Laboratory of Fougères

Unit: AB2R

National Reference Laboratory

EURL for Antimicrobial and Dye Residue Control in Food-Producing Animals

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Analytical method characterisation and validation report

5 ELISA kits from different suppliers for the detection of Chloramphenicol (CAP) residues in meat and aquaculture products

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Contents

Report summary

This study covers the evaluation of performance characteristics and the validation of five commercial ELISA kits for the detection of a prohibited antimicrobial, chloramphenicol (CAP), in muscle tissue and aquaculture products. CAP is banned in European Union since 1994, but is still authorised in some countries across the world. In 2019, the European Union set a new Reference Point for Action (RPA), decreasing the acceptable limit of CAP in animal tissues for human consumption from 0.30 µg/kg to 0.15 µg/kg. The validation was performed in line with the European Regulation (EU) 2021/808 and according to the European Guideline for validation of screening methods (2023). The detection capabilities CCβ were all estimated below the RPA, but were 3 to 15 times higher than the commercially announced limits of detection (LOD). False negative rates and false positive rates were satisfactory for all the kits (≤ 5%). All of them were found out to be applicable to aquaculture products and meat at a common CCβ.

Context of application

Use of chloramphenicol in animal production and aquaculture

Chloramphenicol (CAP) is a broad-spectrum antibiotic. It was widely used in animal production due to its low cost and its large spectrum. However, many reports indicate the toxic effect of CAP on human health, as bone marrow suppression, aplastic anaemia, medullar aplasia (EFSA Journal, 2014). This toxicity induced the ban of CAP in European Union in 1994 (CR 1430/94, 1994), followed by other countries across the world, in order to prevent presence of CAP residues in food of animal origin. However, this substance is still in use sometimes in veterinary medicine due to its commercial availability, its low cost and its efficient antibacterial effect in Gram - and Gram + bacteria (Rimkus et al., 2020).

Regulation

In 2002, a European Commission Decision stated the use of minimum required limits (MRPL) as the “minimum content of an analyte in a sample, which at least has to be detected and confirmed. It is intended to harmonise the analytical performance of methods for substances for which no permitted limited has been established” (2002/657/EC, 2002) for unauthorized substances, including CAP. Later, the Commission Regulation (EU) 2019/1871 (CR 2019/1871, 2019) stated a lower reference point for action (RPA) for CAP, i.e. 0.15 µg/kg instead of 0.3 µg/kg.

Existing analytical methods

The European Regulation 2021/808 defines a screening method as “a method that is used for screening of a substance or class of substances at the level of interest”, whereas a confirmatory method, in the case of unauthorized substances as CAP, is explained as “a method that provides full or complementary information enabling the substance to be unequivocally identified and if necessary quantified […] at the reference points for action (RPA) for prohibited or unauthorised substances, for which a RPA is established”. Thus, screening and confirmatory methods are complementary and can be used altogether in a 2 step analytical strategy (Impens et al., 2003; Jester et al., 2016; Tajik et al., 2010). In fact, screening a non-compliant result requires the use of a

confirmatory method (e.g. LC-MS/MS). As a result, a lot of studies reported the use of different analytical methods, either by physico-chemical analysis (i.e. HPLC-MS/MS) (Jung et al., 2022; McEneff et al., 2013), or by biological immunoassay (Guo et al., 2015; Rimkus et al., 2020; Scortichini et al., 2005; Zhou et al., 2014) or, more recently, by biosensors (Sun et al., 2022) with their own strengths and weaknesses.

Physico-chemical analysis by high-performance liquid chromatography coupled with a mass spectrometer or a tandem mass spectrometer (HPLC-MS; HPLC-MS/MS) are the most commonly used methods, especially for banned substances, due to their sensitivity with low limit of detection (LOD) and limit of quantification (LOQ), to their precision and accuracy of measurement, and in accordance with the RPA. However, they are expensive and need more advanced equipment and well qualified staff. In another way, ELISA kits are very specific to a target substance and can be faster and cheaper screening methods than instrumental methods.

Moreover, ELISA kits are very specific of each substances. And for instance one kit is necessary for each of the 4 major nitrofuran metabolites, and the same for some of the mostly used dyes. Regarding dyes, the most common kits commercially available are detecting Malachite Green (MG) and its leucobase (LMG) and only one kit has been marketed for Crystal Violet (CV). Currently, there is no ELISA kits for the other dyes on the market like Briliant Green or Methylene Blue. In the other hand, there are several kits commercially available for the detection on CAP in feed, food and animal matrices.

Previous evaluations of ELISA kits for chloramphenicol in the laboratory

Several evaluations had been carried out on CAP detection ELISA kits between 2001 and 2007 in the laboratory of Fougères:

- In 2001, 4 ELISA kits marketed by 4 different suppliers (Eurodiagnostica, Netherlands; Randox, UK; R-Biopharm (Ridascreen), Deutschland; Riedel, Deutschland), respectively, were compared to detect CAP in milk. In 2003, the ELISA kit Eurodiagnostica (Abcys) (Netherlands) had been tested on honey and shrimps. However, those previous evaluations are too old to be considered because they had been managed before the implementation of the European Guidance of the validation of the screening test (Crl 2010).

- In 2001-2002, the EURL organised an inter-laboratory proficiency test partly dedicated to these screening methods. Participants had to use the ELISA kit of their choice for CAP screening in milk and in muscle tissues (Gaudin et al. 2003). Matrices had a spiked concentration of CAP between 0.5 and 5.0 µg/kg for milk and between 2.1 and 6.5 µg/kg for muscle. Those concentrations were higher than the actual target amount.

- In 2007, three ELISA kits from three different suppliers (Eurodiagnostica, Netherlands (now Europroxima), R-Biopharm (RIDASCREEN), Deutschland and Transia, France) were evaluated for CAP screening in honey. Among those 3 ELISA kits, the most efficient was Transia kit (CCβ ≤ MMPR = 0.3 µg/kg). The false-positive rate was 15% and false negative rate was at 0% when the screening target was fixed at 0.75 (absorbance unit). CCβ of the Eurodiagnostica kit was also ≤ 0.3 µg/kg. In fact, it was the very same kit, produced by two different manufacturers (Transia and Eurodiagnostica), with different brand names. However, the results were non-compliant with the Ridascreen (R-biopharm) kit. We assume that there has been a problem with the stability of the conjugate but haven't had any feedback from the supplier or from the manufacturer of this kit.

