 10 % of the signal of the native compound. Depending on the amount present in the sample, this may considerably effect the quantification, yielding too low results.

The chromatogram of the blank muscle fortified at 1.0 µg/kg CAP after clean-up (annex IIId) demonstrates this effect. Although no internal standard was added, a signal due to the native CAP is found in the ion traces of $[^{37}Cl_2]$-CAP at a level of about 10 to 15 % of the signal of the native CAP. This is in accordance with the theory.
For correct quantification this effect has to be mathematically corrected.
The response-factor is determined by dividing the sum of the intensity of both product ions of CAP by the sum of the corrected intensity of both product ions of the internal standard.

The quantification was performed using matrix calibrants. Therefore, the influence of matrix effects is avoided. The matrix calibrants were prepared by fortifying blank muscle samples in the range of 0.2 to 5.0 µg/kg. The fortified samples were taken through the sample clean-up procedure, similar to the unknown samples.

Figure 1 presents the relation between concentration and response-factor for the matrix calibrants. There is an almost perfect relation (coefficient of correlation is 0.998) between the response-factor and the fortification level. This points out that the LC-MS/MS analysis procedure is very suitable for the quantitative determination of the amount of CAP present in the unknown samples.

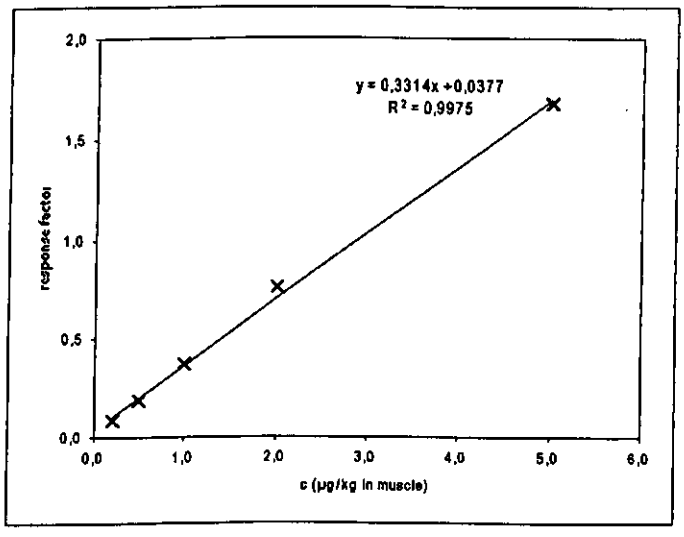


Figure 1. Plot of the response-factor (LC-MS/MS) versus the fortification level of CAP in blank porcine muscle.

Based on the fortified blank muscle samples, the limit of quantification was estimated at 0.2 µg/kg in porcine muscle. A chromatogram of a blank muscle, a blank muscle fortified at 0.2 µg/kg and a blank muscle fortified at 1.0 µg/kg are presented in respectively annex III a, b and c.

The recovery was determined by comparing the blank muscle fortified at 1.0 µg/kg before the clean-up procedure (annex IIIc) with the blank muscle fortified at 1.0 µg/kg after the clean-up procedure (annex IIId). The recovery of CAP was about 65 %.

3.2 Confirmatory analysis

To carry out confirmation of the analytes identity, the relative abundance of the diagnostic ions was determined. The relative abundance of the diagnostic ions of CAP in muscle was found to be identical to the corresponding abundance in standards. The average relative abundance of the diagnostic ions was calculated at 50 % (n=12, RSD=12 %). The spectrum of CAP is presented in annex II.

Confirmation was carried out in accordance with EU criteria [2].
Therefore, two product ions were used for the confirmation. For quantification at least one product ion is required. Therefore, in most cases the LOQ is lower than the level at which confirmation can be performed. However, in this case, the relative abundance of the diagnostic ions is high, meaning that the sensitivity of both diagnostic ions is about the same. The level at which confirmation can be performed is therefore estimated at the same level as the LOQ: 0.2 µg/kg in porcine muscle.

According to the EU criteria, the relative abundance of CAP in the unknown samples may differ from the average relative abundance in the QC samples with a maximum of 20 %. A sample is considered positive when the amount calculated is higher than 0.2 µg/kg and the relative abundance of the diagnostic ions is between 40 and 60 %.

3.3 Proficiency study samples

A duplicate analysis was carried out for all unknown samples.
One chromatogram of each proficiency study sample is presented in annex IV.
The results of the analysis are presented in table 3.

