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Report EUR 21181

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REFERENCE MATERIALS

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ABSTRACT

This report describes the preparation, homogeneity, stability and certification studies of a freeze-dried salmon tissue (BCR-725) certified for its contents of flumequine and oxolinic acid. A description of the analytical procedures used in the homogeneity and stability studies as well as in the certification study is included. All individual results of the certification study are reported. All relevant data from the homogeneity and stability studies and certification measurements are presented.

The certified values (expressed on dry mass basis) are:

Substance	*Certified value* [µg/kg]	*Uncertainty** [µg/kg]
Flumequine	1170	210
Oxolinic acid	600	100

* combined uncertainty with a coverage factor $k = 2$

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BCR	Community Bureau of Reference
BHT	2,6-di-*tert*-butyl-4-methylphenol
CRL	Community Reference Laboratory
CRM	Certified Reference Material
d.f.	Degrees of freedom
DTI	Danish Technological Institute
FLD	Fluorescence detector
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
ISO	International Standardisation Organisation
IRMM	Institute for Reference Materials and Measurements (Geel, Belgium)
k	coverage factor
LC	Liquid Chromatography
MRL	Maximal Residue Limit
MS	Mass Spectrometry
MS_q	Mean of squares
NCVM	Norwegian College of Veterinary Medicine
R	Ratio value
RM	Reference Material
RSD	relative standard deviation
s	standard deviation
SIM	Selected ion monitoring
s_{meas}	measurement uncertainty
SS	Sum of squares
T	Temperature
U	Expanded uncertainty
u_{bb}	uncertainty component from homogeneity
u^*_{bb}	upper limit of inhomogeneity that can be hidden by the method repeatability
u_{b1}	bias contribution to the u_{lts}
u_{b2}	uncertainty associated to the bias contribution to the u_{lts}
u_{char}	uncertainty component from batch characterisation
U_{CRM}	expanded uncertainty of CRM
u_{lts}	uncertainty component from long-term stability
u_{sts}	uncertainty component from short-term stability

1. INTRODUCTION

1.1 Background: need for a CRM

The quinolones group includes flumequine and oxolinic acid, which are used in aquaculture. Both compounds are active at low doses and only slightly metabolized.

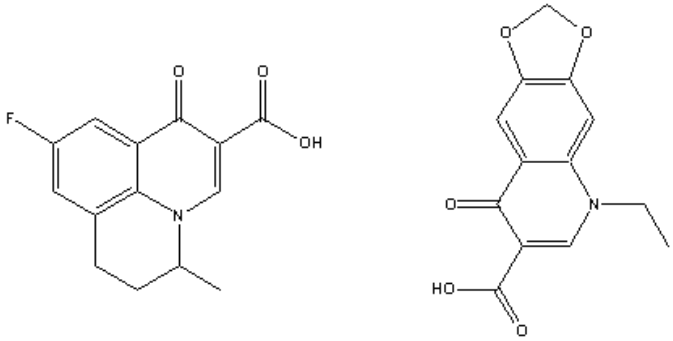
Flumequine is a first generation quinolone. In aquaculture, flumequine is especially used against furunculosis, enteric redmouth disease and pseudotuberculosis. The oral absorption in fish is rather good, a bioavailability from medicated feed on 60-80% is reported. Flumequine seems to be less used in Europe than oxytetracycline and oxolinic acid, but the use is increasing.

Oxolinic acid is an older member of the group of synthetic antimicrobial agents generically termed the quinolones. The compound is particularly active against Gram-negative bacteria including fish pathogens which cause diseases like enteric redmouth disease, furunculosis, cold-water vibriosis and classic vibriosis. Because of its effectiveness and relatively modest cost it has become one of the most widely used drugs in aquaculture. The absorption from medicated fish in seawater is about 20%. In freshwater the bioavailability is somewhat higher.

Flumequine is included in annex 1 of Council Regulation (EEC) No. 2377/90 for salmon species in muscle and skin in a natural proportion with a maximum residue limit (MRL) of 600 µg/kg.

Oxolinic acid is included in annex 1 of Council Regulation (EEC) No. 2377/90 for fish and fins in muscle and skin in a natural proportion with MRL of 100 µg/kg. At the date of the production of the certified reference material, a temporary MRL was established to 300 µg/kg.

Analytical procedures for determining flumequine and oxolinic acid in fish tissue involve extractions and chromatography. Quality and verification of all steps in the analytical procedures require the availability of a matrix reference material. BCR-725 has been produced for this purpose. The property values of flumequine and oxolinic acid in the freeze-dried fish tissue were chosen to be higher than the MRL values. The structures of the two quinolones are shown in fig. 1.



FLUMEQUINE [42835-25-6]

OXOLINIC ACID [14698-29-4]

Figure 1 - Structures of the target compounds and CAS-numbers.

1.2 Choice of the material to be certified

Flumequine and oxolinic acid are not usually simultaneously employed for fish treatment in aquaculture, therefore it is unlikely to find both compounds within the same salmon fish tissue. However one single CRM was finally produced and bottled, as freeze-dried salmon tissue, since the two quinolones can easily be determined using the same analytical procedure.

Salmon fish was treated with flumequine and oxolinic acid, respectively. Muscle tissue plus skin was blended in natural proportions, and non-medicated tissue plus skin was added to obtain the contamination level desired. After throughly blending and mixing the material was freeze-dried. Non-medicated tissue ensured zero blank values.

Water content in fresh fish is approximately 70% w/w. As laboratories usually analyse fresh fish, the water content has to be taken into account. The certified values are given on the basis of dry matter.

1.3 Design of the project and certification procedure

Preparation of medicated fish and procedures for blending, mixing and freeze-drying were developed in a feasibility trial. A test batch was produced and used for preliminary homogeneity and stability studies as well as for training and testing the candidate laboratories for the certification measurements. Details are given in section 3.

Approximately 3.2 kilos of freeze-dried tissue with slightly different target values were prepared and sent to IRMM for final freeze-drying and bottling of 1400 samples with 2.2 g of material each. Random samples were used for homogeneity testing, stability testing and the certification measurements. Details are given in sections 4-7.

Before the training exercise, standard solutions of both quinolones were supplied to each laboratory to verify their calibration procedure. Vials with known contents of both quinolones were supplied together with the samples for certification measurements.

2. PARTICIPANTS

Preparation of medicated and not medicated salmon

- Nutreco A/S, Stavanger NO

Feasibility study, preparation of freeze-dried tissue

- Norwegian College of Veterinary Medicine, NCVM NO

Final homogenization and bottling of glass vials

- Institute for Reference Materials and Measurements, IRMM BE

Homogeneity and stability studies

- Danish Technological Institute, DTI DK

Certification measurements

- AFSSA Fougères FR
- Danish Veterinary and Food Administration, IFSE DK
- Danish Technological Institute, DTI DK
- Ecole Nationale Vétérinaire de Nantes FR
- Institute for Reference Materials and Measurements, IRMM BE
- Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III ES
- National Food Administration, Chemistry Division 3 SE
- National Veterinary and Food Research Institute, Dept. of Chemistry FI
- Niedersächsisches Landesamt für Verbraucherschutz & Lebensmittelsicherheit DE
- Norwegian College of Veterinary Medicine, NCVM NO
- Universitat de Barcelona ES
- Wetenschapelijk Instituut Volksgezondheid - Louis Pasteur, Dept. Voedingswaren BE

Statistical evaluation and uncertainties

- Danish Technological Institute, DTI DK
- Institute for Reference Materials and Measurements, IRMM BE

3. FEASIBILITY STUDY

3.1 General

The certification of the reference material was preceded by an extensive feasibility study performed both on the material preparation and the ability of the participants to measure the substances of interest.

The preparation of a homogeneous muscle tissue material was not a difficult task, but inclusion of the skin in a natural proportion presented problems. It was not possible just to blend muscle plus skin. After several attempts, the procedure described in section 4.1 was developed.

Analytical methods published so far describe the determination of the quinolones in muscle tissue. A method involving a clean-up step with organic solvents [1] and a fast direct method [2] are described. Both approaches, developed by Yndestad and co-workers at the Norwegian College of Veterinary Medicine (NCVM), permit the extraction of the quinolones into a liquid phase in a few minutes. In addition to the work to produce a homogeneous mixture of tissue and skin, NCVM also investigated the extraction of quinolones from skin. Such study, which presents more difficulties than extraction from muscle, ended up on the development of a di-

rect method avoiding any clean-up step with organic solvents [3]. All methods were presented to the participating laboratories.

Analytical work for homogeneity and stability tests was done using the method described in [1]. Water content in the freeze-dried material was ignored in the feasibility study.

3.2 First preliminary interlaboratory study

The experiments including blending, mixing and freeze-drying muscle plus skin tissues gave as a result a portion containing 30-40 g of freeze-dried material. One vial containing 1 g of material was delivered to each laboratory, so they could become familiar with the freeze-dried material. The freeze-dried material, reconstituted by mixing 2 mL of water with 1 g of the powder resembled blended fresh fish. Methods [1], [2] and [3] describe the analysis of fresh tissue.

