Analytical method for food safety

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Method for the detection and confirmatory quantification of chloramphenicol residues in urine by LC-MS/MS

Fougères Laboratory
EUROPEAN UNION and National Reference Laboratory : Veterinary Medicinal Product residues and dyes in foodstuffs of animal origin and animal feed

ANSES/FGE/0139 [version e]
plan de classement PR3/ANSES/7

History of the method

A method may be updated to consider any changes into account.

A change is considered major when it involves the analytical process, the scope, or critical points of the method and is likely to modify the performance of the analytical method and/or the results. A major change requires adaptations. The amended method is then fully or partially revalidated and a new version is released.

A change is considered minor if it provides useful or practical clarifications, reformulates the text to make it clearer or more accurate, or corrects minor errors. A minor change has no impact on the method's performance and does not require revalidation. However, a new version is released as well.

The table below summarises the versions history of this method, including the nature of any changes made.

Version	Nature of the changes (major/minor)	Date	Principales modifications
1	-	2007	Initial method
2	major	2013	Method using molecular imprint cartridges (SupelMIP)
3	major	2023	New method development Updated validation in accordance with Implementing Regulation (EU) 2021/808
4	minor	2024	Modification of the confirmation threshold in §9.2.1

Foreword

This method has been developed by:

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This method has been optimised, characterised and validated under the ARC Unit “Analysis of Residues and Contaminants” at the ANSES-Fougères Laboratory.

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Introduction

The present method has been developed for the control of official samples implemented according to the European Regulation (EU) 2017/625 of 15/03/2017 [1] on the control of certain substances and residues in live animals, animal products and animal feed. This method is used to detect and confirm the presence of chloramphenicol in urine by LC-MS/MS.

Chloramphenicol belongs to group A2a of Delegated Regulation (EU) 2022/1644 of 7/07/2022 [2]. Chloramphenicol is listed in Table 2 (prohibited substances) of European Commission Regulation No. (EU) 2010/37 [3]. The use of chloramphenicol is therefore banned in veterinary medicine in Europe, and this antibiotic must not be found in live animals or their products. A reference control value (RPA) of 0.15 µg/kg is defined in Commission Regulation (EU) 2019/1871 [4].

This method was validated according to the requirements described in the French implementation guide [5] of the European Commission Regulation (EU) 2021/808 of 22 March 2021 [6] associated with the LR-UE's validation guide for confirmation methods (EURL Guidance Document on Confirmation Method Validation, Version 1.1, 25 November 2021 [7]).

Warnings and safety precautions

Users of this method should be fully familiar with common laboratory practices. It is the user's responsibility to establish appropriate health and safety practices and to ensure compliance with the regulations in force.

All actions taken in accordance with this method must be performed by employees who have attended relevant training.

This method assumes that the operator is familiar with the usual rules for handling chemicals and solvents. As far as possible, it should be carried out under a fume hood. All necessary precautions must be taken when handling the standards (weighing done under the hood, gloves worn, etc.). It is important to carefully check the risks associated with each product before use, in particular for the standard substances (see the hazard statements in annexe).

1 Purpose and scope

This method can be used for the detection and confirmatory quantification of chloramphenicol around the level of interest of 0.15 µg/kg in urine (pork, bovine, …). The validated quantification according to the requirements of commission regulation (EU) 2021/808 covers the range 0.075 to 0.6 µg/kg. The detection capacity of the method (CCβ) has been evaluated to be less than or equal to 0.10 µg/kg. The CCα is equal to 0.087 µg/kg.

