Analytical method for food safety

REFERENCE : ANSES/LMV/06/01 v5

April 2024

Method for the detection and confirmatory quantification of chloramphenicol residues in matrices of biological origin 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	-	2006	Initial method
2	major	2007	Modification following initial validation
3	major	2012	Modified following additional validation and inclusion of honey matrix.
4	major	2023	Updated after validation has been completed in accordance with Implementing Regulation (EU) 2021/808
5	Minor	2024	Modification of the confirmation threshold in §9.2.1

Foreword

This method has been developed by:

ANSES – Fougères Laboratory

European Union and National Reference Laboratory for Veterinary Drug Residues

Address : 10B rue Claude Bourgelat – JAVENE

CS 40608

35306 FOUGERES Cedex (France)

Contacts : lnr-rmv@anses.fr

CHOTARD Marie-Pierre

LAGREE Marie Pierre

GAUGAIN Murielle

HURTAUD-PESSEL Dominique

VERDON Eric

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

Contents

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] and Delegated Regulation (EU) 2022/1644 of 7/07/2022 [2] on the control of certain substances and residues in live animals, animal products and animal feed.

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 food matrices of animal origin (muscle, milk, aquaculture products, eggs, honey, royal jelly, casings) and drinking water (see note in §10). The validated quantification according to the requirements of commission regulation (EU) 2021/808 covers range 0.1 and 0.4 µg/kg. The detection capacity of the method (CCβ) has been evaluated at 0.10 µg/kg and estimated to be less than or equal to 0.10 µg/kg. The CCα are as follows:

Milk-Muscle-Egg-Aquaculture products-Casings	CCα = 0.123 µg/kg
Honey-Royal jelly	CCα = 0.131 µg/kg
Drinking water	CCα = 0.104 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

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 (=SE0): Matrix blank quality control sample (with internal standard) serves to prove that no contaminations is present in matrix.

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

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

SE: calibration standard

TR: retention time

4 Principle of the method

The method involves the following steps

- Extraction of chloramphenicol from matrix in ethyl acetate and evaporation of the solvent,
- Purification of the extract by liquid/liquid partitioning
- 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 Ethyl acetate for analysis (Fisher E/0900/17) N°CAS : [141-78-6]

5.3.2 Isooctane for analysis (Supelco-Merck 1.04727) N°CAS : [540-84-1]

5.3.3 Ammonium acetate (Supelco-Merck 1.01116) N°CAS : [631-61-8]

5.3.4 Acetonitrile Optima LC/MS Grade (Fisher A955-212) N°CAS : [75-05-8]

5.3.5 Iso-Hexane (Fisher H/0411/PB17) N°CAS : [73513-42-5]

5.3.6 Ammonia solution 25% for analysis (Supelco-Merck 1.05432) N°CAS : [1336-21-6]

5.3.7 Fuming hydrochloric acid 37 % for analysis (Supelco-Merck 1.00317) N°CAS : [7647-01-0]

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 4.8 mol/L hydrochloric acid solution: take 10 mL of hydrochloric acid 37% (approximately 12N) in a 25 mL volumetric flask already half full of water. Make up to the mark with water.

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 C113S010ANEA	Acetonitrile	10 µg/mL	[56-75-7]
Chloramphenicol-D_5 (CAP-D_5)	Cluzeau 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 1 ng/mL of chloramphenicol : SS-CAP-1.***

In a 20 mL volumetric flask, add 200 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 2 ng/mL of chloramphenicol : SS-CAP-2.***

In a 20 mL volumetric flask, add 400 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 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.

5.5.3.5 ***Working solution at 4 ng/mL of chloramphenicol : SS-CAP-4.***

In a 20 mL volumetric flask, add 800 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.

5.5.3.6 ***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.

5.5.3.7 ***Working solution at 25 ng/mL of chloramphenicol-D_5 : SS-CAP-D_5-25.***

In a 20 mL volumetric flask, add 500 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

6.1.1 Grade A volumetric flasks.

6.1.2 Variable volume solvent dispensers (Socorex).

6.1.3 Nalgene 16 mL polypropylene centrifuge tube (VWR 525-2855) with stoppers (VWR 525-2934)

6.1.4 Disposable round-bottomed glass tube of volume ≥ 5 mL

6.1.5 Automatic laboratory pipettes and corresponding tips (Gilson-Biohit).

6.1.6 Mixer (Vortex type).

6.1.7 Rheax 2 type rotary shaker (Heidolph).

6.1.8 Precision laboratory balance (resolution ≤ 10 mg).

6.1.9 Refrigerated ultra-centrifuge, type Multifuge X1R (Thermo).

6.1.10 Refrigerated centrifuge, type GR 422 (Jouan).

6.1.11 Dry bath for evaporation under N2 (Stuart)

6.1.12 Polypropylene injection bottle of 650 µL (Interchim 963290)

6.1.13 20 mL polyethylene scintillation bottle, type Wheaton (986721)