In addition to the sample, two vials containing blank fish and spiked fish extract, respectively, were delivered to the participant laboratories. Freeze-dried blank fish was produced from non-medicated salmon. For the production of spiked extract several portions of extract were made according to [3] using non-medicated salmon. The extracts were mixed, and spiked with quinolones up to a final concentration of 12 ng/mL flumequine and 24 ng/mL oxolinic acid.

3.3 Results from first preliminary interlaboratory study

Homogeneity test carried out on 6 vials prior to the study was satisfactory, RSD < 3%. Most of the participating laboratories analysed the freeze-dried fish sample either using extraction of the quinolones [2] followed by direct measurements by HPLC with fluorescence detection, or carrying out a clean-up step with organic solvents before the chromatographic stage. A number of laboratories used their own methods, different from those already described.

Excluding very deviating results from two laboratories, the mean ± mean standard deviation of the remaining results were:

- Flumequine: 351 µg/kg ± 45 µg/kg (RSD = 13%);
- Oxolinic acid : 849 µg/kg ± 102 µg/kg (RSD = 12%).

A rough division between methods gave two groups:

- Group I: Methods using extraction at pH > 9 and simple clean-up of the water phase;
- Group II: Methods using extractions involving organic solvents and evaporation.

Methods in Group I demand close matching of the whole analytical procedure and the calibration procedure as mentioned in the NCVM method [2]. Personnel must have experience with the applied method and use it frequently. The addition of quinolones to a sample of blank fish is necessary for calibration, and it is important to ensure complete mixing with the fish material before extraction.

Methods in Group II are more labour work consuming, but may be more robust. It is easier to obtain the same conditions for analysis and calibration at the chromatographic stage.

In most cases the signals corresponding to oxolinic acid and flumequine in blank fish samples were below the limit of detection of the technique employed.

Results from analyses of spiked extracts were generally good, but many participants reported the presence of interfering peaks. Sometimes these peaks could be resolved from oxolinic acid and flumequine peaks, but that was not always the case. The origin of these peaks is not clear – they could be attributed to degradation products.

3.4 Second preliminary interlaboratory study

Approximately 300 vials with 1.2 g of freeze-dried material were produced using the procedure described in section 4.1, followed by bottling into vials. 30 vials with blank freeze-dried material were prepared analogously.

Due to instability problems, vials with spiked extracts were not prepared. It was instead decided to prepare vials with evaporated spiked extracts. An extract was made as described in section 3.2, and the quinolones were extracted with trichloromethane. 1.00 mL $CHCl_3$ was transferred to vials, and the $CHCl_3$ evaporated. The laboratories were instructed to add their normal mobile HPLC phase to the vial, mix throughly and inject into the HPLC system.

Before distribution of the vials to the participating laboratories, homogeneity tests were carried out on 10 vials containing fish material and 6 vials containing evaporated spiked extract. The results of the test indicated negligible inhomogeneity between the units.

When the production was finished, a stability test was initiated, using 41 vials with fish material chosen at random. Vials were successively transferred from the storage temperature to –80 °C. After 150 days all vials were analysed using repeatability conditions. No significant (95% and 99%) alteration was found during 150 days' storage at –18 °C, +4 °C and ambient temperature, respectively.

3.5 Results from second preliminary interlaboratory study

The laboratories were requested to analyse 12 vials with freeze-dried material on 5 different days, in order to calculate the RSD within days and RSD between days.

RSD within days > 15 % and RSD between days > 20 % were considered not acceptable. 14 out of 18 laboratories submitted acceptable results.

10 laboratories used extraction involving clean-up step with organic solvent. One laboratory used MS for detection. No difference was observed between different extraction procedures and detection modes.

The laboratory detecting with MS did not get any improvement using nalidixic acid as internal standard. Nalidixic acid behaved differently from flumequine and oxolinic acid. The outcome of the calculations on all laboratory results is shown in table 1.

Table 1- Summary of second preliminary intercomparison study results for contaminated freeze-dried material.

	Flumequine	*Oxolinic acid*
Overall mean, µg/kg	303	537
RSD of overall mean	7.4	7.9
Mean RSD, within days	4.3	3.2
Mean RSD, between days	5.9	4.3
Mean recovery, %	79	83

In most cases the signals corresponding to oxolinic acid and flumequine in blank fish samples were below the limit of detection of the technique employed.

Many laboratories reported values close to the detection limits for the evaporated spiked extract samples.

3.6 Concluding remarks

The feasibility study demonstrated the suitability of the procedure developed for the production of homogeneous contaminated freeze-dried material of muscle and skin in natural proportions. Furthermore, extraction of the quinolones from fish material was investigated, and the results implemented in analytical methods at all participating laboratories. Essential prerequisites for a successful preparation and certification of a candidate CRM were provided. The feasibility study took more time and resources than planned in the project schedule, which originally did not foresee the inclusion of skin.

4. PREPARATION OF THE MATERIAL

4.1 Preparation of the homogenized batch

Breeding of salmon medicated with flumequine and oxolinic acid, respectively, together with unmedicated salmon was done at Nutreco A/S, Stavanger, Norway. In frozen condition they were brought to NCVM in Oslo, where the fish was analysed using the method described in [3].

Analyses of fish treated with oxolinic acid and analyses of fish treated with flumequine were carried out. On the basis of the results the average values of the two substances in musculature, including skin, were calculated. In addition, analyses of untreated fish were made to ensure that the fish was free from medicine.

In order to get an end-concentration of about 400 µg/kg of flumequine and 200 µg/kg of oxolinic acid in the fish material before freeze-drying, the proper amount of muscle/skin of untreated fish to be added to the treated fish was calculated.

In practice, two mixtures of muscles and the corresponding skin containing the double concentration of the desired end value were stored. Previous to storage, a definite amount of uncontaminated fish was added to fish with a well-known content of oxolinic acid to obtain the desired concentration in the mixtures. In the same way a mixture of flumequine-containing

fish was added to uncontaminated fish to obtain the desired concentration before storage. In such a way two mixtures were obtained, one containing about 800 µg/kg of flumequine and the other about 400 µg/kg of oxolinic acid. Equal amounts of the two mixtures were mixed and homogenized to obtain the desired concentrations.

During the preparation of the material, a skin-to-muscle ratio of 1:9 was used. The calculated amount of skin to be added was treated separately. This procedure applied to skin from both treated and untreated fish.

A weighted amount of skin, 10 g, was cut into small pieces with a pair of scissors. The material was transferred to a blender and 200 mL of distilled water was added. It was homogenized for 3 x 5 minutes and sifted through a sieve (pore size 0.5 mm). Afterwards the blender was flushed and the mixture was sifted 3 times using 10 mL of water each time. The amount of treated and untreated fish contained in the 10 g of skin was calculated in advance. This ratio was calculated on the basis of the results from the analyses which were carried out on treated and untreated fish to begin with.

Afterwards a definite amount of muscle (e.g. 90 g of muscle if the skin fraction constitutes 10 g) was taken. The sample was cut into pieces, approximately 3 x 3 cm, and was transferred to a blender. The ratio of treated to non treated fish to be employed was calculated beforehand.

The homogenized skin fraction of 10 g with water was subsequently added to the blender. This means that the blender contained a certain amount of oxolinic acid per g of fish (e.g. about 400 ng of oxolinic acid per g of wet weight) where the ratio between muscle and skin is 9:1.

The same procedure was carried out on flumequine-containing material, and equal amounts of the two mixtures were then mixed in the blender.

After homogenization all the material was sifted through a sieve (pore size 0.5 mm) to remove residues of connective tissue and other particles. At this stage the material was a liquid homogeneous mixture containing a lot of water. It was necessary to homogenize the fish skin. The liquid mixture was subsequently distributed in 250 mL plastic bottles – 100 mL in each. The bottles were frozen horizontally (to have a large surface) at –80 °C for 6 hours. Afterwards they were placed in a freeze-drier. The freeze-drying process lasted 7 days. Eventually the contents of all bottles were pressed through a sieve with a pore size of 0.5 mm, transferred to a homogenizer and homogenized for 2 x 5 minutes.

4.2 Bottling of samples

Totally 3.2 kg of freeze-dried material was produced for the certification. The material packed in dry ice was sent to European Commission DG Joint Research Centre, Institute for Reference Materials and Measurements, IRMM, (Geel, Belgium) in February 2002.

The final homogenization and bottling were done at IRMM. All salmon was homogenized for 1 hour in a Turbula mixer. A total number of 1400 units were bottled with 2.2 g powder in amber glass vials. After a vacuum treatment and filling with argon, the vials were closed with PTFE protected rubber stoppers and finally sealed with an aluminium cap. The last day of bottling (= production day) was 13-03-2002. Ten representative samples were taken for Karl Fisher water determination. The mean of the moisture content was calculated as 2.20% ± 0.14% and the water activity as 0.12 ± 0.01.