Table 3. Quantitative results for CAP in the proficiency study samples (µg/kg)

Sample code	Cal. Amount	Cal. Avg. Amount	Relative abundance (r.a.)	Deviation from average r.a.	Confirmation
SJN-140	4.6	4.2	46 %	8 %	POS
	3.8		43 %	14 %	POS
SJN-400	1.3	1.6	42 %	16 %	POS
	1.8		51 %	2 %	POS
SJN-454	2.2	2.5	53 %	6 %	POS
	2.7		45 %	10 %	POS
SJN-466	6.7	6.7	46 %	8 %	POS
	6.6		43 %	14 %	POS
SJN-505	< 0.2	< 0.2	-		NEG
	< 0.2		-		NEG
SJN-602	2.1	2.1	49 %	2 %	POS
	2.1		52 %	4 %	POS
SJN-661	4.8	4.9	48 %	4 %	POS
	5.0		43 %	14 %	POS
SJN-935	6.6	7.1	45 %	10 %	POS
	7.5		44 %	12 %	POS

Table 3 shows that the presence of CAP could be confirmed in seven of the proficiency study samples. The amount of CAP in the positive samples ranged from 1.6 to 7.1 µg/kg. This is well above the LOQ and the level at which confirmation can be carried out. One sample proved to be negative (<0.2 µg/kg) for CAP.

4 CONCLUSION

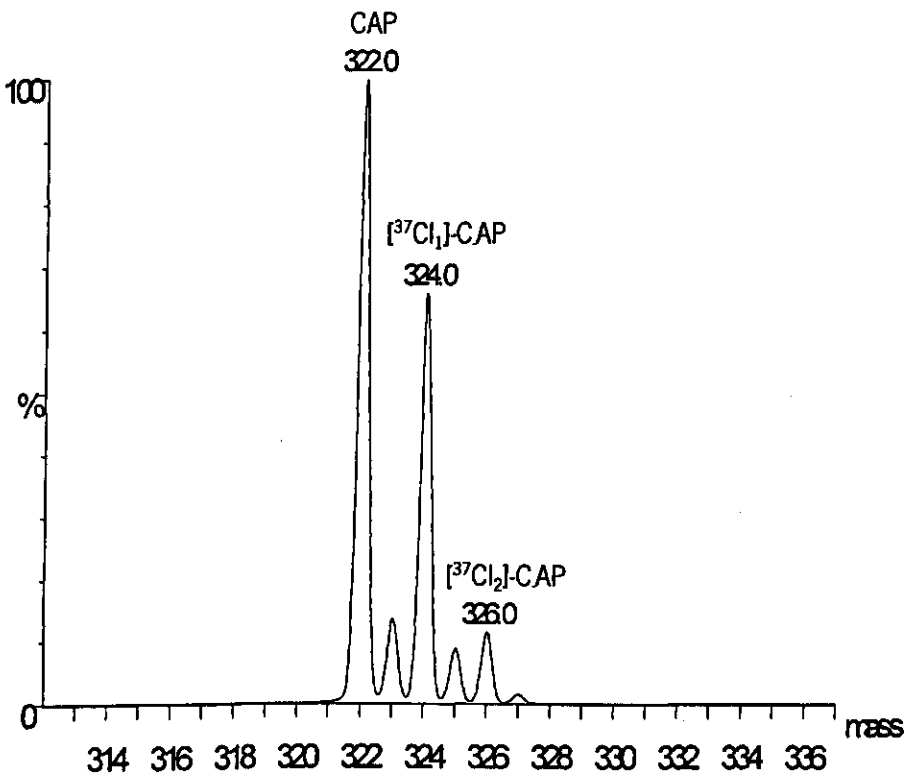
Eight porcine muscle samples were analysed for the presence of chloramphenicol in the framework of the proficiency study for the analysis of chloramphenicol in porcine muscle organised by AFSSA, Fougères.
The samples were analysed using LC-MS/MS. Therefore, the quantitative analysis and the confirmatory analyses were carried out simultaneously. No separate screening analysis was carried out.
The level of quantification and the level at which confirmation could be carried out with the applied method, were both estimated at 0.2 µg/kg.
The presence of chloramphenicol was confirmed in seven out of eight samples in amounts ranging from 1.6 to 7.1 µg/kg. One sample proved to be negative for chloramphenicol.