6.1.14 Bulb pipettes, polypropylene Pastettes

6.1.15 2 mL Microtubes (Eppendorf 0030 120.094)

6.1.16 Filter 0.45 µm diameter 13 mm type Millex HV PVDF (Millipore) et 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 API4000 and 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

7.2.1 Muscle and aquaculture products samples

Samples must be stored in the freezer to at least -18°C until analysis. For analysis, thaw the sample, take a portion and grind it.

7.2.2 Milk samples

Samples must be stored in the freezer to at least -18°C until analysis. For analysis, thaw the sample. Shake well to homogenise the cream in the milk before sampling.

Note 1 : To reconstitute milk replacer, dilute 16 g of powder in 84 mL of hot water at 45°C (16 g solution: 100 mL). Keep at 45°C with stirring until the powder is completely diluted (approximately 2 minutes).

Note 2 : After reconstitution, milk replacer is assimilated to bovine milk.

7.2.3 Egg samples

Liquid egg is a homogeneous mixture of egg white and yolk. These samples are stored in the freezer toat least -18°C until analysis. For analysis, thaw the sample. Shake well to homogenise before sampling.

7.2.4 Honey and royal jelly samples

These samples are stored in a refrigerator at between +2°C and +8°C until analysis. For analysis, bring the sample to room temperature. For liquid honeys, homogenise well before taking the sample. For solid honeys, it is preferable to take a portion and heat for about 1 hour at around 45°C, then homogenise before taking the sample.

7.2.5 Casings samples

These samples are stored in a refrigerator at between +2°C and +8°C until analysis. For the analysis, take a portion of the sample of casings. These are rinsed by soaking for approximately 10 to 15 minutes in a beaker filled with ultra-pure water 3 times for dry casings and once for cured casings. Cut into small pieces with a scalpel or grind with dry ice. The dry ice crush can be stored in the freezer at at least -18°C. The vials should only be hermetically sealed after waiting about 24 hours to allow the carbon dioxide to escape.

7.2.6 Drinking water samples

These samples are stored in the freezer to at least -18°C until analysis. For analysis, thaw the sample. Shake well to homogenise before sampling.

Chloramphenicol in matrix is stable for at least 2 months (under the storage conditions mentioned).

8 Procedure

For a screening analysis: extraction of a QC-BR, a QC1-BM (=SE0), a QC2-CCβ (=SE1) and the samples to be analysed.

For a confirmatory analysis: extraction of a QC-BR, 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 blank control (SE0) and spiked samples at the first level of the range (SE1) are used as quality controls (QC1-BM and QC2-CCβ).

8.1 Procedure for biological matrices except drinking water

8.1.1 Preparation of sample prior to analysis

8.1.1.1 Weight 2 ± 0.04 g of sample into a Nalgene centrifuge tube.

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

8.1.1.3 Add 800 µL of water.

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

8.1.2 Preparation of quality control sample QC

8.1.2.1 Reagent blank quality control sample: **QC-BR**

- Take a Nalgene centrifuge tube, weight 2 ± 0.04 g of water and add 200 µL of CAP-D_5-5 and follow the procedure in 8.1.4.1 or 8.1.5.1 depending on the nature of the sample.

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

- Weight 2 ± 0.04 g of sample of the chloramphenicol free matrix concerned into a Nalgene centrifuge tube.
- Add 200 µL of SS-CAP-1.5 and 600 µL of water
- Mix with vortex and leave in contact for 10 minutes.
- These QC can be stored for a least 2 months to at least -18°C.
- At the time of analysis and before extraction, add 200 µL of SS-CAP-D_5-5 and follow the procedure in 8.1.4.1 or 8.1.5.1 depending on the nature of the sample.