No other recent evaluation were carried out at the EURL on ELISA kits for the screening of CAP in muscle tissues from different species and aquaculture products.

Objectives of the project

The current regulation 2019/1871 (CR 2019/1871, 2019) now requires to control unauthorised substances at lower reference points for action (RPA). Those values are applicable since November 28, 2022. According to the evaluation of the 2020's national residue control plans (NRMP2020), 21 out of 30 members of the European Economic Area used ELISA kits for the screening of CAP. Main sampled matrices were muscles, milk, eggs and honey. From the past two years, many National Reference Laboratories (NRL) from our European network were asking for an evaluation of the ELISA kits capabilities regarding the new RPAs.

Consequently, this project aimed at evaluating the performance of ELISA kits for the screening of CAP in meat and aquaculture products at and below the new RPA (CR 2019/1871, 2019). It will allow to assess the relevance of ELISA kits for the official controls in the European Union.

For that, a survey of the existing ELISA kits available on the market for the detection of CAP was implemented and a synthetic comparison of the announced performances by manufacturers was investigated in line with the new target values (RPA = 0.15 µg/kg). The most relevant kits were chosen in terms of both their claimed analytical performances and their market sales. Then an evaluation of the performances of the collected ELISA kits in aquaculture products and in meat was performed at a first stage prior to the in-lab validation of the kit performance itself at a second stage. The validation was led in line with the European regulation (EU) 2021/808 (CIR (EU) 2021/808, 2021) and according to the new European guidance for validation of the screening methods (*EURL Guidance Document on Screening Method Validation*, 2023). The decision to meet the target concentrations was evaluated according to both the RPA and the kit sensitivity, expressed by the manufacturer.

1 Method for characterisation/validation

a. Origin of the method

Each kit is dedicated to the screening of CAP. The kits are produced and sold by 5 different manufacturers / suppliers:

- Biorex Food Diagnostics (Antrim, United Kingdom),
- Europroxima (Arnhem, The Netherlands)
- Perkin Elmer (Austin, USA)
- Randox (County Antim, United Kingdom)
- R-Biopharm (Saint-Didier-Au-Mont-D'Or, France).

b. Principle of the method

All the kits of this study use the same principle of competitive ELISA methods. In short, an analyte-specific antibody is coated to the surface of each well of a microtitre plate (commonly 96 wells). Standards and samples are added in the wells, as well as analyte-conjugated horseradish peroxidase (HRP). If the analyte is present in the sample, it binds to the coated antibodies, in

competition with the analyte-conjugated to HRP. Then, the plate is washed many times to eliminate unbound components in the wells. A chromogen product (TMB Substrate) is added before a stop solution, prior to the photometrical measurement at 450 nm. The concentration of the target substance is inversely proportional to the colour intensity (optical density (OD)) of the test sample.

c. Exact method reference or detailed description, indication of the critical steps or points (full operating procedure in appendix).

Antibiotic standards

Antibiotic standard of CAP (CAS #: 56-75-7) was purchased from Cluzeau Info Labo SARL (Sainte-Foy-La-Grande, France).

Samples

The matrices used for evaluation and validation of the kits were shrimps, fish (fat-fish and low-fat-fish species) and muscles from different species (ovine, bovine, porcine, poultry and turkey) and from different origins, bought in local supermarkets. Before use, matrices were properly prepared, homogenized then aliquoted into clean tubes, grouped by batches and frozen (-20°C). The day before analysis, sample were thawed in a refrigerator set at + 5°C ± 3°C.

ELISA kits

The references of the kits were:

- CAP Fast ELISA (BXEFB03A) (Biorex Food Diagnostics),
- CAP Fast ELISA (5091CAPF) (manufactured by Europroxima, commercialized by R-Biopharm),
- MaxSignal® CAP ELISA Kit (FOOD-1013-02F) (Perkin Elmer),
- CAP Fast ELISA (CN10171) (Randox),
- RIDASCREEN® CAP (R1511) (R-Biopharm).

Data analysis

The intensity of the colour was measured photometrically at 450 nm (and 630 nm when the protocol advised it to reduce background noise (dual wavelength reading)). The absorbance of the sample (optical density OD) was inversely proportional to the CAP concentration present in the sample.

Each day of analysis, the mean, the standard deviation (SD) and the coefficient of variation (CV %) were calculated for a set of identical samples (blank or spiked samples). Then, a statistical approach, which took into account the β error of 5%, was chosen as it was recommended in the European guideline for the validation of screening methods (*EURL Guidance Document on Screening Method Validation*, 2023).

The positivity Threshold (T) and the Cut-Off value (Fm) were calculated as follows:

$$T = B - 1.64 * SD_B \quad (1)$$

B is the mean and SD_B the standard deviation of the signal of the blank samples.

$$Fm = M + 1.64 * SD \quad (2)$$

M is the mean and SD the standard deviation of the signal of the spiked samples.

The assay was considered valid only if T was higher than Fm. When the signal (optical density (OD)) was lower than the cut-off value Fm, the sample was declared positive. When the signal was higher than the cut-off value Fm, the sample was declared negative.

d. Possible optimisation / development actions carried out upstream

The protocols from each kit were strictly followed in order to be as reproducible as possible with the suppliers.

2 Validation area and target values of performance characteristics

According to the European Regulation 2021/808, (CIR (EU) 2021/808, 2021), the parameters that allow to evaluate the performance of semi-quantitative screening methods like ELISA Kits are the detection capability (CCβ), the selectivity/specificity and the stability and robustness.

CCβ is defined as the smallest content in analyte that can be detected or quantified in a sample, with an error probability of β (here, β=5%). It needs to be lower or equal to the new RPA (i.e. 0.15 µg/kg for CAP).

Selectivity/specificity parameters are checked by assessing the false positive rate and the cross-reactivity. No regulatory criteria are stated for the false positive rate but it is intended to be as low as possible in order to avoid unnecessary confirmatory analysis. The cross-reactivity is determined by a rate between Main Analyte (MA) and Potentially Interfering substances (PI).

According to the European Guideline for validation of screening methods, “The applicability of a newly developed screening method to different matrices (and/or different animal species) should be demonstrated by the determination of specificity and detection capability CCβ for these different matrices.” (*EURL Guidance Document on Screening Method Validation*, 2023). As well, “A common specificity and common CCβ for the tested analytes would be determined if less than 5 % of the spiked samples are negative.” In this study, the applicability and ruggedness determination was combined.

