CHLORAMPHENICOL ELISA

5091CAP[23]04.20

A competitive enzyme immunoassay for screening and quantitative analysis of Chloramphenicol in various matrices

EUROPROXIMA CHLORAMPHENICOL ELISA

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BRIEF INFORMATION

The Chloramphenicol (CAP) ELISA is a competitive enzyme immunoassay for the screening in various matrices on the presence of this broad spectrum antibiotic.
With this ELISA kit 96 analyses can be performed. Samples and standards are measured in duplicate. The kit contains all the reagents, including standards, required to perform the test.

1. INTRODUCTION

The use of the broad-spectrum antibiotic chloramphenicol (CAP) was totally banned within the European Union by modification of Annex IV of council Regulation (EEC) 2377/90 with Commission Regulation 1430/94. In 2003, a minimum required performance limit (MRPL) for CAP residues in food of animal origin was set at 0.3 µg/kg.
This CAP-ELISA is suitable for the detection of CAP and its glucuronide (major metabolite urine). CAP can be detected in urine directly (after a dilution in buffer), or after ethyl acetate extraction for increased sensitivity and specificity. Defatted milk samples can also be applied to the test directly with a detection limit of 0.1 ng/ml. This detection limit can be lowered ten times using an extraction in ethyl acetate. For eggs and tissue samples, including shrimps, crab and fish, the ethyl acetate extraction is followed by a clean-up step using a mixture of iso-octane/ trichloromethane or n-hexane resulting in detection limits down to levels of 0.04-0.02 ng/g.

2. PRINCIPLE OF THE CHLORAMPHENICOL ELISA

The microtiter based ELISA kit consists of 12 strips, each 8 wells, precoated with sheep antibodies to rabbit IgG. A specific antibody (rabbit anti-CAP), enzyme labelled CAP (enzyme conjugate) and CAP standard or sample are added to the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilised antibodies and at the same time free CAP (present in the standard solution or sample) and enzyme conjugated CAP compete for the CAP antibody binding sites (competitive enzyme immunoassay). After an incubation time of 1 hour, the non-bound (enzyme labelled) reagents are removed in a washing step.
The amount of CAP enzyme conjugate is visualised by the addition of a chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme conjugate transforms the colourless chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the CAP concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The CAP-ELISA utilizes a specific antibody raised in rabbits against protein conjugated CAP. The reactivity pattern of this antibody is:

Cross- reactions:	Chloramphenicol	:	100%
	Chloramphenicol-glucuronide	:	65%
	Chloramphenicol-base	:	< 1%
	Thiamphenicol	:	< 1%
	Florfenicol	:	< 1%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	**Procedure**	**LOD ppb**
Urine	8.1.1	0.1
Milk	8.2.1	0.1
Urine	8.1.2	0.01
Milk	8.2.2	0.01
Egg	8.3	0.02
Tissue	8.4	0.02
Liver	8.4	0.01
Honey	8.6	0.02
Feed	8.7	0.1

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

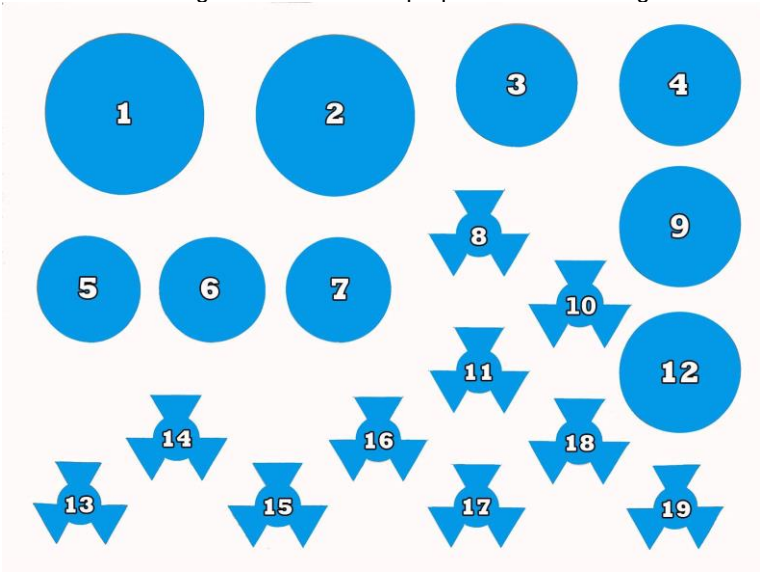
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