2 Reference documents

[1] Commision Regulation (EU) 2017/625 of 15 march 2017, Official Journal of the European Union, L95 : 1-142

[2] Commision Regulation (EU) 2022/1644 of 7 jully 2022, Official Journal of the European Union, L248 : 3-17

[3] Commision Regulation (EU) n°37/2010 of 22 december 2009, Official Journal of the European Union L15/1 (2010) version may 2022

[4] Commision Regulation (EU) 2019/1871 of 7 november 2019, Official Journal of the European Union L289/41 (2019)

[5] French guide to applying the commission regulation (EU) 2021/808 – Version 00 (2022)

[6] Commission regulation 2021/808/CE of 22 march 2021. Official Journal of the European Union. L180 : 84-109

[7] EURL Guidance Document on Confirmation Method Validation, Version 1.1, 25 November 2021

3 Terms, acronyms and definitions

ACN: acetonitrile

CAP: chloramphenicol

CAP-D_5: chloramphenicol-D_5

HPLC: High performance liquid chromatography

IS: Internal standard

LC-MS/MS: Liquid Chromatography coupled with tandem mass spectrometry

QC-BR: reagent blank quality control sample serves to prove that no contaminations is present in reagents

QC1-BM without IS: matrix blank quality control sample (without internal standard) to check the absence of CAP and CAP-D5 contamination in the matrix.

QC1-BM with IS (=SE0): matrix blank quality control sample (with internal standard) to check the absence of CAP contamination in the matrix.

QC2-CCβ (=SE1): fortified quality control sample to 0.1 µg/kg (CCβ level), which is used to validate the analytical series on screening identification criteria.

QC3-RPA: fortified quality control sample to 0.15 µg/kg (level RPA), which is used to validate the analytical series on quantification criteria (verification of the value obtained within the limits defined on validation).

SE: calibration standard

RT: retention time

4 Principle of the methode

The method involves three steps:

- Extraction liquide / liquide on Extrelut column,
- Purification of the extract on a reverse-phase polymer SPE column,
- HPLC separation on a C18 silica column followed by electrospray ionisation in negative mode and detection by tandem mass spectrometry with acquisition in segmented MRM mode.

To guarantee the method's performance and enable quantification, penta-deuterated chloramphenicol (CAP-D5) is used as the internal standard.

5 Reagents

<u>Caution</u>: Trade names or commercial suppliers may be mentioned in the description of the products needed for the implementation of this method. This information is provided for the users of the method and does not in any way presume that ANSES-Fougeres recommends the exclusive use of these products. Similar products may be used if it has been demonstrated that they achieve similar results.

5.1 Water

Use ultrapure water such as water purified by a Milli-Q-IQ-7000 (Millipore) system.

5.2 Gas

5.2.1 Nebulisation and desolvation gas: purified air

5.2.2 Curtain and collision gas: 99.995 % pure nitrogen (nitrogen tank)

5.3 Reagents

The reagents used must be of analytical quality.

5.3.1	β-glucuronidase arylsulfatase of Helix-pomatia (Sigma G0876)	N°CAS : [9001-45-0]
5.3.2	Ethyl acetate (Fisher E/0900/17)	N°CAS : [141-78-6]
5.3.3	Ammonium acetate (Supelco-Merck 1.01116)	N°CAS : [631-61-8]
5.3.4	Sodium acetate anhydrous (Supelco-Merck 1.06268)	N°CAS : [127-09-3]
5.3.5	Acetonitrile (Fisher A/0626/17)	N°CAS : [75-05-8]
5.3.6	Acetonitrile Optima LC/MS Grade (Fisher A955-212)	N°CAS : [75-05-8]
5.3.7	Methanol (Fisher M/4000/17)	N°CAS : [67-56-1]
5.3.8	Acetic acid 100 % (VWR 20104.298)	N°CAS : [64-19-7]
5.3.9	Ammonia solution 25% (Supelco-Merck 1.05432)	N°CAS : [1336-21-6]

5.4 Solutions

5.4.1 0.01 mol/L ammonium acetate solution: prepare a 2 mol/L ammonium acetate solution (15.4 g in 100 mL of ultrapure water) then dilute to 1/200 (5 mL in 1L of ultrapure water).

5.4.2 Buffer solution pH 4.8: weigh 16.4 g of sodium acetate, dissolve in approximately 900 mL of ultrapure water, adjust the pH to 4.8 ± 0.3 with acetic acid, make up to 1L with ultrapure water.