Stability of the analytes spiked in the matrices or in stock solutions has to be evaluated, either by the laboratory itself or throughout a bibliographical study, if identical conditions are applied.

3 Intra-laboratory characterisation of method performance

This project aimed at determining the performance capabilities for the screening of CAP of the different kits collected from several commercial suppliers.

Preparation of standard solution, stability and storage

The purchased CAP standard was supplied in acetonitrile solution. Then the stock solutions were prepared at 100 ng/mL by diluting the appropriate amount of CAP standard with distilled water. The stock solutions of CAP were then stored at +4°C during one year maximum.

Working solutions were then prepared immediately before use, diluting the appropriate amount of the stock solution in double distilled water. Spiked samples were then prepared by adding 300 µL of spiking solution in 3 g of tissue (1/10th).

Sample preparation for evaluation and validation

Before use, matrices were properly prepared, grinded, homogenized and registered in the LIMS (Laboratory Information Management System), according to our internal procedure and finally stored in a freezer (-20°C). One day before analysis, matrices were thawed in a refrigerator (+5°C ± 3°C).

After testing, if we obtained a positive result with a presumed blank matrix, we requested confirmatory analysis by LC-MS/MS.

Each manufacturer supplied an operating procedure detailed within the leaflet of the kits. These were strictly followed. The sample preparation for the analysis of CAP started with an extraction step. The kits' procedures were very similar between manufacturers and allowed a common extraction and assay for all matrices. The extraction procedure of each kits is detailed in Appendix A. The assay procedures for each kit are detailed in the corresponding kit booklet.

After an extraction step, the assay procedure could slightly vary between suppliers. For the first step, x µL of the standards and samples were pipetted in separate duplicate wells with x µL of CAP-HRP conjugate. During this step, only the kit from Europroxima requested the addition of 25 µL of a secondary antibody solution into all wells. Then, for all kits, the plate was shaken and incubated for 30 minutes at room temperature (20-25°C) in the dark. After this, the plate was washed three times and x µL of substrate/chromogen were added to the wells. After an incubation of 15-20 min at room temperature (20-25°C) in the dark, a stop solution was added and the optical density of the wells was read at 450 nm (and 630 nm when the protocol advised it to reduce background noise (dual wavelength reading)).

Evaluation and validation of the ELISA kits

Evaluation of the Screening Target Concentration (STC) before the validation of the ELISA kits

An evaluation of the kit performance was carried out on the muscle tissues and in flesh of aquaculture products. If the extraction protocol was the same for muscle and flesh, the evaluation of the STC could be common for those different matrices (eg. 10 muscle samples from different species and 10 flesh samples from aquaculture products).

The aim was to determine the Screening Target Concentration (STC) for each kit that will lead to determining their detection capability CCβ and their cut-off (Fm). In general, one single kit was necessary to carry out this first evaluation step.

Determination of the Screening Target Concentration (STC)

Every day, three different species were used (muscle, fish, and shrimp). On the first day, the analyte was tested at half the RPA for each kit. The concentration of day 2 was redefined according to the result of day 1. For example:

- Day 1: standard curve in duplicate (≈12-14 wells) + 10 duplicate samples (3 blanks, 3 spiked in duplicate wells = 12 wells) with the target analyte at half the RPA.

- Day 2: standard curve in singlet (≈6-7 wells) + 10 duplicate samples (3 blanks, 3 spiked in duplicate wells = 12 wells) with the target-analyte at half the RPA (or another concentration according to the results at day 1).
- Day 3 was performed only if necessary (eg. when the concentration at half the RPA was not sufficient at day one, it was necessary to confirm the concentration used at day 2).

Results of the evaluation of the STC

When T was higher than Fm, the assay was valid and the STC was considered as equal to the spiked concentration. The evaluations were performed on various matrices, including fishes, shrimps and muscles. A common STC for muscle and flesh from aquaculture products was determined for all of the tested kits. For Biorex, Perkin Elmer, Randox and R-Biopharm, a common STC was determined at 0.075 µg/kg and the four evaluations were compliant. The STC for Europroxima was set at 0.1 µg/kg. The resulting STC were used to determine the CCβ during the validation. Regarding the ratio between the STC and the RPA, the number of analysed samples to determine CCβ would differ (n=20 for Biorex, Perkin Elmer, Randox and R-Biopharm because 0.075 µg/kg is equal to half of the RPA; and n=40 for Europroxima because 0.1 µg/kg is between 50% and 90% of the RPA) (*EURL Guidance Document on Screening Method Validation*, 2023).

Validation of the ELISA kits

The validation could be carried out on the kits when STC values were lower than or equal to RPA in target matrices. The validation step was performed according to the European regulation (UE) ,2021/808 (CIR (EU) 2021/808, 2021) and to the European guideline for screening methods (*EURL Guidance Document on Screening Method Validation*, 2023). The various performance characteristics to determine are the practicability, the specificity (blanks, false positive), the detection capability (CCβ, false negative), the applicability (different species: muscles, flesh of aquaculture products) and the stability.

3.1 Practicability

3.1.1 Evaluated parameter(s)

Practicability is the ease of use associated with the necessary material, reagents, instruments and environmental conditions. The aim was to check if the method and the kit were adapted for routine analysis.

3.1.2 Characterisation methodology: materials and methods

No particular methodology was applied to evaluate the practicability. This parameter is based on the ease of use of the kit and on the supplementary material required (not supplied in the kits).

3.1.3 Results

The practicability of each kit has been assessed during the experiments. In general, the extraction method and the assay procedure were quite similar for all manufacturers' kits. Each kit had different methods for the reagent preparations. For all of them, the rinsing buffer was delivered in powder or in a concentrate solution and it was necessary to dissolve/dilute the content with distilled water. However, the kits from Europroxima and Randox needed a few more preparations. Indeed, both of

them required the dilution of the sample extraction buffer. Moreover, Europroxima supplied lyophilised conjugate and antibody that needed to be reconstituted. In another hand, the kit from Randox needed a dilution of the conjugate solution, as it is furnished 400 times concentrated and the volumes used for this dilution may change according to the batch of the kit (a supplementary sheet is provided to detail the volumes required). Those additional steps could lead to more handling errors and may influence the selection of one ELISA kit.