1 sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Ready to use, do not wash.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Sample dilution buffer** (20 ml, 4x concentrated)
2 **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, Ready to use)
4. **Stop solution** (15 ml, Ready to use)
5. **Conjugate** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. Not in use
8. **Standard solution 1** (1 ml, Ready to use) **100 ng/ml**
9. **Reconstitution/zero standard buffer** (10 ml, Ready to use)
10. Not in use.
11. Not in use
12. Not in use.
13. Not in use.
14. **Standard solution 2** (1 ml, Ready to use) **0.025 ng/ml**
15. **Standard solution 3** (1 ml, Ready to use) **0.05 ng/ml**
16. **Standard solution 4** (1 ml, Ready to use) **0.1 ng/ml**
17. **Standard solution 5** (1 ml, Ready to use) **0.2 ng/ml**
18. **Standard solution 6** (1 ml, Ready to use) **0.5 ng/ml**
19. **Standard solution 7** (1 ml, Ready to use) **2 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Water bath (37ºC)
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 10 – 100 µl
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Centrifuge (4000 x g)
- Rotation mixer
- Glass or polypropylene tubes 4 ml
- Glass tube 10 ml
- 15 ml tubes polypropylene
- Evaporation equipment
- Helix pomatia juice
- Methanol 100%
- Ethylacetate
- Hexane
- Iso-octane
- Trichloromethane
- Distilled water
- Columns, NH2 500 mg, 3 ml
- Tertiaire butylmethylether (TBME)
- Acetic acid 1M

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE PREPARATION

8.1 Urine samples

Urine samples can be analysed after a 5 times dilution step in buffer. A lower detection limit can be obtained using the extraction method. Especially for “dark/dirty” urine’s this extraction method is advised.

8.1.1 Direct method

Add 800 µl of sample dilution buffer to 200 µl of the urine sample and mix. Centrifuge the mixtures for 10 min at 2000 g and use 50 µl portions of the supernatant for the test. Adjust the pH , if necessary, to pH 7 (± 0.5).

8.1.2 Extraction method

Add to 1 ml urine a few drops of 1 M acetic acid to adjust pH to 4.8. Mix and add 25 µl Helix pomatia juice and incubate overnight at 37°C or alternatively for 2 hours at 55°C. Cool to room temperature and adjust pH to 7 ± 0.5. Add 2 ml of ethyl acetate, mix for 1 minute. Wait for 5-10 min. to allow phase separation and evaporate 1 ml of the upper layer (ethyl acetate) at 50°C under a stream of nitrogen. Dissolve the residue in 200 µl of sample dilution buffer. Use 50 µl portions for the test.

8.2 Milk samples

Milk samples can be analysed directly. To skimmed milk powder water has to be added to obtain 'milk'. E.g. to 10 g of skimmed milk powder an amount of 60 ml of distilled water is added.

A ten times lower detection limit can be obtained using an extraction method (paragraph 8.2.2)

8.2.1 Direct method

For undiluted defatted milk samples the matrix effect is a critical factor. To avoid fat residues in the sample, the manual should be strictly followed. The pH of the sample is another potential pitfall. Sour milk disturbs the ELISA, neutralisation of the pH is essential.

Centrifuge cold milk samples for 15 min. at 2000xg and at 4°C. Remove the upper fat layer using a spatula. Use 50 µl portions for the test.

8.2.2 Extraction method

Defat 5 ml of milk as described at 8.2.1. Pipette 2.5 ml of defatted milk in a glass tube, add 5 ml of ethyl acetate and mix carefully head over head for 1 min. (when shaking is too rigorous, the extract becomes jelly). Wait for 5-10 min. to allow phase separation, transfer 4 ml of the upper ethyl acetate layer into a clean tube. Evaporate the 4 ml ethyl acetate at 50°C under a mild stream of nitrogen. Dissolve the residue in 200 µl of sample dilution buffer. Use 50 µl portions for the test.