5.4.3 Methanol/water solution, (5/95): add 5 mL of methanol to a 100 mL volumetric flask and make up to the mark with water.

5.4.4 50% acetic acid solution: add 25 mL of acetic acid to a 50 mL volumetric flask containing water and make up to the mark with water.

5.4.5 0,5 % acetic acid solution: add 0.5 mL acetic acid to a 100 mL volumetric flask containing water and make up to the mark with water.

5.4.6 1% ammonia solution: add 4 mL of 25% ammonia solution to a 100 mL volumetric flask and make up to the mark with water.

5.4.7 Acetonitrile/0.5% acetic acid solution, (5/95): add 5 mL acetonitrile to a 100 mL volumetric flask then make up to the mark with the 0.5% acetic acid solution.

5.4.8 Acetonitrile/1% ammonia solution, (20/80): add 10 mL acetonitrile to a 50 mL volumetric flask then make up to the mark with the 1% ammonia solution.

5.5 Standards and solutions preparation

5.5.1 Stock solutions

Chloramphenicol is preferably purchased in solution form to avoid contamination with the powder.

Analytes	Supplier	Solvent	Concentration solution	N° CAS
Chloramphenicol (CAP)	Cluzeau DRE-L11120000AL	Acetonitrile	10 µg/mL	[56-75-7]
Chloramphenicol-D_5 (CAP-D_5)	Cluzeau DRE-XA11120100AL	Acetonitrile	100 µg/mL	[202480-68-0]

If stock solutions concentrations are different from the table above, it should be taken into account when preparing the intermediate solutions.

5.5.2 Intermediate solutions

5.5.2.1 *Intermediate solution at 100 ng/mL of chloramphenicol: SI-CAP-100.*

In a 20 mL volumetric flask, add 200 µL of SM CAP (10 µg/mL) and make up with water.

This solution can be stored for 1 year at between +2°C and +8°C.

5.5.2.2 *Intermediate solution at 1000 ng/mL of chloramphenicol-D_5: SI-CAP-D_5-1000.*

In a 20 mL volumetric flask, add 200 µL of SM CAP-D_5 (100 µg/mL) and make up with water.

This solution can be stored until depleted at between +2°C and +8°C.

5.5.3 Working solutions

5.5.3.1 *Working solution at 0.75 ng/mL of chloramphenicol: SS-CAP-0,75.*

In a 20 mL volumetric flask, add 150 µL of SI-CAP-100 and make up with water.

This solution can be stored for 1 year at between +2°C and +8°C.

5.5.3.2 *Working solution at 1.5 ng/mL of chloramphenicol: SS-CAP-1,5.*

In a 20 mL volumetric flask, add 300 µL of SI-CAP-100 and make up with water.

This solution can be stored for 1 year at between +2°C and +8°C.

5.5.3.3 *Working solution at 3 ng/mL of chloramphenicol: SS-CAP-3.*

In a 20 mL volumetric flask, add 600 µL of SI-CAP-100 and make up with water.

This solution can be stored for 1 year at between +2°C and +8°C.

5.5.3.4 *Working solution at 6 ng/mL of chloramphénicol: SS-CAP-6.*

In a 20 mL volumetric flask, add 1200 µL of SI-CAP-100 and make up with water.

This solution can be stored for 1 year at between +2°C and +8°C.

5.5.3.5 *Working solution at 5 ng/mL of chloramphenicol-D_5: SS-CAP-D_5-5.*

In a 20 mL volumetric flask, add 100 µL of SI-CAP-D_5-1000 and make up with water.

This solution can be stored until depleted at between +2°C and +8°C.

6 Equipment and materials

Caution : Trade names or commercial suppliers may be mentioned in the description of the equipment and materials needed for the implementation of this method. This information is provided for the users of the method and does not in any way imply that ANSES recommends the exclusive use of these materials. Similar materials may be used if it has been demonstrated that they achieve the same results.