The first step consisted in the matrix extraction by adding ethyl acetate, followed by a dry step under a gentle stream of nitrogen at 50-60°C, and a recovery in n-hexane. For the recovery, two manufacturers (Europroxima and Randox) recommended the use of an isooctane-chloroform (2:3) mix instead of the n-hexane. The major issue here was, due to its carcinogenic, mutagenic and reprotoxic effects and according to the article R4412-15 of the French Labor Code “when it is impossible to eliminate this risk, it is reduced to a minimum by substituting a hazardous chemical agent with another chemical agent or with a non-hazardous or less hazardous process”(*Article R4412-15 - Code du travail*, 2008), chloroform must be substituted. After short discussions with the manufacturers (Europroxima and Randox), it was agreed to replace the mix isooctane:chloroform (2:3) by n-hexane, using the lower layer instead of the upper layer, in the same volume for the assay procedure.

Regarding the assay procedure, the time of incubation was approximately the same (between 45 and 50 min in total). One major difference between the kits is that the protocol from Europroxima requires an extra step consisting in the addition of a secondary antibody solution after pouring the conjugate into the wells. All manufacturers recommended to protect the plate from the light while being at room temperature until the end of the manipulation.

3.2 Specificity/false positive rate

3.2.1 Evaluated parameter(s)

The validation was led on different batches of matrices, from different species (muscles, aquaculture products). At least 20 different batches per kit were analysed, during separate days.

The specificity means the ability of a method to distinguish between the target analyte and other substances. This characteristic is predominantly a function of the measuring technique described, but can vary according to class of compound or matrix.

The false positive rate is the probability that the tested sample is negative, even though a positive result has been obtained. It is a percentage, calculated as the ratio between the number of negative results categorized as positive (false positive) and the total number of actual negative results.

3.2.2 Characterisation methodology: materials and methods

The specificity was determined on shrimp and several species of fish and muscles. When the evaluation allowed harmonising the protocol between the species, a common validation was performed.

The specificity was determined on different batches of blank tissues (free of CAP) analysed on separate days.

3.2.3 Results

Detailed validations can be found in Appendix B. No false positive and false negative were calculated when T was lower than Fm as the result is considered as invalid.

The summary of the results for different batches of matrices from each supplier is represented in **Fig 1 .**

Regarding the kits from Europroxima, Perkin Elmer and R-Biopharm, the positivity threshold (T) value was always higher than the cut-off (Fm) value for inter-days. When Fm is taken as the cut-off value, no false positive were found so the results are satisfactory.

The kits from Biorex and Randox show a T that is lower than the cut-off Fm for aquaculture products, regarding the inter-days values. However, regarding intra-day values, the kits showed satisfactory results (see **Fig 2).** It was decided to go through the evaluation of the results using Fm as the cut-off value for each kit, as it is performed usually with immunoassays.

For the five kits, the false positive rate was always lower when the Fm value was considered as the cut-off value **(**Appendix B**).** It was decided to go through the evaluation of the results using Fm as the cut-off value for each kit, as it is performed usually with immunoassays. As a conclusion, the specificity was satisfactory for each kit (false positive rate of 0%).

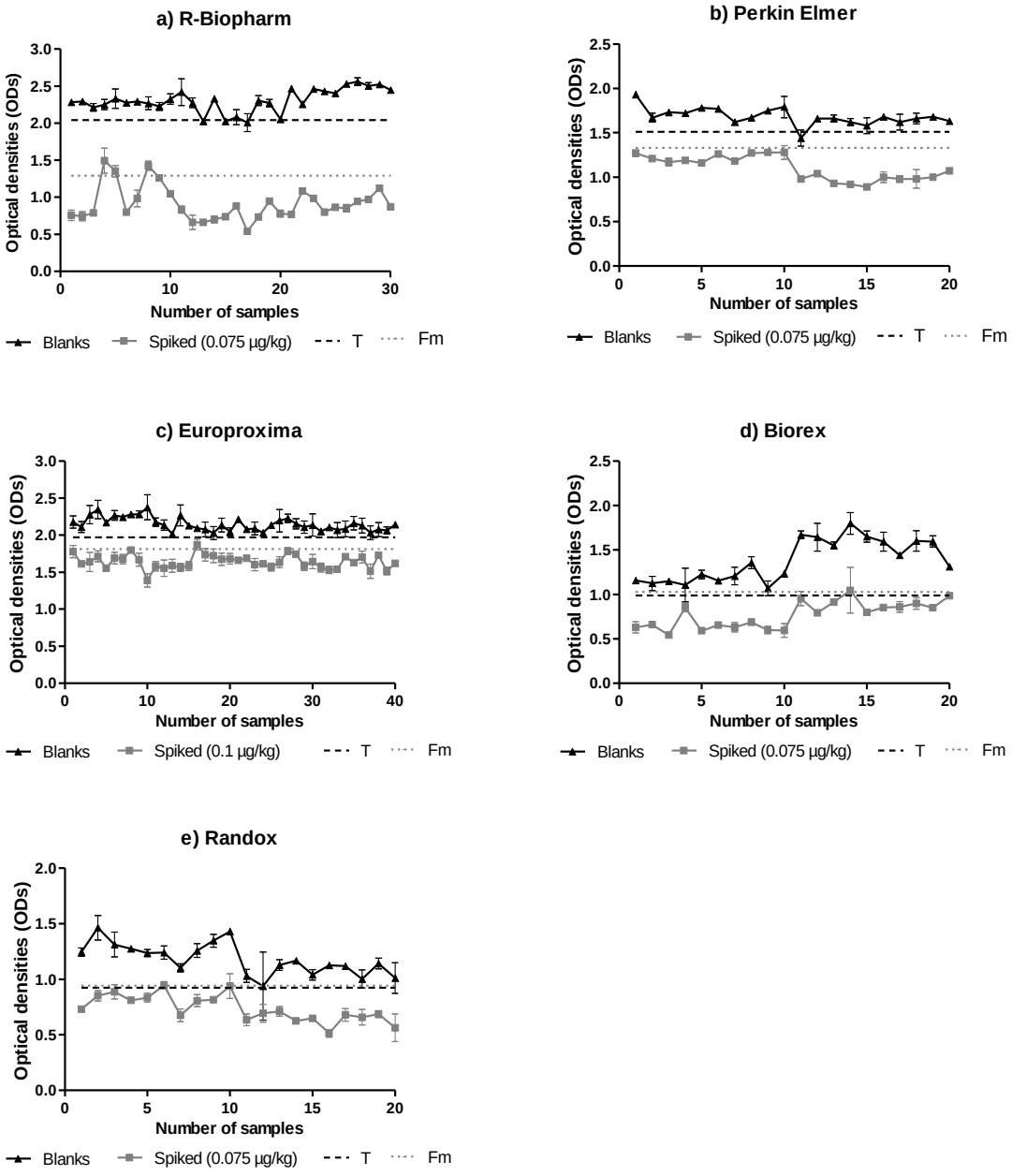

Fig 1 –Optical densities (ODs) for blank and spiked samples (analyzed in duplicate) for each kit.