8.3 Egg samples

Transfer 1 g of the homogenised whole egg, the white or yolk in a test tube, add 6 ml of ethyl acetate and mix carefully (Vortex) for 1 min. When shaking too rigorous, the liquid becomes jelly. After centrifugation (10 min. at 2000xg), 3 ml of the ethyl acetate layer is pipetted into a glass tube and the ethyl acetate is evaporated at 50°C under a mild stream of nitrogen. The fatty residue is dissolved in 1 ml of iso-octane/trichloromethane (2:3; v/v) and 0.5 ml of sample dilution buffer is added. The whole is mixed (Vortex) for 1 min. and centrifuged (10 min. at 2000 g). Remark: In case of an emulsion in the upper layer, the test tube is shortly (about 5 min.) put into a water bath (80°C) and centrifuged again.
50 µl of the upper layer (1g of whole egg, white or yolk/per ml of sample dilution buffer) are pipetted into the test.

N.B. Instead of iso-octane/trichloromethane, n-hexane can be used. When iso-octane/trichloromethane is used, pipette 50 µl of the upperlayer, when n-hexane is used, take 50 µl of the layer underneath

8.4 Tissue samples (meat, liver, shrimp, crab, fish)

8.4.1 Extraction in ethyl acetate and clean up

Homogenise approximately 10 g of tissue. Weight 3 g of the homogenised tissue sample and transfer into a glass tube. Add 6 ml of ethyl acetate and mix (head over head) for 10 min. After centrifugation (10 min., 2000 g), 4 ml of the ethyl acetate is pipetted into a glass tube and the ethyl acetate is evaporated at 50°C under a mild stream of nitrogen.

The fatty residue is dissolved in 1 ml of iso-octane/trichloromethane (2:3; v/v) and 1.0 ml of sample dilution buffer (see chapter 9) is added.

The whole is mixed (Vortex) for 1 min. and centrifuged (10 min. at 2000 g).

Remark: In case of an emulsion in the upper layer, the test tube is shortly (about 5 min.) put into a water bath (80°C) and centrifuged again. 50 µl portions of the upper layer are pipetted into the test.

N.B. Instead of iso-octane/trichloromethane, n-hexane can be used. When iso-octane/trichloromethane is used, pipette 50 µl of the upper layer, when n-hexane is used, take 50 µl of the layer underneath.
A better recovery is obtained when iso-octane/trichloromethane is used instead of n-hexane.

8.4.2 Alternative method for shrimps, crab and fish

Homogenise approximately 50 g of shrimps, crab or fish in a blender. Weight 10 g of homogenised sample and transfer into a glass tube. Add 20 ml of ethyl acetate and mix (head over head) for 10 min. Centrifuge (10 min., 2000xg) or filtrate through a paper disk. Transfer 10 ml of the ethyl acetate layer into a clean glass tube, evaporate the ethyl acetate until dryness using a mild stream of nitrogen.

Dissolve the dry residue in 2 ml of iso-octane/trichloromethane (2:3; v/v) (or 2 ml of n-hexane, see N.B. paragraph 8.4.1) and add 1 ml of sample dilution buffer and mix thoroughly. In case of an emulsion, the tube is heated (5 minutes) in a water bath (80ºC). Centrifuge the solution for 10 min. at 2000xg.

Using a Pasteur pipette, transfer the clear upper layer (or in case of n-hexane, the clear underneath layer) in a clean glass tube.

Add another 1 ml of iso-octane/trichloromethane (2:3; v/v) (or 1 ml of hexane).

Mix the solution thoroughly and centrifuge as described above.

Pipette 50 µl portions of the upper layer (or in case of n-hexane of the layer underneath) for the test.

8.5 Serum/plasma samples

Add to 1 ml serum/plasma 2 ml of ethyl acetate and mix for 1 minute. Wait for 5-10 minutes to allow phase separation, or centrifuge (10 min., 2000xg). Evaporate 1 ml of the upper layer (ethyl acetate) at 50°C under a mild stream of nitrogen. Dissolve the residue in 500 µl of sample dilution buffer. Use 50 µl portions for the test.