Particle size measurements have been carried out on bottles 0023, 0303, 0583, 1003 and 1283. The particle size distribution peaked at 200 µm with a top particle size of < 515 µm.

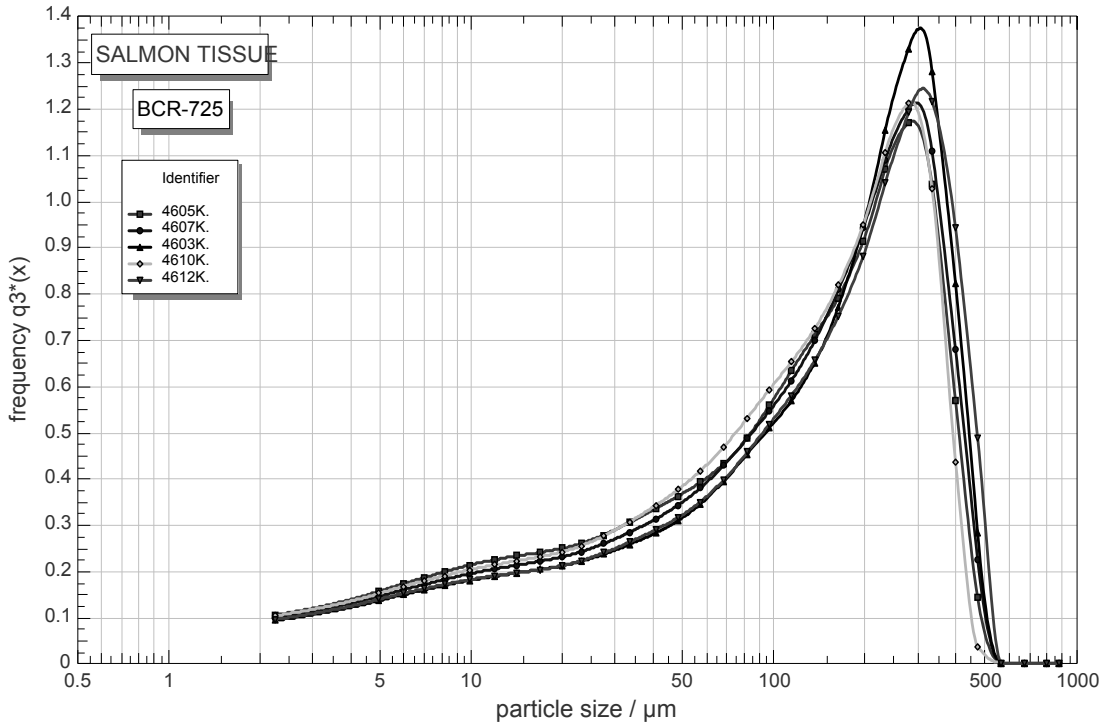


Figure 2 - Particle size distribution of freeze-dried material in bottles.

4.3 Dispatching of samples

Samples were dispatched to DTI for homogeneity and stability studies.

For the certification exercise 14 samples were dispatched to each of the 15 participants. Two separate vials closed with a screw cap, marked A and B, of evaporated spiked fish extract were also sent to the participants. These samples were supplied by Yndestad, NCVM, and spiked with oxolinic acid and flumequine. They were prepared analogously as described in section 3.4.

All these samples were packed in special containers filled with dry ice and dispatched within 24 hours by a private courier to the various laboratories.

5. TESTING OF THE MATERIAL

5.1 Homogeneity study

40 samples, out of all 1400 bottles, were selected for the evaluation of the between-bottle and within-bottle homogeneity. The samples were selected by IRMM to represent the whole production and had previously been stored at –80 °C.

From each bottle 2 sub-samples were analysed under repeatability conditions. Sample intake was 1.00 g.

5.1.1 Analytical method used for the homogeneity tests

The analytical work for homogeneity test and stability test for certification samples was done using the method described in [1], including 5 min in ultrasonic bath for the extraction. Detection limits were 10 µg/kg for flumequine and 5 µg/kg for oxolinic acid.

5.1.1.1 Extraction technique

1 g of freeze-dried material was reconstituted with 2 mL of water. 1 mL of 25% w/w NH_3 and 6 mL of acetonitrile were added. The sample was mixed for 1 min in a whirly mixer and 5 min in an ultrasonic bath. The homogenate was centrifuged and 3 mL of 5 mol/L NaCl was added to 5 mL of supernatant. To the lower layer were added 1 mL of 85% w/w H_3PO_4 and 4 mL of $CHCl_3$. After mixing and centrifugation, $CHCl_3$ was evaporated and the residue redissolved in the mobile phase.

5.1.1.2 HPLC analysis

The chromatographic separation of the quinolones was performed on a programmable HPLC system with a fluorescence detector and a 3 µL flow cell.

HPLC conditions were:

- Column: Phenomenex, Ultracarb, 5 ODS;
- Gradient eluent: A: 0.02 mol/L H_3PO_4/Acetonitrile/Tetrahydrofurane (650/200/150, v/v/v);
  B: Acetonitrile/H_2O (60/40, v/v);
- Column temperature: 25 °C;
- Injection volume: 50 µL;
- Flow rate: 0.8 mL/min;
- Fluorescence detection: Excitation: 325 nm; Emission: 360 nm.

Calibration was performed with flumequine and oxolinic acid supplied by Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of flumequine were prepared in acetonitrile/H_2O, (50/50, v/v) and of oxolinic acid in 0.5 mol/L NaOH. Working solutions were prepared in mobile phase. Correction was made for water content (1.7 % w/w) and recovery (82.4 % for flumequine and 81.0 % for oxolinic acid).

5.1.2 Results

The results of the homogeneity study are given in table 2.

Table 2 - ANOVA table for homogeneity of flumequine and oxolinic acid. Measurement unit: µg/kg.

	Source of variation	*SS*	*d.f.*	MS_q	*St. dev.*	*F*
Flumequine	Between Units	70,281.564	39	1,802.091	15.678	1.375
	Within Units	52,418.685	40	1,310.467	36.200	
	Total	122,700.249	79			
Oxolinic acid	Between Units	22,544.519	39	578.065	10.868	1.691
	Within Units	13,673.845	40	314.846	18.489	
	Total	36,218.364	79			

The measurements showed no relevant inhomogeneity among the samples.

5.2 Stability study

The isochronous layout for stability studies was applied to both analytes in BCR-725. Storage of samples was started in May 2002, immediately after the production of 1400 samples. The reference temperature for this study was –80 °C. At this temperature no impact on the analyte contents over a long time period can be expected. The analytical method used is analogous to the method used for the homogeneity study.

5.2.1 Short-term stability study

5.2.1.1 Design of the short-term stability study

The stability study mentioned in section 3.4 was done on material that essentially was the same as the BCR-725 material. The results of this study are complemented with additional data obtained as part of the stability study on the BCR-725 material produced in 2002. First the old data will be presented and subsequently the new data will be shown in the last paragraph of section *5.2.1.2*.

The reference temperature in all cases was –80 °C.

For the first stability study, a certain number of samples were stored at various temperatures: -18 °C, +4 °C and at room temperature in the dark. While the temperatures –18 °C and +4 °C were automatically kept constant, the room temperature varied from +20 °C to +30 °C. The study started on March 2001.

All samples stored at the different temperatures were put back to the reference temperature, -80 °C, after a pre-determined time period (see table 3).

Table 3 - Analytical scheme for the short-term stability study.

	Temperature in °C	*Number of analysed vials*			
Storage	*-80*	6			
temperature T	*-18*	2	3	3	3
	+4	3	3	3	3
	Ambient T	3	3	3	3
Duration of storage in weeks		21	6	2	1

All 41 samples were analysed under repeatability conditions using the method described in [1] and not taking water content into account. Visually, alterations could be seen on vials stored at 20-30 °C after 150 days. The material in these vials had turned brownish, while material in all other vials had maintained its original appearance.

In the new study, a temperature of +40 ºC was chosen, and three measurements were performed on time points 0, 1 and 2 months.

5.2.1.2 Results of the short-term stability study

The results of the first stability study are shown in figure 3 and 4. All analyte contents are given as relative contents corresponding to the average of the three measurements at temperature T, versus the average content for the six measurements of the reference sample (stored at –80 °C for 21 weeks). The value of 1.0 for the reference temperature of –80 °C shows the uncertainty of the method. In figure 3 and 4 the relative contents are plotted versus the storage time in weeks.