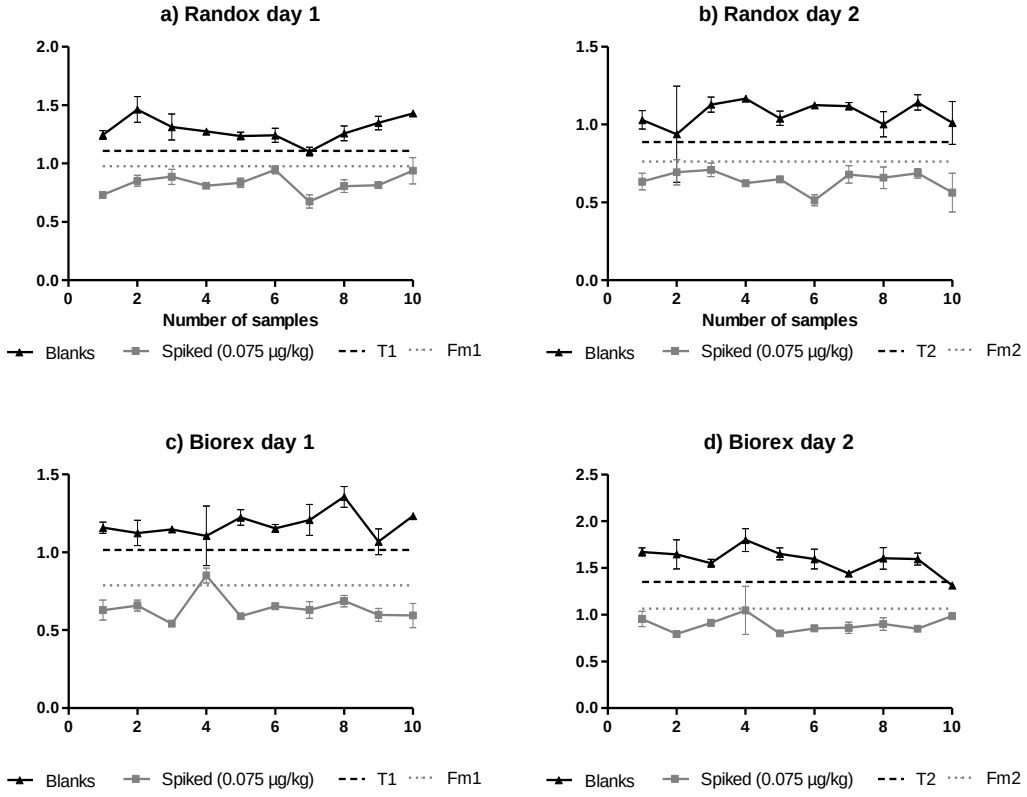

Fig 2- Optical densities (ODs) for blank and spiked samples (analyzed in duplicate) from Randox and Biorex kits, obtained each day of validation.

3.3 Detection capability (CCβ)

3.3.1 Evaluated parameter(s)

The Commission Decision 2021/808 stated the definition of CCβ as “the smallest content of the analyte that may be detected or quantified in a sample with an error probability of β: in the case of prohibited or unauthorised pharmacologically active substances, the CCβ is the lowest concentration at which a method is able to detect or quantify, with a statistical certainty of 1 – β, samples containing residues of prohibited or unauthorised substances” (CIR (EUR) 2021/808, 2021). The β error is the probability that the tested sample is truly non-compliant even though a compliant measurement has been obtained. For the validation of screening tests, the β error (i.e. false compliant rate) should be ≤ 5%. In the case of banned substances, CCβ must be lower than or equal to the RPA.

At least two different batches of kits were used for the determination of detection capability.

3.3.2 Characterisation methodology: materials and methods

The number of samples for the validation (spiked samples to the target concentration) for each substance depends on the statistical trust level required in the result and on the screening target concentration (determined during evaluation) and regulated limit. A minimum of 20 samples was necessary if STC was set at half the regulatory limit (RL) (with one or less false-compliant result (i.e. β error 5%)) and 60 samples if STC was close to the RL (10% below the RL) (with 3 or less than 3 false-compliant results).

3.3.3 Results

The STC used to determine the CCβ were chosen following the evaluation step. **Fig 1** shows the detailed results obtained during the validation of each kit. All the kits showed very satisfactory results regarding the false negative rate, as each of them was less than or equal to 5%. Two different batches of kits were used for each validation.

Biorex. The kit was evaluated at a common STC of 0.075 µg/kg for aquaculture products and muscles during 2 days (n=20). Inter-days validation was not valid due to high signal variability. But the intra-day results were valid (T>Fm) (**Fig 2**) for all matrices and showed a 5% false negative rate. So the common CCβ was equal to **0.075 µg/kg** when intra-days results were taken into account.

Europroxima. It was decided to evaluate this kit at a common STC of 0.1 µg/kg, as the evaluation showed unsatisfactory results at 0.075 µg/kg for aquaculture products. Four days of validation were necessary to analyse 40 blank and 40 spiked samples. The kit showed a T value higher than the Fm value (cut-off) for each matrix. The conclusion was that the CCβ was equal to **0.1 µg/kg**, with a false negative rate of 5%.

Perkin Elmer. The kit was evaluated at a common STC of 0.075 µg/kg for aquaculture products and muscles during 2 days (n=20). No false negative results were obtained and the T value was always higher than the cut-off value Fm in inter-days and intra-day data. As a conclusion, this kit was validated at a common CCβ of **0.075 µg/kg with a false negative rate of 0%**.

Randox. The kit was evaluated at a common STC of 0.075 µg/kg for aquaculture products and muscles during 2 days (n=20). The inter-days evaluation was not valid for aquaculture and global results. However, intra-day results were all valid (T > Fm) for each matrix and global results (**Fig 2**). Therefore a common CCβ was determined, equal to **0.075 µg/kg with a false negative rate of 0%**.