5 REFERENCES

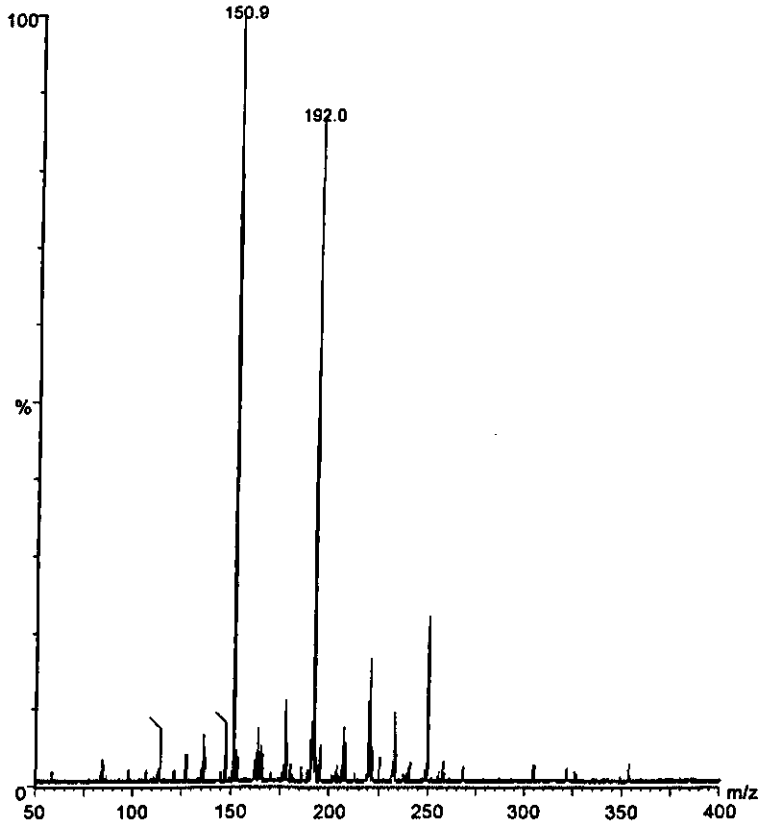
[1] Masslynx software, version 3.4, Micromass, Manchester, UK.

[2] Final Draft Version of Revision of EC Directive 93/256/EC, SANCO/1805/2000, version 1, December 12, 2000.

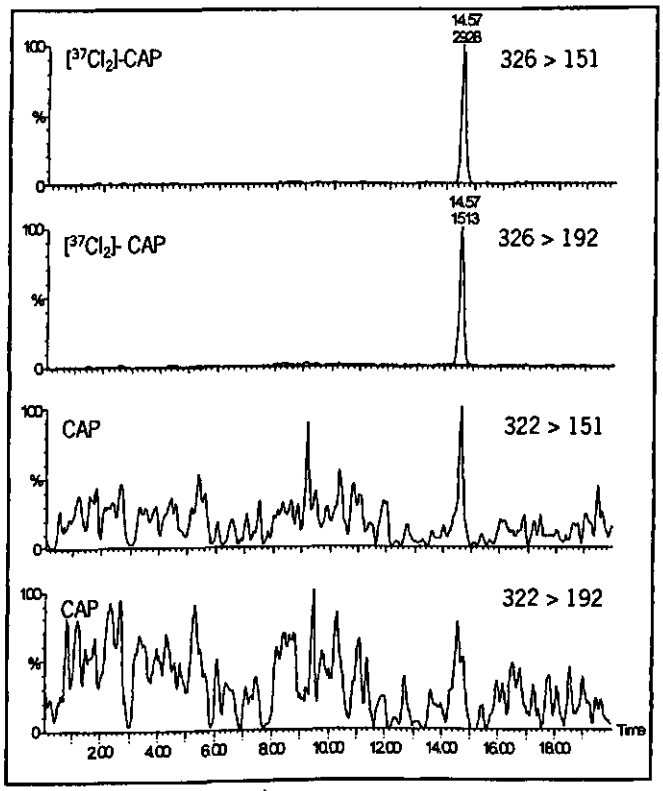
Annex I : Natural abundance of chloramphenicol isotopes



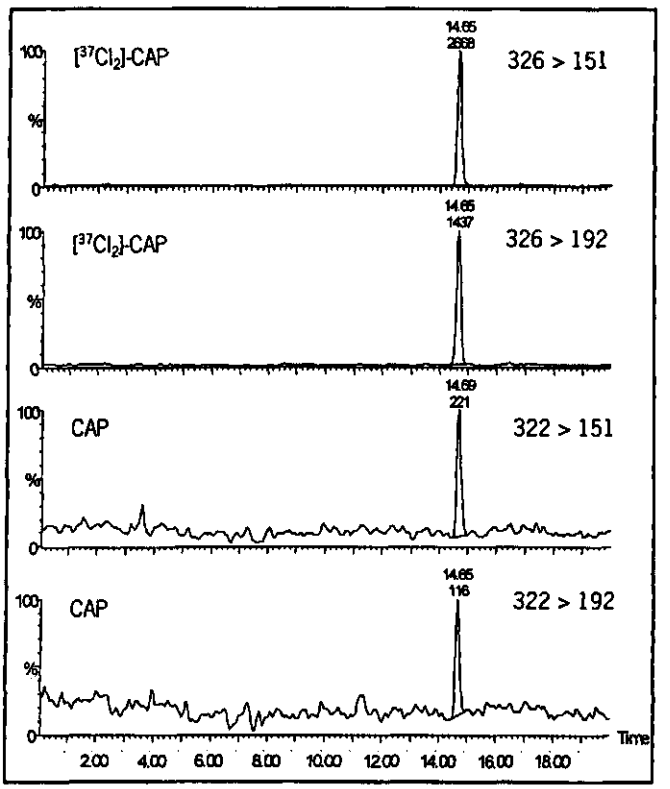
Annex II : MS/MS-Spectrum of chloramphenicol in negative ion mode