The results are also shown in tables 4 and 5 where:

$$R_{(T)} = \frac{X_T}{X_{\mathrm{Re}f}}$$

$$U_{(T)} = \left(RSD^2_{\mathrm{Re}f} + RSD^2_T\right)^{½} * R_{(T)}$$

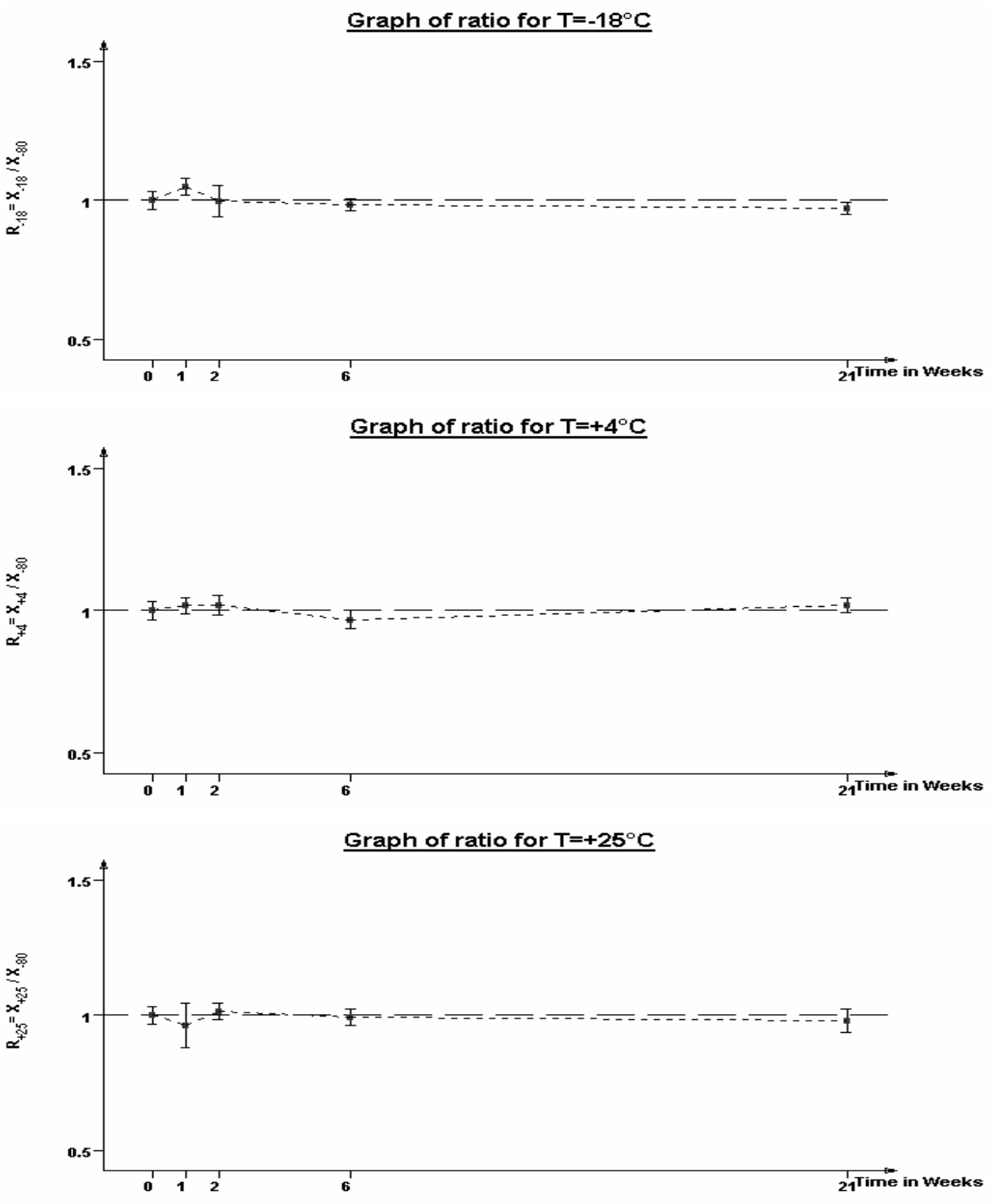


Figure 3 - Results of short-term stability study, flumequine

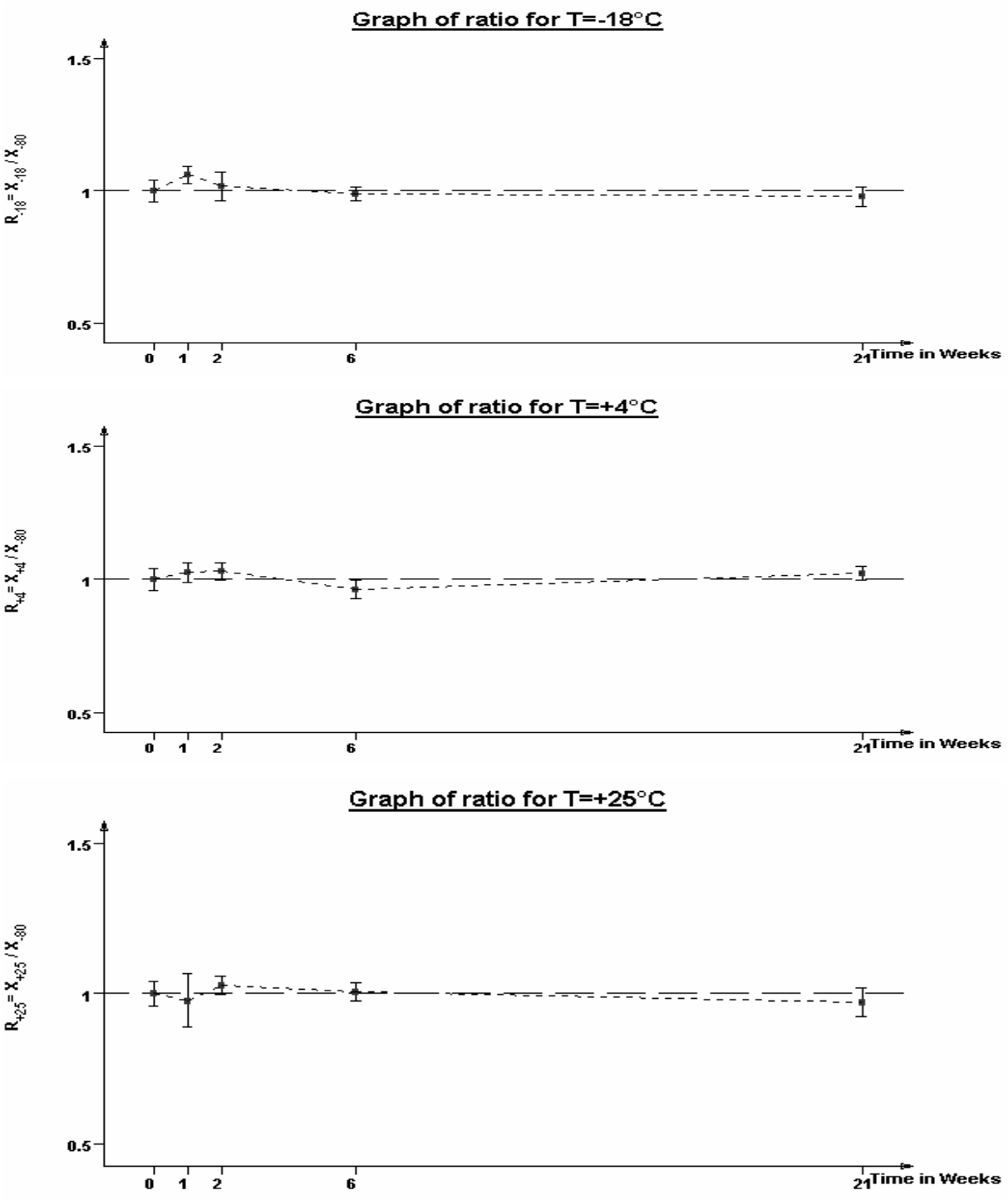


Figure 4 - Results of short-term stability study, oxolinic acid

Table 4 - Ratio of means, flumequine. Measurements are expressed in µg/kg

Weeks	*0*	*1*	*2*	*6*	*21*
R(-18) ± U(-18)	1.000 ± 0.033	1.050 ± 0.030	0.998 ± 0.056	0.985 ± 0.023	0.973 ± 0.021
R(+4) ± U(+4)	1.000 ± 0.033	1.017 ± 0.028	1.019 ± 0.033	0.968 ± 0.032	1.020 ± 0.028
R(25) ± U(25)	1.000 ± 0.033	0.965 ± 0.082	1.016 ± 0.031	0.995 ± 0.030	0.981 ± 0.044

Table 5 - Ratio of means, oxolinic acid. Measurements are expressed in µg/kg

Weeks	*0*	*1*	*2*	*6*	*21*
R(-18) ± U(-18)	1.000 ± 0.040	1.061 ± 0.032	1.017 ± 0.056	0.988 ± 0.026	0.979 ± 0.036
R(+4) ± U(+4)	1.000 ± 0.040	1.026 ± 0.037	1.031 ± 0.031	0.963 ± 0.036	1.024 ± 0.028
R(25) ± U(25)	1.000 ± 0.040	0.977 ± 0.088	1.027 ± 0.031	1.006 ± 0.029	0.974 ± 0.047

From T = -80 °C measurement the RSD of the method was calculated:

- RSD flumequine: 2.3 %
- RSD oxolinic acid: 2.8 %

The slopes corresponding to the data obtained at each temperature considered were calculated for both compounds. No statistically significant trend was observed according to the trend analysis described in [7], therefore no instability was associated to the flumequine and the oxolinic acid content of BCR-725. All relative contents, determined within the temperature range of interest and over the whole time period, are within the range of measurement uncertainty of the reference sample. A slight decrease of contents at room temperature is observed for both quinolones, but not outside the 5% and 1% uncertainty range.