R-Biopharm. The R-Biopharm kit was validated on different species of fishes, shrimps and muscles at a common STC of 0.075 µg/kg during 3 days. It showed unsatisfactory results with a false negative rate of 6.6% (2 false negative for 30 samples). However, taking each matrix apart, the results showed a false negative rate of 0%. As a conclusion, the kit is valid with a CCβ **of 0.075 µg/kg for muscles with a false negative rate of 0%** and with a CCβ of **0.075 µg/kg for aquaculture products with a false negative rate of 0%.**

3.4 Applicability

3.4.1 Evaluated parameter(s)

The applicability of a kit for muscles from different animal species and from different aquaculture products was evaluated during specificity and detection capability (CCβ) tests. An ELISA kit could be considered as applicable to different matrices when two conditions were fulfilled: firstly the different matrices (aquaculture products and muscles) strictly followed the same protocol (extraction, assay on the microplate). During this project, this condition was applicable for each kit manufacturer. Secondly specificity and CCβ validated were the same for all matrices.

Applicability was demonstrated if the specificity and CCβ were the same for different species (muscle and aquaculture products), using the same protocol.

3.4.2 Characterisation methodology: materials and methods

Specificity and Detection capability (CCβ) were determined for CAP in 5 kits from 5 different manufacturers (Europroxima, R-Biopharm, Randox, Perkin Elmer and Biorex). At least two different batches of kits were used for the determination of detection capability and specificity.

Each supplier has already performed an evaluation of the applicability of the kits. The kits from Biorex, Europroxima, Perkin Elmer and R-Biopharm were all validated on fish, shrimps and muscles. For those kits, the same protocol was used for aquaculture products and muscles, so the applicability could be demonstrated and the announced LOD was the same for all matrices. However, the kit from Randox was validated by the manufacturer only on aquaculture products. It doesn't mean that this kit is not applicable to muscle matrices, but only that it was not validated by the manufacturer in other matrices than shrimps.

3.4.3 Results

Biorex. This kit has been validated at a common concentration of 0.075 µg/kg with 20 different batches of muscles (ovine, bovine, poultry) and aquaculture products (fish and shrimps) (20 blanks, 20 spiked) with the same protocol. A false positive rate of 0% and a false negative rate of 5% were reported. Thus, **the kit is applicable to meat, fish and shrimps at a common CCβ of 0.075 µg/kg.**

Europroxima. This kit has been validated at a spiked concentration of 0.1 µg/kg with 40 different batches of muscles (ovine, bovine, poultry) and aquaculture products (fish and shrimps) (40 blanks, 40 spiked) with the same protocol. No false positive and one false negative (5%) results were found in the global validation. As a conclusion, **the kit is applicable to meat, fish and shrimps at a common CCβ of 0.1 µg/kg**.

Perkin Elmer. This kit has been validated at a spiked concentration of 0.075 µg/kg with 20 different batches of muscles (ovine, bovine, poultry) and aquaculture products (fish and shrimps) (20 blanks, 20 spiked) with a common protocol. No false negative and no false positive results were recorded during validation. **The kit was applicable to aquaculture products and muscles at a common CCβ of 0.075 µg/kg.**

Randox. It is important to note that this kit was only validated on aquaculture products by the manufacturer and not on meat. To compare this kit to the 4 others, the kit has been validated on 20 different samples (20 blanks, 20 spiked) from different species (meat and aquaculture products) with a common protocol and a target concentration of 0.075 µg/kg for all matrices. No false negative and no false positive results were obtained during validation. **This kit was applicable to aquaculture products and muscles at a common CCβ of 0.075 µg/kg**.

R-Biopharm. This kit has been validated at a spiked concentration of 0.075 µg/kg with 30 different batches of muscles (ovine, bovine, poultry) and aquaculture products (fish and shrimps) (30 blanks, 30 spiked), with a common protocol. The validation showed a T value largely higher than Fm values at the target concentration. However, two false negative samples (6.6%) were found with the validation. In another hand, no false negative and no false positive results have been found for intra-matrix validation. As a result, **this kit was applicable to meat at a CCβ equal to 0.075 µg/kg, and to aquaculture products at a CCβ of 0.075 µg/kg. However, a quality control will be necessary to discriminate muscle and aquaculture products if they are analysed on the same run (one for aquaculture product, one for muscles).**

3.5 Cross-reactions (CR %)

3.5.1 Evaluated parameter(s)

The rate of cross-reactions (CR) between the main analyte and potentially interfering substances is determined to analyze the specificity/selectivity of an ELISA kit.

3.5.2 Characterisation methodology: materials and methods

Due to previous evaluations of ELISA kits, the cross-reactions analyses were not performed but were compared according to the data furnished by manufacturers and a short bibliographical study has been led.

3.5.3 Results

Suppliers data regarding cross-reactions are presented in Appendix C. The five ELISA kits are very specific for the detection of CAP.

CAP has four stereoisomers but only two of them are mainly found in industrial chemical synthesis: RR-CAP (chloramphenicol) and SS-CAP (dextramycine) (Yanovych et al., 2018) and only one of them (RR-CAP) is bioactive, has antimicrobial activity and is used in human and veterinary medicine. However, RR-CAP and SS-CAP can be found in food samples (Rimkus et al., 2020). A recent study tested two different CAP standards with ELISA kit (Ridascreen® CAP, R-Biopharm). One of the standard – the same as the one supplied in the kits, RR-CAP - was correctly determined whereas the other (SS-CAP) could not be detected by the method. Thus, they suggested the hypothesis that not all stereoisomers can be detected by ELISA methods as it could be stereoselective (Sykes et al., 2017). They also showed that immunoaffinity columns (IACs, EASIEXTRACT® Chloramphenicol immunoaffinity columns) do not bind SS-CAP to the antibodies. A study from Rimkus et al., 2020, had the same conclusion with a radioimmunoassay (RIA) test (Charm® II Chloramphenicol Test for Honey) (Rimkus et al., 2020). Those methods showed that even high amounts of SS-CAP can't be detected by immunological methods for now, inducing a risk of systematically false-compliant (i.e. false-negative results) in samples contaminated with SS-CAP (Rimkus et al., 2020). Finally, Rimkus & Hoffmann developed a LC-chiral method to discriminate the four CAP stereoisomers in honey. In this study, they proved the bioactive effect of RR-CAP but pointed the need to determine the toxicological effect of each stereoisomer, in particular SS-CAP (Rimkus & Hoffmann, 2017).