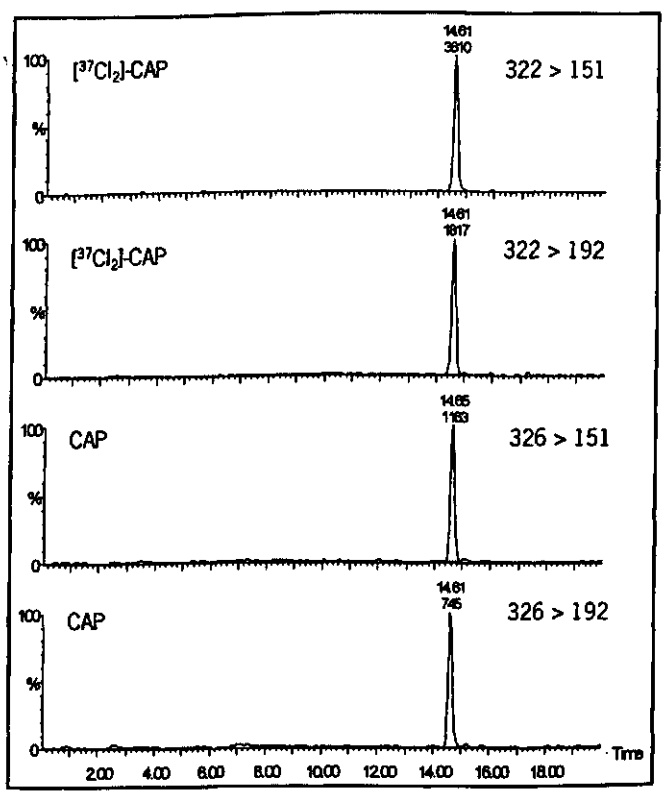
Annex III: LC-MS/MS chromatograms of QC samples consisting of blank and fortified porcine muscle samples



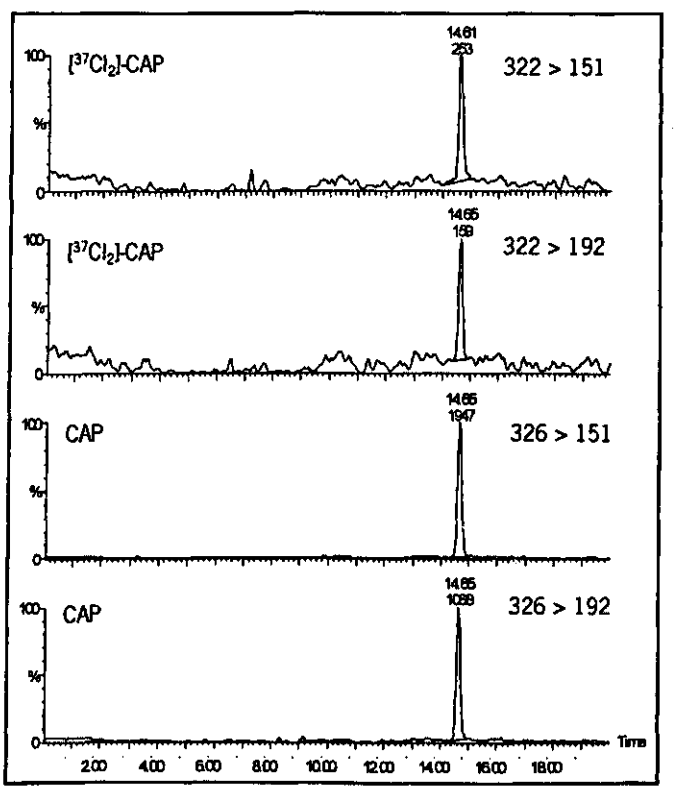
a: blank porcine muscle



b: blank muscle fortified at 0.2 µg/kg (LOQ)