8.6 Honey samples

Weigh in 3 g of honey and transfer into a glass tube. Add 3 ml of distilled water and mix. Add 6 ml of ethyl acetate and mix (head over head) for 10 min. After centrifugation (10 min., 2000xg), pipette 4 ml of ethyl acetate in a glass tube. Evaporate at 50°C under a mild stream of nitrogen. The residue is dissolved in 1 ml of sample dilution buffer. Use 50 µl portions in the ELISA.

N.B. Non purified honey tends to be fatty. When after evaporation a fatty residue is obtained, dissolve the residue in 1 ml of iso-octane/trichloromethane (2:3; v/v) (or in 1.0 ml of n-hexane, see N.B. paragraph 8.4.1), add 1.0 ml of sample dilution buffer and centrifuge (10 min. 2000xg). An aliquot of 50 µl of the dilution buffer (upper layer in case of iso-octane/trichloromethane and the layer underneath in case of n-hexane) is used in the ELISA.

8.7 Feed samples

Grind 10 to 100 g of feed samples.
Homogenise 5 g of the grinded feed in 20 ml of distilled water.
Pipette 5 ml of this mixture into a glass tube.
Add 10 ml of ethyl acetate and mix (head over head) during 30 minutes.
Centrifuge for 10 min at 2000xg.
Pipette 5 ml of ethyl acetate (upper layer) into a glass tube and evaporate at 50ºC under a mild stream of nitrogen.The fatty residue is dissolved in 0.5 ml of iso-octane/trichloromethane (2:3; v/v) and 0.5 ml of sample dilution buffer is added.
The whole is mixed (Vortex) for 1 min. and centrifuged (10 min. at 2000 g).
An aliquot of 50 µl of the upper layer is used in the ELISA test.

Remark: In case of an emulsion in the upper layer, the test tube is shortly (about 5 min.) put into a water bath (80°C) and centrifuged again. 50 µl portions of the upper layer are pipetted into the test.

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature.

Keep standard solutions in the dark and store at +2°C to +8°C.

Microtiter plate
Return unused strips into zip lock bag with desiccant and store at +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution
The substrate solution (ready to use) precipitates at 4°C. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.

Conjugate solution
Reconstitute the vial of lyophilised conjugate (CAP-HRPO) with 4 ml of reconstitution/zero standard buffer, mix thoroughly and keep in the dark until use.

Antibody solution
Reconstitute the vial of lyophilised antibodies with 4 ml of reconstitution/ zero standard buffer, mix thoroughly and keep in the dark until use.

Sample dilution buffer
The sample dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should be at room temperature and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water. The 4 times diluted buffer can be stored in a refrigerator (+2°C to +8°C) until the expiry date stated on the kit label.

Standard solution (100 ng/ml)
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng CAP per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 2, 0.5, 0.2, 0.1, 0.05, 0.025 ng/ml (Optionally: and 0.0125 ng/ml). Also the zero standard should be of the same matrix.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, un-bound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results. Basically, manual rinsing or rinsing with automatic plate wash equipment can be done as follows:

Manual rinsing

1. Empty the contents of each well by turning plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.
   Microtiter plate is ready to use, do not wash.

2. Pipette 100 µl of reconstitution/zero standard buffer in duplicate (well H1, H2).
   Pipette 50 µl of reconstitution/zero standard buffer in duplicate (well A1, A2)
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2 i.e. 0.025, 0.05, 0.1, 0.2, 0.5 and 2 ng/ml).
   Pipette 50 µl of each sample solution in duplicate into the remaining wells of the

microtiter plate.

3. Add 25 µl of conjugate (CAP-HRPO) into all wells, except wells H1 and H2
4. Add 25 µl of antibody solution into all wells, except wells H1 and H2.
5. Seal the microtiter plate and shake the plate for 1 min.
6. Incubate for 1 hour in the dark at 4°C (2°C to 8°C).
7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipette 100 µl of substrate solution into each well. Incubate 30 min. at room temperature (+20°C to + 25°C).
9. Add 100 µl of stop solution into each well.
10. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) value of the blank wells H1 and H2 from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (wells A1 and A2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard}} \times 100 = \text{percentage of maximal absorbance}$$

Calibration curve:
The values (% of maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the CAP equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.025 - 2 ng/ml range.