The references are given as an indication. Any equivalent material may be used.

6.1 Laboratory materials

6.6.1 Grade A volumetric flasks.

6.6.2 Variable volume solvent dispensers (Socorex).

6.6.3 50 mL centrifuge tubes, type SARSTEDT 62.547.254.

6.6.4 15 mL centrifuge tubes, type FALCON 352095

6.6.5 12 mL tubes in polypropylene, type Corning TR 95-23

6.6.6 5 mL tubes in polypropylène, type VWR 216-1167

6.6.7 Automatiques laboratory pipettes and corresponding tips (Gilson-Biohit).

6.6.8 Mixer (Vortex type).

6.6.9 Precision laboratory balance (resolution ≤ 10 mg).

6.6.10 pH meter.

6.6.11 Refrigerated centrifuge, type GR 422 (Jouan).

6.6.12 Dry bath for evaporation under N2 (Stuart)

6.6.13 Solid phase extraction system type Vac Elut (Supelco), suitable needles, adapters and taps.

6.6.14 Diaphragm pump 0.4 bar (Bioblock)

6.6.15 Polypropylene injection bottle of 650 µL (Interchim 963290)

6.6.16 20 mL polyethylene scintillation bottle, type Wheaton (986721)

6.6.17 Bulb pipettes, polypropylene pastettes

6.6.18 Extraction cartridges liquide / liquide EXTRELUT NT3 (MERCK, 1.15095.0001)

6.6.19 SPE cartridges OASIS HLB 3cc 60 mg (WATERS, WAT094226)

6.6.20 Filter 0.45 µm diameter 13 mm type Millex HV PVDF (Millipore SLHVX13NK) and 1 mL syringes for filtration.

6.2 Chromatography apparatus

6.2.1 HPLC system of the UFLC type from Prominence (Shimadzu), or equivalent, comprising the following components:

- Solvent selector FCV-11AL
- Two pumps HPLC (binary system) LC-20AD XR
- Degasser DGU 20A5
- Autosampler SIL-20AC XR
- Column oven CTO-20AC
- Controller CBM-20A

6.2.2 Pre column security guard C18, 4.0 x 3.0 mm (ref AJO-4287, Phenomenex)

6.2.3 Analytical column Symmetry C18, 5 µm, 150 X 3.9 mm (WAT 046980, Waters)

6.3 Mass spectrometer

6.3.1 Valco switching valve or equivalent.

6.3.2 Quadrupole tandem mass spectrometer API5500 (SCIEX) or equivalent with control and configuration software Analyst (or equivalent).

7 Samples

7.1 Conditions for acceptance of samples

It is important that the laboratory receives a truly representative sample that has not been damaged or modified during transport or storage.

7.2 Storage and preparation of samples prior to analysis

Samples must be stored in the freezer to at least -18°C until analysis.

The stability of chloramphenicol in its glucuronide form in urine was tested over 3 months and no degradation was observed over this period (under the storage conditions mentioned).

The following protocol is applied to the control urine that will be used for the calibration range, the QCs and the urine samples to be analysed:

7.2.1 Thaw the urine, collect a volume greater than 20 mL and place in a 50 mL centrifuge tube.

7.2.2 Centrifuge 5 minutes at 3000 g at approximately 4°C.

7.2.3 Take 20 ± 0.2 mL of urine.

7.2.4 Adjust pH at 4.8 ± 0.3 (50 % acetic acid).

7.2.5 Place all the urine adjusted to pH 4.8 in a 25 mL volumetric flask and make up to the mark with pH 4.8 buffer.

7.2.6 Freeze the prepared urine and the rest of the sample if the analysis is not immediate.

8 Procedure

For a screening analysis: extraction of a QC-BR, a QC1-BM without IS, a QC1-BM with IS (=SE0), a QC2-CCβ (=SE1) and the samples to be analysed.