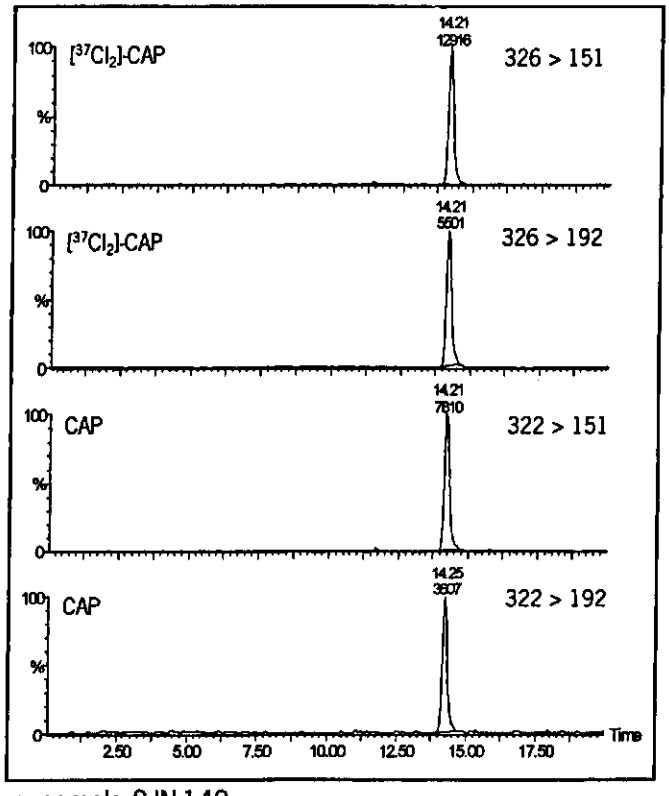


c: blank muscle fortified at 1.0 µg/kg before clean-up

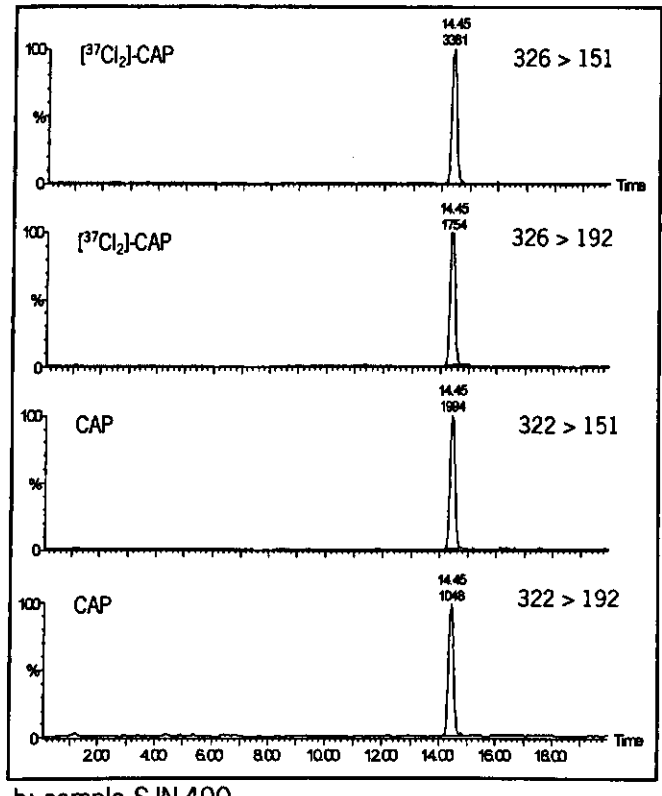


d: blank muscle fortified at 1.0 µg/kg after clean-up
no internal standard was added to the blank sample

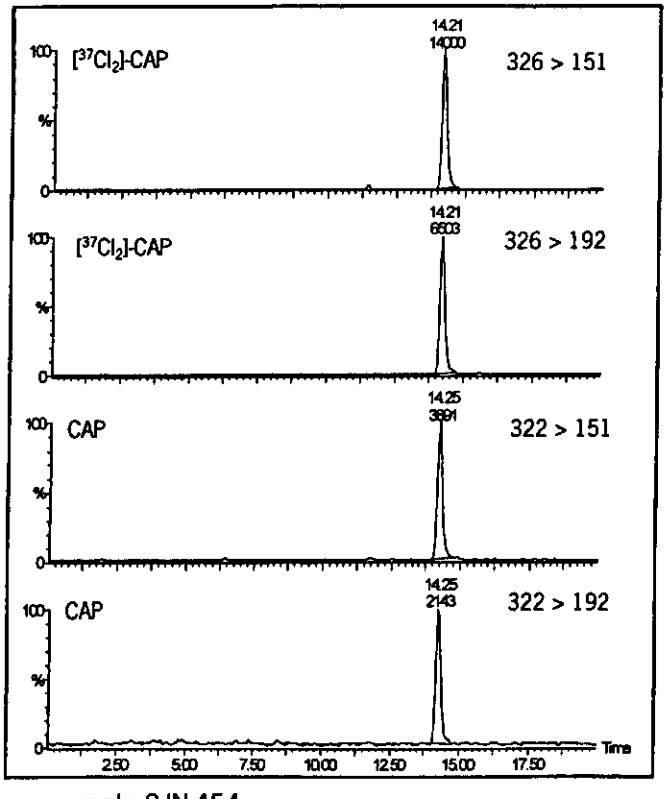
Annex IV : LC-MS/MS chromatograms of proficiency study samples