8.1.3 Preparation of the range of spiked sample (SE) for confirmation (0; 0.1; 0.2; 0.3; 0.4 µg/kg)

8.1.3.1 Weight 2 ± 0.04 g of sample of the chloramphenicol free matrix concerned into a Nalgene centrifuge tube.

8.1.3.2 Spiking is done as follows:

		Add water	Add spiked solution	Add of SS-CAP-D_5-5
SE0	*Blank control (=QC1-BM)*	800 µL	/	200 µL
SE1	*Spiked at 0.1 µg/kg (=QC2-CC6)*	600 µL	200 µL of SS-CAP-1	200 µL
SE2	*Spiked at 0.2 µg/kg*	600 µL	200 µL of SS-CAP-2	200 µL
SE3	*Spiked at 0.3 µg/kg*	600 µL	200 µL of SS-CAP-3	200 µL
SE4	*Spiked at 0.4 µg/kg*	600 µL	200 µL of SS-CAP-4	200 µL

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

8.1.4 Procedure of extraction– purification for all matrices except milk

8.1.4.1 Add 6 mL of ethyl acetate, mix with vortex.

8.1.4.2 **For royal jelly only, add 200 µL of ammonia solution (5.3.6) then mix.**

8.1.4.3 **For liquid egg only, add 2 mL of iso-hexane.**

8.1.4.4 Cap the tubes and mix in the Heidolph 10 minutes at 100 tours/minute.

8.1.4.5 Centrifuge 5 minutes at 14000 g at approximately 4°C.

8.1.4.6 Using a pastette, recover approximately 5 mL of the upper organic phase**.**

8.1.4.7 Evaporate under nitrogen at around 40°C.

8.1.4.8 Dissolve the residue in 0.6 mL isooctane and mix with vortex.

8.1.4.9 Add 0.6 mL of water and shake slowly by hand.

8.1.4.10 Centrifuge 5 minutes at 3000 g.

8.1.4.11 Inject 50 µL of the lower phase**.**

8.1.5 Procedure of extraction– purification for milk

8.1.5.1 Add 100 µL of hydrochloric acid at 4.8 mol/L, mix.

8.1.5.2 Add 8 mL of ethyl acetate.

8.1.5.3 Cap the tubes and mix in the Heidolph 10 minutes **at minimum speed**.

8.1.5.4 Centrifuge 5 minutes at 14 000 g at approximately 4°C.

8.1.5.5 Continue extraction-purification as described in 8.1.4.6 to 8.1.4.11.

8.2 Procedure for drinking water

To prepare the calibration range and QCs, the laboratory's tap water is used as an equivalent to drinking water.

8.2.1 Preparation samples for analysis

Even if chloramphenicol was deliberately added to animal drinking water in the form of chloramphenicol palmitate, some of the chloramphenicol would be in free form in the water solution. Concentrations are likely to be high and, to avoid contamination problems, it is strongly recommended to inject intermediate dilutions of the sample beforehand. If no traces of chloramphenicol are detected, a direct injection can be carried out.

8.2.1.1 Preparation of intermediate dilutions for drinking water samples *:*

Dilution	Volume of sample to be taken	Flask
1/10000ème (dilution 1)	10 µL	100 mL
1/1000ème (dilution 2)	10 µL	10 mL
1/10ème (dilution 3)	1000 µL	10 mL

8.2.1.2 Remove 1 mL of sample (diluted or not) in a microtube of 2 mL, add 100 µL of SS-CAP-D_5-25 and 100 µL of water.

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

8.2.1.4 Continue in §8.2.4.

8.2.2 Preparation QC sample for drinking water

8.2.2.1 Reagent blank quality control sample: **QC-BR**

Note: The QC-BR is useless for drinking water because there is no reagent.

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

- Remove 1 mL of chloramphenicol free laboratory's tap water in a microtube of 2 mL.
- Add 100 µL of SS-CAP-1.5.
- Mix with vortex and leave in contact for 10 minutes.
- These QC can be stored for a least 2 months to at least -18°C.
- At the time of analysis and before centrifugation and filtration, add 100 µL of SS-CAP-D_5-25.

8.2.3 Preparation of the range of spiked drinking water sample (SE) for confirmation (0 ; 0.1 ; 0.2 ; 0.3 ; 0.4 µg/kg)

8.2.3.1 Remove 1 mL of chloramphenicol free laboratory's tap water in a microtube of 2 mL.

8.2.3.2 Spiking is done as follows:

		Add water	Add spiked solution	Add SS-CAP-D_5-25
SE0	*Blank control (=QC1-BM)*	100 µL	/	100 µL
SE1	*Spiked at 0.1 µg/kg (=QC2-CCß)*	/	100 µL of SS-CAP-1	100 µL
SE2	*Spiked at 0.2 µg/kg*	/	100 µL of SS-CAP-2	100 µL
SE3	*Spiked at 0.3 µg/kg*	/	100 µL of SS-CAP-3	100 µL
SE4	*Spiked at 0.4 µg/kg*	/	100 µL of SS-CAP-4	100 µL