For a confirmatory analysis: extraction of a QC-BR, a QC1-BM without IS, a range of spiked samples (SE0-SE1-SE2-SE3-SE4), a QC3-RPA and the samples to be confirmed in duplicate. Determination of concentration in the samples and in the QC3-RPA against the calibrating spiked samples. The spiked samples at the first level of the range (SE1) are used as quality controls (QC2-CCβ).

8.1 Preparation of sample prior to analysis

8.1.1 The day before analysis, prepare tubes containing 2 ± 0.04 g each (1 for screening and 2 for confirmation) from urine samples adjusted to pH 4.8.

8.1.2 Add 50 µL of β-glucuronidase to each tube and mix with a vortex.

8.1.3 Incubate overnight at around 37°C.

8.1.4 Add 200 µL of SS-CAP-D5-5 to 2 ± 0.04 g of urine sample.

8.1.5 Mix with vortex and leave in contact for 10 minutes.

8.2 Preparation of quality control sample QC

8.2.1 Quality control sample: QC-BR and QC1-BM without IS

8.2.1.1 The day before the analysis, prepare a tube containing 2 ± 0.04 g of water for the QC-BR and a tube containing 2 ± 0.04 g of urine adjusted to pH 4.8 for the QC1-BM without IS.

8.2.1.2 Add 50 µL of β-glucuronidase to each tube and mix with a vortex.

8.2.1.3 Incubate overnight at around 37°C.

8.2.2 Spiked control sample at 0.15 µg/kg : QC3-RPA

These QCs can be stored for at least 3 months at at least -18°C.

8.2.2.1 Add 200 µL of **SS-CAP-1.5** at 2 ± 0.04 g of urine chloramphenicol free adjusted to pH 4.8.

8.2.2.2 Mix with vortex and leave in contact for 10 minutes. Place in the freezer.

8.2.2.3 The day before analysis, thaw a QC3-RPA

8.2.2.4 Add 50 µL of β-glucuronidase to this tube, mix with a vortex.

8.2.2.5 Incubate overnight at around 37°C.

8.2.2.6 Add 200 µL of **SS-CAP-D_5-5**.

8.2.2.7 Mix with vortex and leave in contact for 10 minutes.

8.3 Preparation of the range of spiked sample (SE) for confirmation (0; 0.075; 0.15; 0.3; 0.6 µg/kg)

8.3.1 The day before analysis, prepare 5 tubes containing 2 ± 0.04 g of urine adjusted to pH 4.8

8.3.2 Add 50 µL of β-glucuronidase to each tube and mix with a vortex.

8.3.3 Incubate overnight at around 37°C.

8.3.4 Spiking is done as follows:

		Add water	Add spiked solution	Add of SS-CAP-D_5-5
SE0	*Blank control*	200 µL	/	200 µL
SE1	*Spiked at 0.075 µg/kg (=QC2-CC6)*	/	200 µL of SS-CAP-0.75	200 µL
SE2	*Spiked at 0.15 µg/kg*	/	200 µL of SS-CAP-1.5	200 µL
SE3	*Spiked at 0.3 µg/kg*	/	200 µL of SS-CAP-3	200 µL
SE4	*Spiked at 0.6 µg/kg*	/	200 µL of SS-CAP-6	200 µL

8.3.5 Mix with vortex and leave in contact for 10 minutes.

8.4 Procedure of extraction– purification

From the outset, fit PP tubes containing approximately 12 ml and needles into the solid phase extraction system.

8.4.1 Transfer all the samples prepared in 8.1, 8.2 et 8.3 to the Extrelut NT 3 cc column.

8.4.2 Leave to equilibrate for 15 min.

8.4.3 Place 3 ml of ethyl acetate on the Extrelut column.

8.4.4 Leave to equilibrate for 15 min.

8.4.5 Place 8 ml of ethyl acetate on the Extrelut column.

8.4.6 Collect the organic phase in the 12 ml polypropylene tube.

8.4.7 Slightly dry the cartridge.

8.4.8 Evaporate under nitrogen at around 40°C.

8.4.9 Dissolve the residue in 3 ml methanol/water solution, (5/95)

8.4.10 Install new needles and taps on the extraction system then OASIS HLB 3cc 60mg cartridges.

8.4.11 Condition cartridges with 2 mL of methanol and 4 mL of water. Pass it through at a rate of approximately 1 drop per seconde.