The short-term stability study at +40 ºC, performed in 2002, gave as a result the data shown in Table 6 and 7, which are illustrated in Figures 5 and 6. The statistical evaluation on the slopes of the regression lines revealed a significant trend only in the case of flumequine.

Table 6 - Ratio of means, flumequine.

Weeks	*0*	*1*	*2*
R(+40) ± U(+40)	1.000 ± 0.0035	0.943 ± 0.032	0.946 ± 0.027

Table 7 - Ratio of means, oxolinic acid.

Weeks	*0*	*1*	*2*
R(+40) ± U(+40)	1.000 ± 0.041	0.955 ± 0.047	0.954 ± 0.029

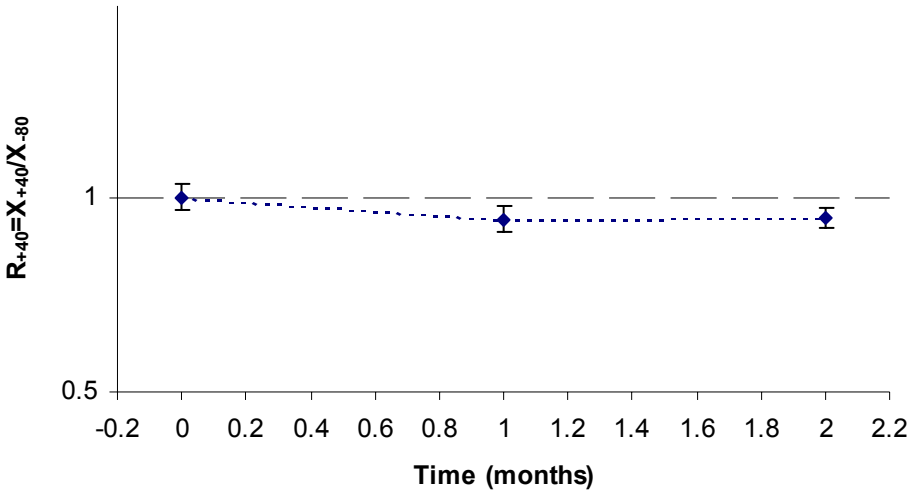


Figure 5- Results of short-term stability study 2002, flumequine.

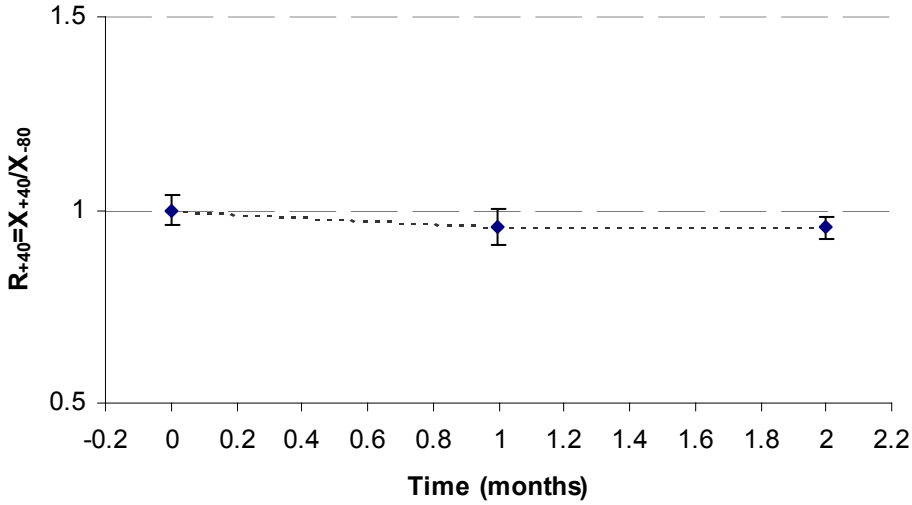


Figure 6- Results of short-term stability study 2002, oxolinic acid.

5.2.1.3 Conclusion on short-term stability

Flumequine and oxolinic acid contents were determined to be stable over the whole time period up to a temperature of +30 °C. No analyte losses outside the uncertainty level of the measurement (reference content at –80 °C) up to room temperature occurred. Oxolinic acid showed stability even up to +40 ºC. Based on these considerations and preventing from possible extreme conditions, it would be recommendable to refrigerate the reference material at least down to +4 ºC during the dispatch.

5.2.2 Long-term stability study

The long-term stability study was conducted over a period of 16 months, following an isochronous design. Three samples were analysed at temperatures of –18 ºC, +4 ºC and +21 ºC

ºC and at time points 0, 4, 8 and 16 months. The reference temperature employed was –80 ºC. The results obtained for flumequine and oxolinic acid, expressed as ratios, are illustrated in Tables 8 and 9, respectively.

Table 8 - Ratio of Means for flumequine concentrations, $R(T)=X_T/X_{ref} \pm$ Uncertainty(T). Time in months

	0	*4*	*8*	*16*
R(-18) ± U(-18)	1.000 ± 0.035	0.961 ± 0.035	0.949 ± 0.032	0.952 ± 0.027
R(+4) ± U(+4)	1.000 ± 0.035	0.950 ± 0.029	0.907 ± 0.067	0.918 ± 0.050
R(+21) ± U(+21)	1.000 ± 0.035	0.968 ± 0.026	0.922 ± 0.030	0.908 ± 0.045

Table 9 - Ratio of Means for oxolinic acid concentrations, $R(T)=X_T/X_{ref} \pm$ Uncertainty(T). Time in months

	0	*4*	*8*	*16*
R(-18) ± U(-18)	1.000 ± 0.041	0.969 ± 0.040	0.961 ± 0.033	0.960 ± 0.029
R(+4) ± U(+4)	1.000 ± 0.041	0.961 ± 0.041	0.962 ± 0.037	0.949 ± 0.051
R(+21) ± U(+21)	1.000 ± 0.041	0.983 ± 0.032	0.927 ± 0.030	0.935 ± 0.051

The statistical evaluation of the slope was performed for each temperature individually, -18 ºC, +4 ºC and +21 ºC. The calculations were carried out taking into account all the individual measurements included within the ratio values showed in Tables 8 and 9. The trends were found not significant either for flumequine or for oxolinic acid at each temperature considered. However the comparison of the negative slope values between different temperatures indicates an increase in the absolute value of the slope with the increase in temperature, which suggests certain level of degradation of the material.

A significant trend of the slope at the 95% confidence level was found when all individual measurements, regardless the temperature, were plotted as a single regression line *versus* time. Again, this is an indication of certain degree of degradation of the material, as already suggested. As a consequence, an additional uncertainty, which covers the possible maximum degradation during the shelf life of the material, is included as a contribution to the long-term stability uncertainty (see section 8.1.).

Due to the slow degradation rate observed, even at a temperature of –18 ºC, a long-term storage temperature of –70 ºC is established. As a preventive measure and waiting for the final assessment on the stability of the material, samples were kept at –70 ºC, from the finalisation of the production to the present time, to avoid any possible degradation.

6. CERTIFICATION MEASUREMENTS

Measurements for the certification exercise were performed according to the BCR Guidelines [4]:

- At least six independent measurements of flumequine and oxolinic acid content had to be performed, at least spread over two different days.
- The water content had to be determined the same day as the analyte measurements from the same bottle, but on a separate test portion.
- A blank determination had to be made at each day of analysis.
- The following materials and quantities were distributed for the certification of BCR-725:
  - 14 bottles of approximately 2.2 g of BCR-725
  - 1 vial of evaporated spiked extract containing known amounts of flumequine and oxolinic acid.
  - 1 vial of evaporated spiked extract containing unknown amounts of flumequine and oxolinic acid.

The analytical methods used for the determination are described in detail in section 6.6. Each laboratory used its own optimized procedure for sample preparation, extraction, clean up (where appropriate), method of injection, chromatographic separation and detection. These methods were optimized after the feasibility study. All procedures and measurements were recorded and reported by each laboratory according to a given protocol in reporting sheets provided by the co-ordinator of the study.

Twelve of the participating laboratories delivered the results in the framework of the certification exercise. One laboratory withdrew from the exercise, and another laboratory withdrew flumequine results.

6.1 Calibration standards

Each laboratory prepared separate calibration solutions according to its own laboratory procedures. These solutions were used for calibrating the relevant detector within its dynamic range.

The calibration solutions were prepared from chemicals from Sigma-Aldrich, Fluka and ICN, and taking purity into account. Being pharmaceutical products, certificates of analysis for the particular lot number of the chemicals were available with purities and other informations. Different lot numbers were used, and neither with FLD nor with MS, peaks from impurities were detected.

6.2 Internal standards

No laboratories reported use of internal standards.