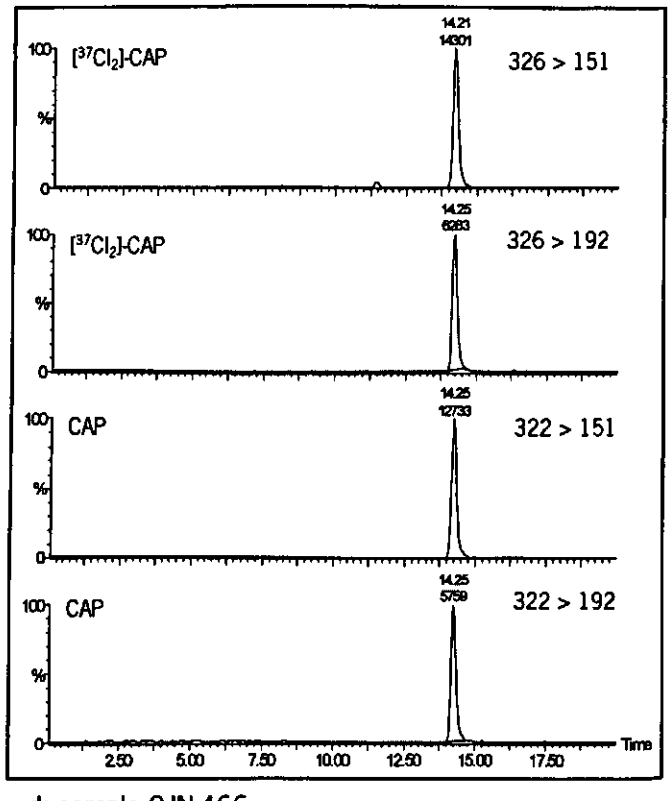
a: sample SJN-140



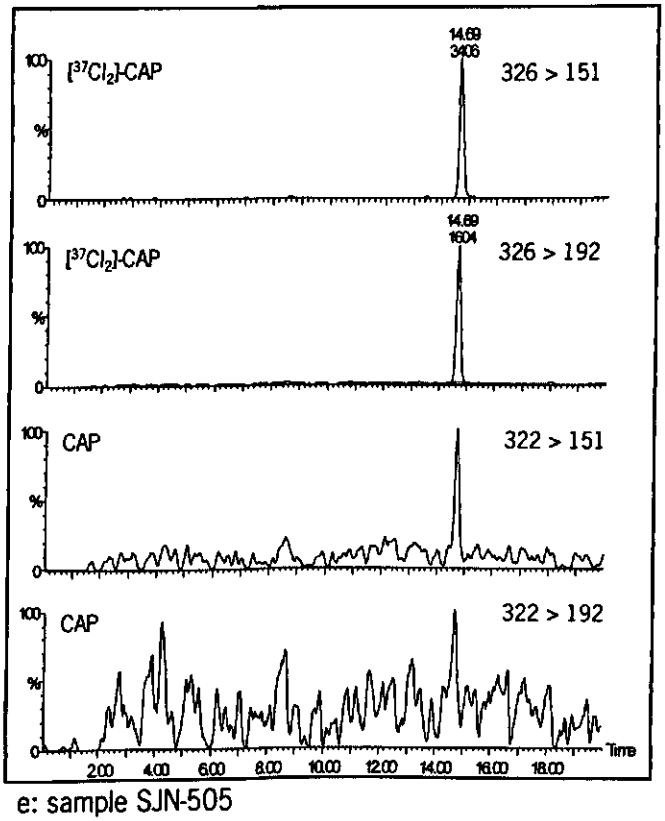
b: sample SJN-400



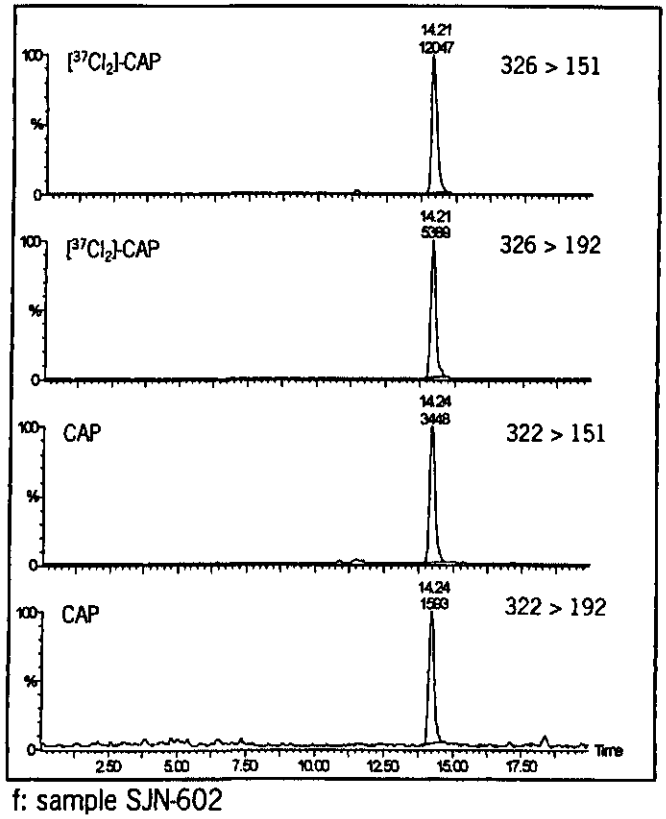
c: sample SJN-454



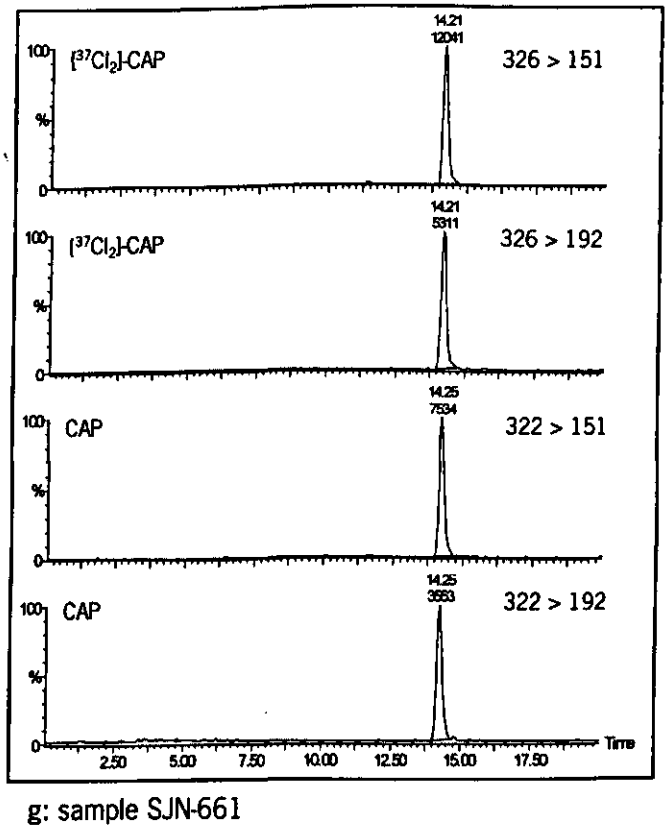
d: sample SJN-466



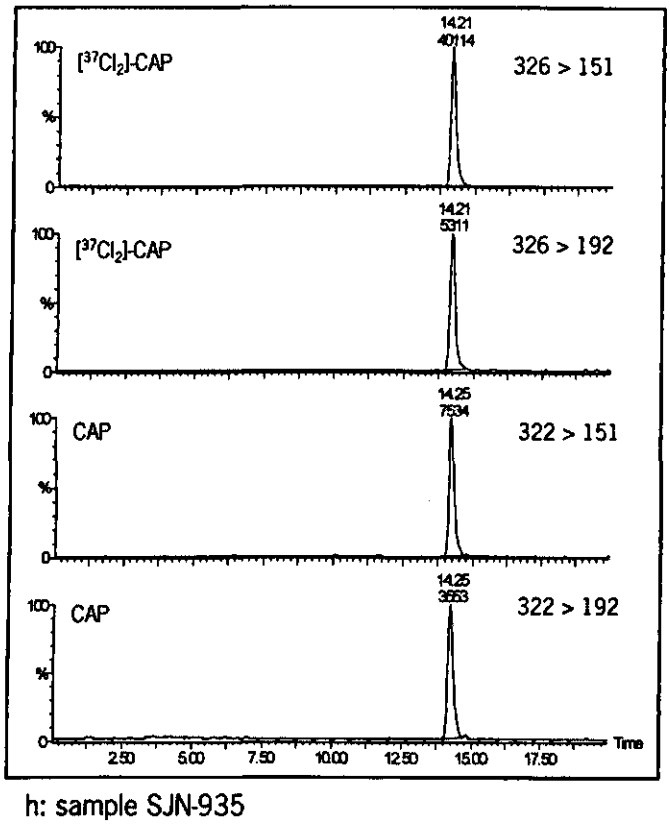
e: sample SJN-505



f: sample SJN-602



g: sample SJN-661



h: sample SJN-935

Annex V : Result forms

Proficiency Study for The Analysis of Chloramphenicol in Pig Muscle

RESULTS FORM 1/3

Laboratory Code: N

Date(s) of the analysis: 13-07-2001

SAMPLE CODE	DETERMINED CONCENTRATIONS (µg/kg)	
	1st value	2nd value
SJN-140	4.6	3.8
SJN-400	1.3	1.8
SJN-454	2.2	2.7
SJN-466	6.7	6.6
SJN-505	<0.2	< 0.2
SJN-602	2.1	2.1
SJN-661	4.8	5.0
SJN-935	6.6	7.5