Appendix C shows that, among the kits tested in our study, only R-Biopharm (with the Ridascreen® CAP kit) stated that there is no cross reactivity with SS-p-CAP. To compare the different kits on the market, the other manufacturers should specify the cross-reaction of ELISA kits with the four stereoisomers.

3.6 Stability

3.6.1 Evaluated parameter(s)

The stability of CAP in analyzed matrices needs to be evaluated as it might influence the test results.

3.6.2 Characterisation methodology: materials and methods

According to the European Commission 2021/808, “If stability data for analytes in the matrix are available (e. g. on the basis of information from the EURLs, published data, etc.), these data do not need to be determined by each laboratory” (CIR (EUR) 2021/808, 2021). Following this, the stability of CAP in different matrices and in solutions was evaluated in a bibliographical study.

3.6.3 Results

A stock solution of CAP (1 mg/mL) in methanol was proven to be stable at least 3 months when stored at 4°C (Li et al., 2006). The stability of three different incurred materials, two from shrimps and one from crayfish, supplied for a proficiency test organized in Germany was determined (Polzer et al., 2006). For stability testing, two aliquots of each material were stored for each of condition (ie. different temperatures (-80°C, -30°C,+4°C, +20°C) combined with different storage periods ((2 days, 1 month, 1 year). The storage of aliquots at +20°C was stopped after one month. Each aliquot was analysed in double. No degradation of CAP could be observed in all conditions.

The degradation of CAP has often been studied in various matrices such as shrimps or water. In a study of Shalika et al., microbiological assays were led on the stability of residuals from CAP in shrimps tissues subject to a heat processing of 100°C (Shakila et al., 2006). They showed that CAP is degradated with increasing heat temperature and time processing. As a conclusion, CAP was unstable with a heat treatment process (ie. destroyed or degraded).

4 Inter-laboratory characterisation of method performance

Not performed.

5 Measurement uncertainty

During this study, several points and critical steps were significantly relevant for uncertainty. It was demonstrated that, for two kits having the same brand reference but not the same batch (i.e. that has not been manufactured at the same date), the optical density OD at the final read can vary a lot. When it was the case, the results taken into account were the intra-batch results instead of inter-batch results.

The other identified critical steps were the nitrogen drying stage and the washing step during the assay procedure.

6 Conclusion: validation

6.1 Summary and validation of technical characteristic results

Table 1 – Validation of technical characteristic values

Performance characteristic	Parameter	Result per kit	Predetermined target value (specifications)	Conclusion(s)
Specificity	False positive rate	0% for all kits	As low as possible (no regulatory criterion)	All kits are valid
False negative rate	False negative rate at the STC	0% for all kits	Lower than or equal to 5% at the STC	All kits are valid
Detection capability CCβ	Value of CCβ	Biorex: 0.075 µg/kg Perkin Elmer: 0.075 µg/kg R-Biopharm: 0.075 µg/kg Randox: 0.075 µg/kg Europroxima: 0.1 µg/kg	Lower than or equal to the RPA (0.15 µg/kg)	The kits from Biorex, Perkin Elmer, R-Biopharm and Randox are all valid at half the RPA The kit from Europroxima is valid below the RPA

The detection limits announced by the manufacturers (LOD) were always lower than the estimated CCβ (Table 2). This can easily be explained because the LOD and the CCβ are determined in different ways: in their leaflets, the manufacturers explained that the LOD was determined “as equal to the mean calculated concentration of at least 20 blank samples, plus 3 times their standard deviation” while “the CCβ is the lowest concentration at which a method is able to detect or quantify, with a statistical certainty of 1 – β, samples containing residues of prohibited or unauthorised substances” (CIR (EU) 2021/808, 2021).

Table 2 – Comparison between the determined CCβ and manufacturers' data on kit performance (LOD)

Manufacturer	LOD (µg/kg)	Measured CCβ (µg/kg)	Difference LOD/CCβ
Biorex	0.05-0.1	0.075	CCβ from 0.75 to 1.5 times the LOD
Europroxima (sold by R-Biopharm)	0.02	0.1	CCβ equals to 5 times the LOD
Perkin Elmer (sold by Novakits)	0.025	0.075	CCβ equals to 3 times the LOD
R-Biopharm	0.005-0.008	0.075	CCβ from 9.4 to 15 times the LOD
Randox	0.02	0.075	CCβ equals to 3.75 times the LOD

6.2 Summary and validation of non-technical characteristic results

The practicability was discussed in section 3.1. Two kits were reported to recommend the use of toxic chemicals but could be replaced by less hazardous alternatives. Some kits required more preparation steps than others prior to the experiment. Those supplementary manipulations could lead to errors and those kits that do not required extra steps could be preferred, as they permit to save time and obtain more reliable results.

6.3 General conclusion

Previous studies from early 2000's had validated ELISA kits for the screening of CAP (Europroxima reference 5091CAP1p) in different matrices (eg. muscle, eggs, honey) according to the Commission Decision 2002/657/EC and in relation to the previous MRPL (0.3 µg/kg) (Scortichini et al., 2005).

But, to our knowledge, this new study is the first comparative evaluation of five different ELISA kits for the screening of CAP in muscle and flesh tissues of food–producing animals and aquaculture products, according to the Regulation (EU) 2021/808 and in relation to the new RPA (0.15 µg/kg) (CIR (EU) 2021/808, 2021; CR (EU) 2019/1871, 2019). The five different ELISA kits tested were all applicable to the screening of CAP in different species of fish, shrimps and muscles of reared animals, with a harmonised protocol. The detection capabilities CCβ of all the kits were determined

under the RPA in both the muscles and the flesh of aquaculture products (between half RPA to 0.67 times the RPA). The specificity was also satisfactory, with false positive rates equal to 0% for all kits. **As a conclusion, all the kits evaluated are reliable for official controls and applicable to muscles from different animal species and to flesh of aquaculture products (fish, shrimps, prawns), and all below the new RPA of 0.15 µg/kg.**

7 References

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8 Appendix/Appendices.

Appendix A. Extraction procedures

Europroxima:

- Weight 3 g
- 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 is added.
- The whole is mixed (Vortex) for 1 min. and centrifuged (10 min. at 2,000 g).
- Use 50 µL of the lower aqueous phase per well.