Table 10- Purity of flumequine calibrant

Lab. code	*% C (~ % Purity)*	*% N (~ % Purity)*	*% Purity HPLC*	*% Purity Titration*	*% Purity Used in calculations*
0			95,7	98,6	97,2
1			94,6	100,7	94,6
2			94,6	100,7	100
3	64,2 (99,7)	5,4 (100)	99,9		99,9
4	64,2 (99,7)	5,4 (100)	99,9		99
5	64,2 (99,7)	5,4 (100)	99,9		99,9
6	64,2 (99,7)	5,4 (100)	99,9		99,9
7			94,6	100,7	94,6
8	64,2 (99,7)	5,4 (100)	99,9		99,9
9			99,9		100

Table 11- Purity of oxolinic acid calibrant.

Lab. Code	*% C (~ % Purity)*	*% N (~ % Purity)*	*% Purity Thin Layer Chromatography*	*% Purity Titration*	*% Purity Used in calculations*
0	59,7 (100)	5,4 (100)	> 99		100
1	60,0 (100)	5,4 (100)	> 99		100
2	59,7 (100)	5,4 (100)	> 99		100
3	59,65 (99,8)	5,33 (99,4)	99		98
4	59,65 (99,8)	5,33 (99,4)	99		98
5			99	100,5	99
6	59,65 (99,8)	5,33 (99,4)	99		99
7	59,65 (99,8)	5,33 (99,4)	99		99
8	59,65 (99,8)	5,33 (99,4)	99		99
9	59,7 (100)	5,4 (100)	> 99		99
10				> 97	100

6.3 Calibration

All standards and samples were prepared on a mass basis using calibrated balances. Working solutions were prepared by mass controlled dilution. Laboratories code 4 and 8 used dilutions by weight. The agreement of the measurement results of the laboratories was assessed using a ”known” and ”unknown” sample of differing compositions. As a quality check of the calibration, the ”known” solution had to be measured by each participant before the measurements of the rest of the samples.

The dynamic range of the detector was established for each analyte individually from calibra-

tion solutions of different concentrations. The dynamic range of the detector is defined as the range where the detector response per mass unit versus the mass injected shows a linear response. At least 4 calibration points had to be used. The calibration of the detector was verified at least once in a sequence of injections on each measurement day.

6.4 Analytical blanks

Analytical blanks were performed at each day of analysis. All solvents used were of the highest purity grade.

6.5 Determination of the water content

The water content was determined by heating in an oven at a temperature of 102 °C or 105 °C until constant weight. The reported mean water contents for eight samples ranged between 1.0 and 3.3 % w/w with an overall mean of 2.0 % w/w.

In agreement with BCR Guidelines [4] the analyte contents determined in the certification exercise were corrected for the water content as reported by the participants.

Table 12 - Water content in the eight freeze-dried samples.

Lab code	*Water content, % w/w, used for result calculation of eight samples*								*Mean*	*Std. dev.*
0	1,7	1,6	1,7	1,8	1,8	1,6	2,0	1,7	**1,8**	**0,1**
1	2,4	1,9	2,3	2,2	2,0	2,2	2,2	2,6	**2,3**	**0,2**
2	1,8	1,8	1,8	1,9	1,9	1,9	1,8	1,7	**1,8**	**0,1**
3	2,4	2,4	2,3	2,5	2,1	2,1	2,0	2,5	**2,3**	**0,2**
4	2,0	2,0	2,0	2,2	2,4	2,2	1,8	1,6	**2,0**	**0,3**
5	1,0	1,7	1,5	1,0	1,6	1,7	1,4	1,6	**1,5**	**0,3**
6	3,4	2,8	3,3	3,2	3,3	3,5	2,8	4,3	**3,3**	**0,5**
7	2,5	2,4	2,2	1,8	2,1	5,3	1,4	6,4	**3,0**	**1,8**
8	1,8	1,7	2,0	1,8	1,9	2,1	2,0	2,0	**1,9**	**0,1**
9	0,9	0,9	1,4	1,1	1,0	1,2	1,2	0,9	**1,1**	**0,2**
10	1,8	1,4	1,5	1,0	1,0	1,8	1,6	1,9	**1,5**	**0,4**

6.6 Analytical methods used for certification

The analytical procedures used for the determination of analyte contents in BCR-725 consist in four distinct steps of analytical work:

1. Sample preparation and extraction
2. Sample clean up
3. Chromatographic separation by HPLC
4. Calibration and detection

6.6.1 Sample preparation and extraction

The evaporation of centrifugate liquid used for clean up is listed in table 13.

Participants adjusted the solvent extraction step following conclusions drawn from the feasibility study, where all solvents and extraction procedures used were evaluated for their extraction efficiency (reproducibility and recovery). Most laboratories used reconstitution of the freeze-dried material by adding 2 parts of water. The extraction solvents and extraction times applied are listed in table 13.

Table 13 - Preparation of centrifugate liquid

Lab. code	*Reconstitution*	*Extraction solvent* *Type*	*Amount*	*Extraction*	*Extraction time*	*Centrifugation time*
0	1 g sample + 2 g water, M	Acetonitrile + 25%w/w NH_3 (6+1)	7 mL	V,U	6 min.	3 min.
1	1 g sample + 2 g water C : 10 min., M C : 10 min.	Acetonitrile + buffer, pH 9.1 (2+3)	1 mL	U,V	3 min.	3 min.
2	1 g sample + 2 g water, M C : 15 min.	0.5 mol/L NaOH + 67%w/w acetone in water (1+4)	5.5 mL	H,U,C,V	3 hours	10 min.
3	0.5 g sample + 2 mL 0.2 mol/L HCl	Ethyl acetate	2 x 4 mL and 2 mL	3 x HH	3 x 10 min.	3 x 5 min.
4	0.5 g sample	0.05 mol/L potassium phosphate buffer pH 7.4	3 x 10 mL	3 x U	3 x 10 min.	3 x 5 min.
5	1 g sample + 2 mL water C : 15 min.	Ethyl acetate	2 x 10 mL	2 x V	2 x 1 min.	2 x 5 min.
6	1 g sample + 2 mL water + 1 mL of (0.002 mol/L H_3PO_4/acetonitrile/tetra-hydrofuran, 64 /21/15, v/v/v)	Acetone + 25%w/w NH_3 (5+1)	6 mL	H,U,C,V	16 hours	5 min.
7	1 g sample + 2 mL water, V, C : 1 hour	Acetonitrile + 25% NH_3 (6+1)	7 mL	V,U,C,V	16 hours	10 min.
8	0.5 g sample + 1 mL water, M	Dichloromethane	2 x 10 mL	2 x (M,C)	2 x 5 hours	2 x 10 min.
9	1 g sample + 2 g water, H	Methanol	5 mL	2 x (M,C,H)	1 x 12 hours + 2 min.	2 x 10 min.
10	1 g sample + 2 g water, M	Ethyl acetate	2 x 10 mL	2 x V	2 x 10 min.	2 x 10 min.

C = Contact time, standing
H = Ultraturrax
HH = Homogenisation with Heidolph
M = Mix, manually
U = Ultrasonication
V = Vortex

6.6.2 Clean-up

Clean-up of extracts did range from protein precipitation + centrifugation to several back extractions or use of C_{18}-cartridge. The techniques applied are included in table 14.

Table 14 - Preparation of solution to inject into HPLC from centrifugate liquid.

Lab. code	*Clean-up*
0	5 mL supernatant was mixed with 3 mL 5 mol/L NaCl, and centrifugated 1 min. Lower layer was mixed with 1 mL 85%w/w H_3PO + 4 mL chloroform. After centrifugation the chloroform phase was evaporated at 10 °C under N_2 stream. The residue was re-dissolved in 3 mL mobile phase and injected after filtration through 0.5 µm cellulose acetate filter.
1	Supernatant was evaporated at 50 °C under N_2 for 15 min. 0.5 mL buffer, pH 9.1 + 300 µl hexane was added. V: 20 sec. Centrifugation 3 min. and lower phase injected.
2	1 mL supernatant was mixed with 1 mL acetonitrile. V, C: 10 min. (protein precipitation). After centrifugation 10 min. the upper phase was injected.
3	Combined supernatants were mixed with 4 mL 1 mol/L NaOH. HH: 10 min. and centrifugation 5 min. Repeated, and combined aqueous phase was mixed with 1 mL 85%w/w H_3PO_4. 3.5 mL chloroform was added, HH: 10 min. and centrifugation 5 min. Repeated, and combined chloroform phase was evaporated at 40 °C under N_2 stream. The residue was redissolved in 1 mL mobile phase and injected.
4	Combined supernatants were filtered through 2 µm filter and 15 mL loaded on a conditioned Discovery C18 cartridge. After wash with 3 mL water the quinolones were eluted with 5 mL of a mixture of methanol and 25%w/w NH_3 (75/25, v/v). The eluate was evaporated under N_2 stream (< 50 °C). The residue was redissolved to 1.0 g with 0.05 mol/L potassium phosphate buffer, pH 7, before injection.
5	Combined supernatants were evaporated at 45 °C under N_2 stream. The residue was redissolved in 2 mL mobile phase (V: 1 min., U: 3 min.) and 2 mL hexane. V: 1 min. Centrifugation 5 min., and injection after discarding upper hexane phase.
6	1 mL supernatant was mixed with 0.5 mL 3 mol/L H_3PO_4 and 1.0 mL acetone was added. V and centrifugation 3 min. 1 mL supernatant was mixed with 1 mL water, and injected after filtration through Spin-X-filter and centrifugation 5 min.
7	2.5 mL supernatant was mixed with 3 mL hexane. V, centrifugation and hexane phase removed. The other phase was evaporated at 50 °C under N_2 stream. The evaporated sample was redissolved in 0.5 mL mobile phase and injected after filtration through Spin-X-filter.
8	Combined organic phases were back-extracted with 5 mL 0.01 mol/L NaOH. After centrifugation the acqueous phase was injected.
9	Combined supernatants were mixed with 2 mL 0.1 mol/L NH_3 + 2 mL hexane. After centrifugation 10 min. hexane phase was discarded. Hexane washing was repeated 2 times. Residual phase was reduced to approx. 2 mL with a rotary evaporator. 6 mL ethyl acetate was added, and after centrifugation 10 min. the upper phase was evaporated to dryness using rotary evaporator. The residue was redissolved in mobile phase and injected.
10	Combined supernatants were filtered and evaporated at 50 °C under N_2 stream. The residue was dissolved in 2 mL 0.01 mol/L oxalic acid, pH 3, and washed with 2 mL hexane. Aqueous layer was filtered before injection.