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

8.2.3.4 Continue in §8.2.4.

8.2.4 Procedure to follow before injecting drinking water extracts

8.2.4.1 Centrifuge microtubes at 14 000g for 5 min at approximately 4°C.

8.2.4.2 Filter the extracts through 0.45 µm filters.

8.2.4.3 Inject 50 µl.

8.3 LC-MS/MS analysis and detection

8.3.1 Chromatography condition

8.3.1.1 Flow rate: 0.6 mL/min

8.3.1.2 Injection volume: 50 µL

8.3.1.3 Temperature in the column oven: 25°C

8.3.1.4 Auto sampler temperature: ambient

8.3.1.5 Elution gradient:

T (min)	Track A %	Track B %
0	80	20
2	40	60
5	40	60
6	80	20
8	80	20

A: 0.01 mol/L ammonium acetate solution

B: Acetonitrile Optima LC/MS Grade.

8.3.1.6 Valco switching valve: opening time 3 min to 7 min.

8.3.2 Mass spectrometry for API5500 or API4000 system

Ionisation: ESI (turbospray) in negative mode

Electrode height: 3 mm

Source temperature: 700°C

Curtain gas: 35 (API5500) ou 20 (API4000)

GS1: 50

GS2: 50

IS: - 4200V

CAD: 4

EP: -10

Type of scan: MRM (Multiple Reaction Monitoring)

Acquisition time: 8 min

8.3.3 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) API5500	Dwell time (msec) API4000	DP	CE	CXP	TR (min)
Chloramphenicol	1	321.1	152	200	300	-45	-24	-7	3.9
	2	321.1	257	200	300	-45	-16	-17	
	3	323.0	152	200	300	-55	-24	-9	
Chloramphenicol-D_5		326	157	200	300	-50	-28	-15	3.9

Notes :

- For honey, a co-extract (or interference) sometimes gives a peak for the 321.1/152 transition at the CAP retention time. In this case, we recommend using transitions 321.1/257 and 323.0/152.
- For royal jelly, a co-extract (or interference) sometimes gives a peak for transition 321.1/257 at the CAP retention time. In this case, it is recommended to use transitions 321.1/152 and 323.0/152.
- For casings, co-extraction (or interference) sometimes gives a peak for transition 321.1/257 at the CAP retention time. In this case, we recommend using transitions 321.1/152 and 323.0/152. In addition, the CAP-D_5 transition can be noisy. Increasing the CAP-D_5 concentration (for example using SS- CAP-D_5-25 instead of SS- CAP-D_5-5) can help to better integrate the CAP-D_5 peak.

8.3.4 Acquisition sequence

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

- Sample QC-BR
- Sample QC1-BM = SE0,
- Sample QC2-CCβ = SE1,
- Water injection,
- Extracts to be analysed,
- Sample QC2-CCβ = SE1 (possibly if large series)
- Water injection,
- Sample QC1-BM = SE0
- Sample QC2-CCβ = SE1

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

- Sample QC-BR
- Sample SE0 (=QC1-BM)
- Spiked calibration standard (SE1 à SE4),
- Water injection,
- Sample QC3-RPA
- Water injection,
- Extracts to be analysed,
- Sample QC3-RPA (possibly if large series)
- Water injection,
- Sample SE0 (=QC1-BM)
- Spiked calibration standard (SE1 à SE4)

The blank control sample (=SE0) and the first level of the range (=SE1) are also used as QC1-BM and QC2-CCβ samples to check the validity of the results.
The extracts can be stored for at least 4 days in the autosampler at room temperature and for at least 1 month 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 will be chosen as a priority for the different matrices. In some cases, there is co-extraction (or interference), see details in § 8.3.3 for choosing the 2 transitions best suited to the matrix.

9.1.1 Screening analysis

- QC-BR and QC1-BM (=SE0) are free of chloramphenicol.
- The presence of internal standard (IS) in each sample must be checked (QC sample and samples to be controlled).
- 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 and QC1-BM (=SE0) are free of chloramphenicol.
- The presence of internal standard (IS) in each sample must be checked (QC sample and samples to be controlled).
- 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 retention time of chloramphenicol in 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 relative retention 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 of Regulation 2021/080 (point 1.2.4.1).