R-Biopharm:

- Add 3 ml of distilled water and 6 ml ethyl acetate to 3 g of homogenized sample and mix
- Shake for 10 min upside down
- Centrifuge: 10 min / 3,000 g / room temperature (20 - 25 °C)
- Transfer 4 ml of supernatant into a new vial and evaporate at 60 °C to complete dryness by nitrogen or air
- Reconstitute the dried residue in 1 ml n-hexane
- Add 500 µl wash buffer and vortex for 1 min
- Centrifuge: 10 min / 3,000 g / room temperature (20 - 25 °C)
- Use 50 µl of the lower aqueous phase per well in the assay

Novakits (perkin Elmer):

- Weigh 3.0 g (+/- 0.05 g) of the sample
- Add 6.0 mL of ethyl acetate using a glass pipette
- Vortex the sample for 3 minutes at maximum speed or 10 minutes in a multi-vortexer
- Incubate the sample at 50°C for 30 minutes
- Centrifuge the sample a 4,000 g for 10 minutes at RT (20-25°C)
- Transfer 4.0 mL of the upper, ethyl acetate layer to new 15-mL tube and dry the ethyl acetate layer with a nitrogen evaporator at 60°C
- To the dried residue, add 2 mL of hexane, swirl the tube for 15 seconds and add 1.0 mL of 1X sample Extraction Buffer

- Vortex the sample for 1 min at max speed
- Centrifuge the sample at 4,000 g for 10 minutes at RT (20-25°C)
- Discard the upper, hexane layer by careful aspiration or pipetting
- Use 100 µL of the lower layer per well in the ELISA

Biorex:

- To 3 g sample with 3 mL double distilled water, add 6 mL ethyl acetate and agitate vigourously for 10 min
- Centrifuge for 10 min at 3,000 g at room temperature
- Transfer 4 mL of the upper phase to a clean glass vial and evaporate at 50-70°C under a nitrogen or airstream to dryness
- Add 1 mL hexane to the residue
- Add 500 µL diluted wash buffer to the mixture and vortex for 1 min
- For phase separation centrifuge for 10 min at 3,000 g (room temperature)
- Use 25 µL of the lower layer in the ELISA plate

Randox:

- To 3 g tissue, add 6 ml ethyl acetate
- Homogenize for 1 minute
- Centrifuge at 2000 rpm for 15 minutes.
- Remove 4 ml of upper phase and reduce to dryness at+70°C
- Dissolve residue in 2 ml of isooctane/chloroform (2:3), vortex for 1 minute.
- Add 0.5 ml of diluted Tissue Extraction Buffer and vortex for 2 minutes.
- Centrifuge at 2000 rpm for 15 minutes.
- Upper phase is now ready for application to microtitre plate.

Appendix B. Detailed results of the validation of each kit

	n	[CAP] (µg/kg)	Matrix	Mean blanks	SD blanks	Mean spiked	SD spiked	T	Fm	Conclusion	False +	False -
Europroxima	40	0.1	Aquaculture	2.124	0.088	1.630	0.099	1.979	1.792	OK	0	1
			Muscles	2.172	0.120	1.651	0.111	1.975	1.833	OK	0	0
			Global	2.148	0.108	1.640	0.105	1.972	1.813	OK	0	1
R-Biopharm	30	0.075	Aquaculture	2.265	0.168	0.786	0.135	1.989	1.007	OK	0	0
			Muscles	2.344	0.145	1.038	0.242	2.107	1.434	OK	0	0
			Batch 1	2.228	0.131	0.906	0.273	2.013	1.354	OK	0	1
			Batch 2	2.459	0.087	0.926	0.115	2.317	1.114	OK	0	1
			Global	2.305	0.161	0.912	0.232	2.041	1.292	OK	0	2
Perkin Elmer	**20**	0.075	Aquaculture	1.675	0.072	1.117	0.142	1.558	1.351	OK	0	0
			Muscles	1.688	0.130	1.089	0.136	1.476	1.312	OK	0	0
			Batch 1	1.743	0.087	1.227	0.055	1.600	1.317	OK	0	0
			Batch 2	1.621	0.081	0.979	0.061	1.488	1.079	OK	0	0
			Global	1.682	0.104	1.103	0.138	1.512	1.330	OK	0	0
Biorex	20	0.075	Aquaculture	1.400	0.250	0.796	0.200	0.989	1.125	KO	/	/
			Muscles	1.369	0.237	0.750	0.125	0.980	0.955	OK	0	1
			Batch 1	1.178	0.100	0.643	0.088	1.014	0.787	OK	0	1
			Batch 2	1.585	0.144	0.895	0.103	1.350	1.063	OK	0	0
			Global	1.382	0.240	0.769	0.159	0.988	1.029	KO	/	/
Randox	20	0.075	Aquaculture	1.144	0.183	0.708	0.142	0.844	0.941	KO	/	/
			Muscles	1.216	0.119	0.761	0.104	1.022	0.932	OK	0	0
			Batch 1	1.290	0.111	0.829	0.090	1.108	0.977	OK	0	0
			Batch 2	1.070	0.111	0.641	0.075	0.888	0.763	OK	0	0
			Global	1.180	0.156	0.735	0.126	0.923	0.941	KO	/	/

Appendix C. Suppliers data for cross-reactivity

Supplier	Molecule	Cross-reactivity (%)
Perkin Elmer	Chloramphenicol	100
	Thiamphenicol	0.4
	Tetracyclines, Gentamicin, Ampicillin, Florfenicol	<0.01
	Gentamicin	<0.01
	Ampicillin	<0.01
	Florfenicol	<0.01
Biorex	Chloramphenicol	100
	Chloramphenicol glucuronide	>100
Europroxima	Chloramphenicol, Chloramphenicol-glucuronide	100
	Thiamphenicol, Florfenicol	<1
Randox	Chloramphenicol, Chloramphenicol glucuronide	100
	Chloramphenicol base	<0.1
	Chloramphenicol Palmitate	0.5
	Chloramphenicol Stearate	0.1
	Thiamphenicol	<0.5

	Sulphamethazine, Monensin, Chlortetracycline, Penicillin, Tylosin, Avoparcin, Nitrofurazone, Nitrofurantoin, Furazolidone	<0.01
	Fluorophenicol	<0.05
Ridascreen	Chloramphenicol (RR para-stereoisomer)	100
	Dextramycin (SS-para-stereoisomer), Chloramphenicol base, Florfenicol, Thiamphenicol, Nitrofurantoin AHD, NP-AHD, Furaltadone, AMOZ, NP-AMOZ, Furazolidone, AOZ, NP-AOZ, Nitrofurazone, SEM, NP-SEM,	<1
	All other stereoisomers	Not determined
	Chloramphenicol glucuronide	Approx. 68