C = Contact time, stading
HH = Homogenisation with Heidolph
V = Vortex

6.6.3 High performance liquid chromatography

The HPLC conditions as used by the participants are summarized in tables 15, 16 and 17.

Table 15 - HPLC conditions, I.

Lab. Code	*Analytical column*
0	Phenomenex, ultracarb, 5 ODS
1	PLRP-S-100, 5 µm, 150 x 4.6 mm
2	Phenomenex C18 ultracarb 5 ODS (30) 150 x 4.60 mm, 5 micron
3	PuroSpher 180 RP-18 E 125 x 4 mm i.d., 5 µm particle size + guard column 4 x 4 mm i.d. (Merck)
4	Symmetry C18, 5 micra, 4.6 x 150
5	Lichrosorb RP-8, 5 µm, 250 mm x 4.6 mm + guard-column. All-Guard C8, 5 µm, 7.5 mm x 4.6 mm
6	PLRS 15 cm x 4.6 mm, 5 mm
7	PLRP-S, 100Å, 5 µm, 150 x 4.6 mm
8	Inertsil C8 (150 x 4.6 mm) + guard column Inertsil C8
9	Phenomenex aqua 250 x 3 mm
10	Lichrospher 60 RP – select B (5 µm) Merck

Table 16 - HPLC conditions, II

Lab. code	*Mobile phase*
0	A : 0.02 mol/L H_3PO_4/acetonitrile/tetrahydrofurane (650/200/150, v/v/v) B : Acetonitrile/H_2O (60/40, v/v)
1	0.02 mol/L H_3PO_4/acetonitrile/tetrahydrofurane (72/16/12, v/v/v)
2	0.02 mol/L phosphoric acid (pH = 2.5)/acetonitrile (55/45, v/v)
3	Orthophosphoric acid solution 0.02 mol/L (67%) /acetonitrile (67/33, v/v)
4	Acetonitrile/0.02 mol/L phosphate buffer pH 3.0 (34/:66, v/v)
5	Oxalic acid 0.01 mol/L at pH 3/acetonitrile (60/40, v/v)
6	0.01 mol/L H_3PO_4 / acetonitrile/ tetrahydrofurane (64/21/15, v/v/v)
7	0.02 mol/L H_3PO_4 /acetonitrile/tetrahydrofurane /62.5/ 22.5/15.0, v/v/v)
8	0.01 mol/L oxalic acid/acetonitrile (55/ 45, v/v)
9	0.01 mol/L oxalic acid/methanol (30/70, v/v)
10	Acetonitrile/N,N-dimethylformamide/0.01 mol/L oxalic acid (27/6/67, v/v/v)

Table 17 - HPLC conditions, III

Lab. code	*Amount injected (µl)*	*Flow rate (mL/min.)*	*Column temp. (°C)*	*Column in thermostated oven*
0	50	0.8	25	+
1	100	0.8	50	+
2	20	0.8	30	+
3	10	0.8	27	+
4	20	1	Room temperature	+
5	50	1	Room temperature	-
6	50	0.7	Room temperature	-
7	20	1	30	-
8	20	1.5	Room temperature	+
9	5	0.35	35	-
10	20	1	40	+

6.6.4 Detection

Most laboratories used fluorescence detection with a linear range defined by at least 4 calibration standards.

Table 18 - Wavelengths used for fluorescence detection and linear calibration range.

Lab. code	*Exitation wavelength (nm)*	*Emission wavelength (nm)*	*Linear calibration range, flumequine µg/kg dry tissue*	*Linear calibration range, oxolinic acid µg/kg dry tissue*	*Number of calibration standards used*
0	325	360	2400	2200	5
1	320	360	1800	1800	4
2	325	360	5000	2500	6
3	325	365	5000	5000	6
4	312	366	8000	8810	5
5	327	369	1200	1200	7
6	325	360	4000	2000	6
7	325	380	3000	3000	6
8	328	365	4000	4000	6
9	-	-	4000	4000	7
10	246	360	-	500	5

Laboratory code 9 used mass spectrometry for detection and a 7-points calibration. MS conditions were:

- Ionisation: Electrospray, positive mode
- Ions: 262.1 (MH) and 244.0 ($MH-H_2O$); SIM scan mode

6.7 Recovery experiments

All participating laboratories were requested to perform recovery experiments in order to obtain quantitative information on the extraction efficiency and recovery after clean-up. The method of standard addition was applied to obtain recovery data. The spiked amounts should equilibrate with the fish material for 1 hour at least. Recovery figures were used to calculate the final results.

The recommended method of standard addition involved spiking of the freeze-dried material at four different levels of analyte concentration: at 50, 100, 150 and 200 % of the target value of these analyte content. Recovery figures were calculated by means of linear regression.

Recovery figures are shown in table 19. Recovery experiments were made on different days. Two laboratories used the same day recovery to correct for the final result, and nine laboratories used recovery based on all recovery measurements in the final result calculation.

Table 19 - Recovery % and standard deviation of the recovery range %.

	Flumequine			*Oxolinic acid*		
Lab. code	*R1 ± Std.dev. R1*	*R2 ± Std.dev.R2*	*R(1+2) ± Std.dev. R(1+2)*	*R1 ± Std.dev. R1*	*R2 ± Std.dev.R2*	*R(1+2) ± Std.dev. R(1+2)*
0			82,4 ± 3,1			81,0 ± 2,6
1	96,4 ± 2,8	97,8 ± 6,0		100,2 ± 4,3	105,3 ± 3,8	
2	67,8 ± 0,5	67,1 ± 0,8		67,9 ± 0,5	66,8 ± 0,7	
3			100,1 ± 0,9			100,5 ± 1,2
4			94,2 ± 2,2			86,2 ± 1,7
5			91,4 ± 2,3			99,6 ± 2,9
6			84,6 ± 6,7			98,7 ± 4,1
7			53,2 ± 1,8			56,9 ± 1,0
8			85,5 ± 3,4			92,9 ± 4,1
9			82,2 ± 3,1			86,9 ± 3,3
10						65,9 ± 2,7

R1 = Recovery % day 1

R2 = Recovery % day 2

R(1+2) = Recovery % using experiments for both days.

7. TECHNICAL DISCUSSION

The results of the certification study were discussed at an evaluation meeting of all participants on 23 September 2002. All steps of the procedure were reviewed in detail and the respective data were carefully discussed and scrutinised.

The outcome of this technical discussion of the results is summarised in the following paragraphs:

Need for a blank reference fish material

During the technical discussion the potential need for a blank reference material was discussed, e.g. as produced in case of similar reference materials for banned veterinary drug such as clenbuterol. However, it was felt, that it is easy to buy uncontaminated fish, which would be fit for this particular purpose. Moreover, the current MRL values for oxolinic acid and flumequine are significantly higher than the detection limits that can be achieved by the analytical techniques in the certification campaign, so there is no real need for such material.

Water content

Some results from laboratory code 7 were higher than the average level. However, according to proofs provided by the laboratory, all samples had been handled correctly, e.g. samples were taken to a desiccator to equilibrate to room temperature before opening. No technical reasons were found to reject the higher results and they were consequently kept for the establishment of the certified values.

Extraction of the target compounds:

The certification campaign showed that no correlation between the type of solvent used for extraction or extraction time and extraction yields was found. Nevertheless, it should be emphasised that good extraction yields require reconstitution of the freeze-dried material or repeated extractions with aqueous solutions.