8.4.12 Transfer the extract on the cartridges with a flow of approximately 1 drop per seconde.

8.4.13 Wash cartridges with a flow of approximately 1 drop per seconde, using the pump if necessary, by :

- 2 mL of water
- 1 mL of acetonitrile/0.5% acetic acid solution, (5/95)
- 2 mL of 1% ammonia solution
- 1 mL of acetonitrile/1%ammonia solution, (20/80)

8.4.14 Dry the cartridges for 1 min under vacuum.

8.4.15 Install 5 ml polypropylene tubes under the cartridges.

8.4.16 Elute with 1 mL of methanol with a flow rate of about 1 drop/second, using the pump if necessary.

8.4.17 Dry the cartridge using the pump for a few seconds.

8.4.18 Evaporate under nitrogen at around 50°C.

8.4.19 Dissolve the residue with 0.5 mL of 0.01 mol/L ammonium acetate.

8.4.20 Filter the extracts through 0.45 µm filter.

8.5 LC-MS/MS analysis and detection

8.5.1 Chromatographic conditions

8.5.1.1 Flow rate : 0.6 mL/min

8.5.1.2 Injection volume : 50 µL

8.5.1.3 Temperature in the column oven : 25°C

8.5.1.4 Autosampler temperature : ambient

8.5.1.5 Elution gradient :

T (min)	Track A %	Track B %
0	90	10
2	45	55
5	45	55
6	90	10
8	90	10

A: 0.01 mol/L ammonium acetate

B: Acetonitrile Optima LC/MS Grade.

8.5.1.6 Valco switching valve: openning time 3 min to 7 min.

8.5.2 Mass spectrometry for API5500

Ionisation: ESI (turbospray) in negatif mode

Electrode height: 3 mm

Source temperature: 700°C

Curtain gas: 35

GS1: 50

GS2: 50

IS: - 4200V

CAD: Medium

EP: -10

Type de scan: MRM (Multiple Reaction Monitoring)

Acquisition time: 8 min

8.5.3 Transitions and retention time

Transitions and retention times are given as an indication. These may vary according to the chromatographic equipement used.

	Transition	Parent ion (m/z)	Daughter ion (m/z)	Dwell time (msec)	DP	CE	CXP	RT (min)
Chloramphenicol	1	321.1	152	200	-45	-24	-7	4.3
	2	321.1	257	200	-45	-16	-17	
	3	323.0	152	200	-55	-24	-9	
Chloramphenicol-D_5		326	157	200	-50	-28	-15	4.3

8.5.4 Acquisition sequence

In routine screening analysis, samples should preferably be injected as follows:

- Sample QC-BR
- Sample QC1-BM without IS,
- Sample QC1-BM with ISI (equivalent to SE0),
- Sample QC2-CCβ (equivalent to SE1),
- Water injection,
- Extracts to be analysed,
- Sample QC2-CCβ (equivalent to SE1) (possibly if large series)
- Water injection,
- Sample QC1-BM without IS,
- Sample QC1-BM with IS (equivalent to SE0),
- Sample QC2-CCβ (equivalent to SE1)

In routine confirmation analysis, samples should preferably be injected as follows:

- Sample QC-BR
- Sample QC1-BM without IS,
- Sample SE0,
- Spiked calibration standard (SE1 to SE4),
- Water injection,
- Sample QC3-RPA
- Water injection,
- Extracts to be analysed,
- Sample QC3-RPA (possibly if large series)
- Water injection,
- Sample SE0,
- Spiked calibration standard (SE1 to SE4)

The first level of the range (=SE1) is used as QC2-CCβ sample to check the validity of the results.