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. In some cases for honey, there is interference on the 321.1/152 transition (see §8.3.3), in which case one of the other 2 transitions 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).

A linear regression model was chosen for chloramphenicol. The levels of the calibration spiked samples are : 0 – 0.1 – 0.2 – 0.3 and 0.4 µg/kg

$$y=ax+b$$

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. For drinking water, the unit µg/kg is assumed to be equivalent to µg/L. 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 concentration is above or equal to the CCα decision limit.

Milk-Muscle-Egg-Aquaculture products-Casings	CCα = 0.123 µg/kg
Honey-Royal jelly	CCα = 0.131 µg/kg
Drinking water	CCα = 0.104 µg/kg

Additional Information :

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

- If the concentration of the sample is above 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 above 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
Milk-Muscle-Egg-Aquaculture products	none	✓	✓	CAP1/CAP2 :✓
Casings	CAP2	✓	✓	CAP1/CAP3 :✓ SV 0.1 µg/kg - CAP1/CAP2 : ✕
Honey-Royal jelly	Honey : CAP1 Jelly : CAP2	✓	✓	CAP1/CAP2 :✓ CAP1/CAP3 :✓ CAP2/CAP3 :✓
Drinking water	none	✓	✓	CAP1/CAP2 :✓

* interference observed systematically or on some batches

CAP1 : 321.1/152

CAP2 : 321.1/257

CAP3 : 323.0/152

For casings, an interfering peak at transition 321.1/257 was observed on at least one batch of casings, resulting in non-compliance of the relative ionic intensity with the validation standard of 0.1 µg/kg.

For honey and royal jelly, the interfering peaks observed had no impact on the relative ionic intensity, but it is recommended that the transitions be chosen without interferers.

The detection capacity of the method (CCβ) was evaluated according to method 2 of regulation 2021/808 [6] (§2.7) at 0.10 µg/kg and estimated to be less than or equal to 0.10 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
Milk-Muscle-Egg-Aquaculture products-Casings CAP1	0.1	5.9	9.52	0.009791	0.1 - 0.4	✓
	0.15	-0.58	4.90	0.007759		
	0.2	-0.82	8.12	0.01918		
	0.4	1.78	4.63	0.01905		
Honey-Royal jelly CAP1	0.1	-6.4	12.6	0.01346	0.1 - 0.4	✓
	0.15	3.3	ND	ND		
	0.4	3.8	7.54	0.03213		
Honey-Royal jelly CAP2	0.1	1.3	12.2	0.01277		
	0.15	7.4	ND	ND		
	0.4	4.5	11.5	0.04926		
Honey-Royal jelly CAP3	0.1	-3.7	7.90	0.008065		
	0.15	1.05	ND	ND		
	0.4	3.7	6.17	0,02643		
Drinking water CAP1	0.1	-4.7	1.74	0.001811	0.1 - 0.4	✓
	0.15	0.44	2.81	0.004556		
	0.4	0.86	2.93	0.01255		

CAP1 : 321.1/152
CAP2 : 321.1/257
CAP3 : 323.0/152

Note : The study of matrix effects was carried out on 59 batches of different matrices:

- chicken, turkey, pork, bovine, caprine, ovine muscles
- bovine, ovine, caprine milk,
- chicken and quail eggs,
- aquaculture products: trout, sea bream, sea bass, salmon, prawns,
- spring, all-flower, heather, mountain and chestnut honeys, royal jelly,
- cured or dried pork and sheep casings,
- drinking water.

Chloramphenicol-D5 corrects matrix effects correctly. The diversity of matrices/species tested in this study shows that the method is generally applicable to biological matrices (except urine) and therefore potentially to other species not tested.

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
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
Isooctane		H225	Highly flammable liquid and vapour
		H304	May be fatal if swallowed or enters the respiratory tract.
		H315	Causes skin irritation
		H336	May cause drowsiness or dizziness
		H410	Toxic to aquatic organisms, causes long-term adverse effects.
Iso-hexane		H225	Highly flammable liquid and vapour
		H304	May be fatal if swallowed or enters the respiratory tract.
		H315	Causes skin irritation
		H336	May cause drowsiness or dizziness
		H411	Toxic to aquatic organisms, causes long-term adverse effects.
Ammonia solution 25%		H314	Causes skin burns and serious eye damage
		H335	May irritate the respiratory tract.
		H400	Very toxic to aquatic organisms.
Hydrochloric acid 37 %		H290	May be corrosive to metals.
		H314	Causes skin burns and serious eye damage.
		H335	May irritate the respiratory tract.

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