Alternative for calibration curve:
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the CAP equivalent concentration on a logarithmic X-axis.

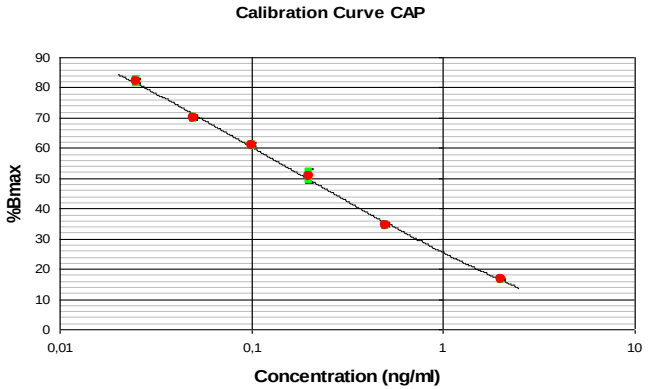


Figure 1: Example of a calibration curve

Urine samples
Using the direct method; the amount of CAP in the urine samples is expressed as CAP equivalent (ng/ml). The CAP equivalents in the urine (ng/ml) corresponding to the % of maximal absorbance of each sample can be read from the calibration curve. These calculated CAP equivalents have to be multiplied by 5 to obtain the CAP equivalents (ng/ml) in the urine.
Using the extraction method; urine samples treated with glucuronidase and extracted (0,5 ml urine/200 µl buffer) are 2.5 times concentrated and the concentration read from the calibration curve has to be divided by 2.5.

Milk samples
Using the direct method and undiluted milk; the CAP equivalents in the milk (ng/ml) can be read directly from the calibration curve (factor 1).
Using the extraction method, milk samples are ten times concentrated and the concentrations calculated from the calibration curve have to be divided by 10.

Milk powder samples
Using the method as described in paragraph 8.2 of this manual (10 g of powder and 60 ml of water), and using the extraction method, the CAP equivalents, as read from the standard curve, have to be multiplied by a factor 0.7 to obtain the results in ng CAP per g of milk powder.
Using the direct method, the CAP equivalents, as read from the standard curve, have to be multiplied by 7 (i.e. 10 g of powder and 60 ml of water).

Egg samples
The CAP equivalents in the whole egg, white or yolk (ng/g) can be read directly from the calibration curve (factor 1).

Tissue samples (meat, shrimps, crab, fish), method 8.4.1
Using the extraction in ethyl acetate followed by the clean up, the CAP equivalents calculated from the calibration curve have to be divided by 2 to express the concentration (ng/g) in tissue.

Shrimps, crab and fish (alternative method 8.4.2)
The CAP equivalent read from the calibration curve has to be divided by a factor 5 to obtain ng/g shrimps, crab or fish.

Serum/plasma samples
Using the extraction method serum/plasma samples, the CAP equivalents can be read directly from the calibration curve.

Honey samples
To calculate the CAP concentration in the sample the CAP equivalent read from the calibration curve is divided by a factor 2.

Feed samples
The CAP equivalents can directly be read from the calibration curve.

12. LITERATURE

Council Regulation (EEC) No. 2377/90 (26 June 1990) Off. J. Eur. Comm. L224/1.
Commission Regulation (EC) No. 1430/94 (22 June 1994) Off. J. Eur. Comm. L156/6.
Commission Decision 2003/181 (13 March 2003) Off. J. Eur. Union L71/17.
Cazemier, G. Haasnoot, W. and Stouten, P. Screening of chloramphenicol in urine, tissue, milk and eggs in consquence of the prohibitive regulation. Proceedings Euroresidue III 1996, Eds. N. Haagsma and A. Ruiter, pg. 315.
P. Maris and V. Gaudin. Report: Proficiency study for the analysis of chloramphenicol residues in milk by ELISA. AFSSA Fougères, January 2002. Available on request.

13. ORDERING INFORMATION

For ordering the Chloramphenicol ELISA kit please use cat. code 5091CAP.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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