The extracts can be stored for at least 12 days in the freezer.

9 Resultats

9.1 Checking the validity of the results

Only 2 transitions associated with the retention time are required to obtain a minimum of 5 confirmation identification points. Transitions 321.1/152 and 321.1/257 are chosen as a priority. If an interference is observed on transition 321.1/152 (see §10), it is preferable to choose transitions 323/152 and 321.1/257.

9.1.1 Screening analysis

- QC-BR, QC1-BM without IS and QC1-BM with IS are free of chloramphenicol.
- The presence of internal standard (IS) must be checked in QC1-BM with IS, QC2-CCβ and extracts to be analysed.
- All chromatographic peaks of interest are present for each transition with a signal-to-noise ratio ≥ 3 in QC2-CCβ (=SE1) at chloramphenicol retention time.

9.1.2 Confirmatory analysis

- QC-BR, QC1-BM without IS and SE0 are free of chloramphenicol.
- The presence of internal standard (IS) must be checked in SE and extracts to be analysed.
- All chromatographic peaks of interest are present for each transition with a signal-to-noise ratio ≥ 3 in QC2-CCβ (=SE1) at chloramphenicol retention time.
- The coefficient of determination r^2 of the calibration curves for the transition chosen for quantification is higher than 0.97. This criterion is an in-house criterion of the Anses-Fougères Laboratory related to the results of the validation.
- The estimated concentration for QC3-RPA should be within 0.132 and 0.168 µg/kg. The limits applied are related to the expanded uncertainty calculated during validation, multiplied by the expansion factor 2.33.

9.2 Calculation and expression of results

9.2.1 Identification criteria for screening analyses for sending confirmation

The presence of chloramphenicol in the sample to be controlled is suspected when the criteria below is met :

- The chromatographic peaks of interest are present with a signal/noise ratio ≥ 3 on at least 2 transitions at the relative retention time of chloramphenicol with a tolerance of ± 1% compared to QC2-CCβ.

The sample is sent for confirmation when its concentration estimated using the QC2-CCβ is greater than or equal to the CCβ.

9.2.2 Identification criteria for confirmation analyses

The presence of chloramphenicol in the sample to be controlled is confirmed when the criteria defined in the Commission Regulation (UE) 2021/808 [6] are met, that is:

- The chromatographic peaks of interest are present with a signal/noise ratio ≥ 3 on at least 2 transitions.
- The relative retention time of chloramphenicol in the controlled sample shall correspond to the relativeretention time of the spiked calibration samples with a tolerance of ± 1 %.
- The relative ion intensities of the two chromatographic peaks of interest in the controlled samples are compared to the relatives ion intensities of the two peaks of interest in the spiked calibration samples and comply with the 40% tolerance.

9.2.3 Determination of the concentration

9.2.3.1 *Internal standard used*

The chloramphenicol-D_5 standard will be used as an internal standard to quantify chloramphenicol.

9.2.3.2 *Calibration curve*

Quantification is carried out based on the transition 321.1/152. If an interference is observed on the 321.1/152 transition (see §10), the transition 323/152 can be used for the quantification. The regression curve is established from the spiked calibration samples areas including the blank control sample (but without forcing through the zero).

The levels of the calibration spiked samples are : 0 – 0.075 – 0.15 – 0.3 and 0.6 µg/kg.

A linear regression model was chosen:

$$y=ax+b$$

y: analyte peak area / internal standard peak area
x: concentration in µg/kg
a: slope of the regression curve
b: the y-axis at the origin

After identification, the chloramphenicol content (µg/kg) is calculated from the regression line equation established above. **Note that this calculated concentration corresponds to the concentration of chloramphenicol in the urine after adjustment to pH 4.8 and must be corrected by the dilution factor (20/25ths) if you want to have the real concentration in the urine sample before dilution** according to the following equation: $[C]_{sample} = [(y-b)/a] * 25/20$

In practice, the data processing software is used to create an automatic quantification method.