Equipment used: LC-MS/MS

Calibration: internal standard
Substance: [$^{37}Cl_2$]-chloramphenicol

Limit of identification: 0.2 µg/kg

Limit of quantification: 0.2 µg/kg

Recovery Rate (%): 65 %

Results to be returned to:
D. HURTAUD-PESSEL . AFSSA-Fougères - BP 90203 - 35302 Fougères (France)
Fax: Int. 33.2.99.94.78.80

RESULTS FORM 2/3

Laboratory Code: N

Indicate a short description of the method (extraction) and the equipment:

Materials

All solvents and reagents used were of analytical grade or better. All chemicals, including the Extrelut-3® columns, were obtained from Merck (Darmstadt, Germany). The chloramphenicol reference standard (batchno. 60740) was provided together with the samples by AFSSA (Fougères, France) and was obtained from Sigma (St. Louis, MO, USA). [$^{37}Cl_2$]-Chloramphenicol was used as internal standard and was obtained from RIVM (Bilthoven, The Netherlands).
A Waters (Milford, MA, USA) Symmetry C18 column (L=15 cm, ID=3.0 mm) was used to establish the separation.
Porcine muscle samples were received in a frozen condition from AFSSA and were coded: SJN-140, SJN-400, SJN-454, SJN-466, SJN-505, SJN-602, SJN-661 and SJN-935.

Blank porcine muscle for the preparation of QC samples, was thoroughly minced. An aliquot of 5 g of the blank muscle or the provided muscle samples was taken and transferred to a stomacher bag.
[$^{37}Cl_2$]-Chloramphenicol was used as internal standard and was added to all samples and QC samples at a level of 5 µg/kg. Chloramphenicol was added to the blank muscle to prepare the QC samples. The samples were thoroughly homogenised. After 30 min, 10 ml water was added to the samples in the stomacher bag and the muscle homogenate was extracted in a stomacher apparatus during 3 minutes. The content of the stomacher bag was transferred to a centrifuge tube and was centrifuged for ten minutes (3500 g, 15°C). Three ml of the supernatant was transferred to an Extrelut-3® extraction column. After 30 minutes of equilibrating, chloramphenicol was eluted with 15 ml dichloromethane. The dichloromethane was evaporated to dryness under nitrogen at 30 °C. The residue was dissolved in 0,5 ml water. An aliquot of 50 µl was injected in the LC-system without further purification.

Separation was established on a Waters Symmetry C18 (L=15 cm, ID=3.0) column using a gradient in methanol. The column was connected to a Micromass (Manchester, UK) Quattro Ultima triple quadrupole mass spectrometer equipped with an Atmospheric Pressure chemical Ionisation (APcI) interface operating in the negative ion mode.

Results to be returned to:
D. HURTAUD-PESSEL . AFSSA-Fougères - BP 90203 - 35302 Fougères (France)
Fax: Int. 33.2.99.94.78.80

Proficiency Study for The Analysis of Chloramphenicol in Pig Muscle

RESULTS FORM 3/3

Laboratory Code: N

Comments concerning the way of identification of the detected substances and the applied criteria:

Confirmation is carried out according to EU criteria (Final Draft Version of the Revision of EC Directive 93/256/EC, SANCO/1805/2000, version 1, December, 12, 2000). The confirmation assay is operated in multiple reaction monitoring mode (MRM), recording the transition of the precursor ion into two diagnostic product ions (table 1). Those transitions are specific for chloramphenicol and yield a total of 4 identification points which is sufficient for confirmatory analysis.

table 1: Characteristic MRM ions of chloramphenicol and [$^{37}Cl_2$]-chloramphenicol, the most abundant ion is underlined.

Compound	Parent ion (m/z)	Daughter ion (m/z)	Coll. Energy (eV)
CAP	322	<u>151</u>	15
		192	15
[$^{37}Cl_2$]-CAP	326	<u>151</u>	15
		192	15

Results to be returned to:
D. HURTAUD-PESSEL . AFSSA-Fougères - BP 90203 - 35302 Fougères (France)
Fax: Int. 33.2.99.94.78.80