9.3 Final decision – Compliance of the controlled sample

The sample is declared non-compliant if all confirmatory identification criteria (retention times, 2 transitions with S/N ≥ 3, ion ratios) are met and if the concentration (average of estimated concentrations of the 2 test samples) is greater than or equal to the CCα decision limit of 0.087 µg/kg.

Additionnal Information :

According to Article 5 of Regulation (EU) 2019/1871, there are two possible scenarios:

- If the concentration of the sample is greater than or equal to the RPA (0.15 µg/kg) then the sample is non-compliant and management measures to withdraw or destroy the batch are applied.
- If the concentration of the sample is greater than or equal to the CCα but less than the RPA (0.15 µg/kg) then the sample is non-compliant with notification of presence but without management action.

10 Performance characteristics of the method

Summary of qualitative data processing results.

Interferent peak	Retention time	Signal to noise	Ion relative intensity
CAP1: 2 out of 24 batches showed interference at CAP RT CAP2: no interference CAP3: no interference	✓	✓	CAP1/CAP2:✓ CAP1/CAP3:✓ CAP3/CAP2:✓

CAP1: 321.1/152

CAP2: 321.1/257

CAP3: 323.0/152

The detection capacity of the method (CCβ) was evaluated according to method 2 of regulation 2021/808 [6] (§2.7) and estimated to be less than or equal to 0.075 µg/kg.

Summary of quantitative data processing results.

	Level tested (µg/kg)	Relative biais %	Intermediate precision CVR %	u Expanded incertainty µg/kg	Validated range (µg/kg)	Conclusion
Urine CAP1	0.075	-1.301	4.255	0.003428	0.075 - 0.6	✓
	0.15	1.525	3.225	0.005309		
	0.6	1.701	3.027	0.02013		
Urine CAP3	0.075	-3.341	5.931	0.004967	0.075 - 0.6	✓
	0.15	0.4855	3.096	0.004978		
	0.6	0.6737	2.709	0.01745		

CAP1: 321.1/152

CAP3: 323.0/152

Note: Matrix effects were studiedon 24 different batches of pork and bovine urine. Chloramphenicol-D5 corrects matrix effects correctly.

11 References

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Annexe : Hazard statements

Analytes	Hazard symbols	Code	Hazard statements
Chloramphenicol		H302	Harmful if swallowed
		H312	Harmful in contact with skin
		H319	Causes severe eye irritation
		H332	Harmful by inhalation
		H225	Highly flammable liquid and vapour
Chloramphenicol-D_5		H302	Harmful if swallowed
		H312	Harmful in contact with skin
		H319	Causes severe eye irritation
		H332	Harmful by inhalation
		H225	Highly flammable liquid and vapour

Reagents	Hazard symbols	Code	Hazard statements
β-glucuronidase		H317	May cause skin allergy
		H334	May cause allergic or asthma symptoms or inhalation difficulties
Ethyl acetate		H225	Highly flammable liquid and vapour
		H319	Causes severe eye irritation
		H336	May cause drowsiness or dizziness
		EUH066	Repeated exposure may cause skin dryness or cracking.
Acetonitrile		H225	Highly flammable liquid and vapour
		H302	Harmful if swallowed
		H312	Harmful in contact with skin
		H319	Causes severe eye irritation
		H332	Harmful by inhalation
Methanol		H225	Highly flammable liquid and vapour
		H301	Toxic if swallowed
		H311	Toxic in contact with skin
		H331	Toxic by inhalation
		H370	Proven risk of serious organ damage
Ammonia solution 25%		H314	Causes skin burns and serious eye damage
		H335	May irritate the respiratory tract.
		H400	Very toxic to aquatic organisms.
Acetic acid 100%		H226	Flammable liquids and vapours
		H314	Causes skin burns and serious eye damage

Analytes and reagents present in the method and not noted in these tables are not hazardous.