It could also be shown that extraction of freeze-dried fish material requires generally a shorter extraction time than extraction of fresh fish, where at least 5 hours' extraction is required [3].

Recovery percentages as low as 50 were accepted provided that the uncertainty of the recovery % (95 % interval) is within ± 10 % rel.

Laboratory code 10 obtained a recovery % too low for flumequine and therefore withdrew those results.

8. EVALUATION OF UNCERTAINTIES AND CERTIFIED VALUES

The evaluation of uncertainties in the context of certification exercises has evolved over the past decade. Nowadays, certified values should be accompanied by uncertainty statements in compliance with the requirements set by GUM [5]. While the design of new certification projects consider the needs for a proper estimation of the various uncertainty sources such as stability and homogeneity, older campaigns aimed only on qualitative statements (yes/no decisions) whether a material was stable and homogeneous.

The evaluation described hereafter is based on a concept described by Pauwels *et al.* [6 and literature cited] and uses available data discussed in the previous chapters.

8.1 Uncertainty evaluation

8.1.1 Conceptual considerations

Based on the findings obtained in the stability and homogeneity studies as well as on the scattering of results in the batch characterisation, estimates for u_{bb} (homogeneity), u_{lts} (long-term-stability) and u_{char} (batch characterisation) were obtained and combined according to the following equation:

$$U_{CRM} = 2 \cdot \sqrt{u_{bb}^2 + u_{lts}^2 + u_{char}^2}$$

Due to the selected transport conditions established for dispatch, the uncertainty constituent for short-term stability (u_{sts}) is negligible and consequently not included in the overall uncertainty. The estimation of the other uncertainty sources is described below.

8.1.2 Uncertainty source "homogeneity"

The homogeneity study is exhaustively described in section 5.1 and results have been evaluated by means of one-way ANOVA. From this data (Table 2), an estimation of u_{bb} was derived from the homogeneity study as described by Linsinger *et al* [7].

Values for u^*_{bb} and s_{bb} were calculated accordingly:

$$u_{bb}^* = \sqrt{\frac{MS_{within}}{n}} \cdot \sqrt[4]{\frac{2}{\nu_{MSwithin}}}$$

where n is the number of replicates per unit and $\nu_{MSwithin}$ the degrees of freedom of MS_{within}; and

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}}$$

As a principle the higher value between s_{bb} and u^*_{bb} is adopted as u_{bb}. The results of these calculations are shown in Table 20.

8.1.3 Uncertainty source "stability"

For both flumequine and oxolinic acid a slow degradation rate was observed upon storage of 16 months, as described in section 5.2.2. At –18 ºC the slope of the regression line indicated an almost negligible degradation, therefore it was decided to use the data corresponding to this temperature for the estimation of the u_{lts}. These data was used and evaluated using the approach employed by Linsinger *et al* [7].

The u_{lts} comprises two main contributions. A term due to the degradation mentioned above corresponding to a bias (u_{b1}), calculated as a rectangular distribution of the slope (b). And a second term, which considers the uncertainty associated to such bias (u_{b2}). The u_{lts}, within the chosen shelf life of the material (x_{shelf}), is estimated as follows:

$$u_{lts} = \sqrt{{u_{b1}}^2 + {u_{b2}}^2} \cdot x_{shelf}$$

Where,

$$u_{b1} = \frac{b}{\sqrt{3}}$$

$$u_{b2} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}}$$

As a result u_{lts} values of 5.4 % for oxolinic acid and of 6.0 % for flumequine were calculated for a shelf-life of 36 months.

8.1.4 Uncertainty source "batch characterisation"

An estimate for u_{char} was derived from the standard error obtained on the mean of laboratories means.

8.1.5 Uncertainty budget

Based on the uncertainty contributions mentioned in sections 8.1.2 to 8.1.4 the following uncertainty budget is established (Table 20):

Table 20 - Uncertainty budget for BCR-725

	Oxolinic acid	*Flumequine*
u_{bb} (s_{bb}) *[in rel. %]*	1.82	1.34
u^*_{bb} *[in rel. %]*[a]	0.81	0.84
u_{lts} *[in rel. %]*	7.35	8.59
u_{char} *[in rel. %]*	1.80	1.73
coverage factor k	2	2
Shelf life [months]	36	36
U_{CRM} *[in rel. %]*	15.56	17.73
Mean [in µg/kg]	***596.91***	***1171.32***
Uncertainty [in µg/kg]	***92.91***	***207.63***
Rounded according to ISO 31-0 [8] and expressed in µg/kg	**600 ± 100**	**1170 ± 210**

[a] not used for combined uncertainty

8.2 Certified values

The certified flumequine mass concentration (dry mass) for BCR-725 is:

1170 ± 210 µg/kg.

The certified oxolinic acid mass concentration (dry mass) for BCR-725 is:

600 ± 100 µg/kg.

9. TRACEABILITY

All measurements are traceable to HPLC. Different extraction, clean-up methods and two detection methods were employed, FLD and MS.

10. INSTRUCTIONS FOR USE

10.1 Transport and storage

The reference materials are supplied in lyophilised form sealed under vacuum in brown glass vials. Shipment by postal services or carrier should be done using appropriate cooling elements. On receipt, the materials should be stored in the freezer. The materials can be handled as non-hazardous substances.

10.2 Recommendations for analysis

As already mentioned in section 7 it should be emphasised that good extraction yields require reconstitution of the freeze-dried material or repeated extractions with aqueous solutions. Test portions of BCR-725 should therefore be treated accordingly.

The homogeneity of BCR-725 was demonstrated at the sample intake of 1.0 g. The certification study did show excellent results with sample intake of 0.5 g. Therefore, a minimum sample intake of 0.5 g is recommended. Extracts from BCR-725 should be prepared immediately before analysis, and should be prepared from reconstituted material, e.g. 1 g BCR-725 + 2 mL water.

The water content of the reconstituted material is approximately equal to the water content in fresh fish. It has to be noticed that extraction of fresh fish may require more than 5 hours [3]. Determination of the minimum fresh fish extraction time for the particular analytical method used is recommended.

If the detection limits of the analytical method are high compared to the MRL values, it may be necessary to prepare and analyse in-house blank fish material.

BCR-725 has a water content of approximately 2 % w/w. Whether BCR-725 is stored at frozen conditions, or at 4-6 °C, the bottles should be taken to a desiccator to equilibrate to room temperature before opening.

10.3 Use of the certified values

This material may be used to check the precision and the trueness of the laboratory measurement process according to ISO Guide 33 [9].

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12. ANNEX – TABLE OF INDIVIDUAL RESULTS AND GRAPHICAL PRESENTATIONS

Table 21 - Mass fraction of flumequine – BCR - 725 (µg/kg)

Lab code (technique)	*Mean*	*STDev*	*H.W. CI (95%)*	*Sample #1*	*Sample #2*	*Sample #3*	*Sample #4*	*Sample #5*	*Sample #6*	*Sample #7*	*Sample #8*
0 (HPLC/FLD)	**1,116.0**	30.3	**25.3**	1,160.0	1,107.0	1,112.0	1,070.0	1,082.0	1,138.0	1,141.0	1,118.0
1 (HPLC/FLD)	**1,242.3**	43.3	**36.2**	1,241.6	1,209.3	1,284.4	1,195.8	1,182.0	1,254.0	1,268.9	1,302.7
2 (HPLC/FLD)	**1,160.9**	43.8	**36.6**	1,103.0	1,129.0	1,128.0	1,128.0	1,221.0	1,184.0	1,205.0	1,189.0
3 (HPLC/FLD)	**1,194.2**	14.4	**12.0**	1,197.4	1,189.3	1,200.6	1,195.5	1,188.6	1,179.7	1,224.1	1,178.9
4 (HPLC/FLD)	**1,135.0**	35.1	**29.4**	1,118.2	1,095.2	1,125.8	1,104.4	1,112.1	1,191.8	1,161.9	1,170.6
5 (HPLC/FLD)	**1,105.9**	27.9	**23.3**	1,104.0	1,148.7	1,140.2	1,114.6	1,097.8	1,078.3	1,068.1	1,096.0
6 (HPLC/FLD)	**1,239.8**	28.6	**23.9**	1,246.0	1,229.0	1,293.0	1,233.0	1,190.0	1,231.0	1,249.0	1,247.0
7 (HPLC/FLD)	**1,269.3**	36.6	**30.6**	1,274.0	1,217.0	1,253.0	1,248.0	1,269.0	1,345.0	1,266.0	1,282.0
8 (HPLC/FLD)	**1,168.7**	17.9	**15.0**	1,166.6	1,137.6	1,180.3	1,165.9	1,182.9	1,147.9	1,187.1	1,181.3
9 (LC/MS)	**1,081.1**	35.3	**29.5**	1,107.0	1,118.0	1,024.0	1,070.0	1,069.0	1,110.0	1,042.0	1,109.0
95% H.W. Confidence Interval	*45,7*										
95% H.W. Tolerance Interval	*215,9*										

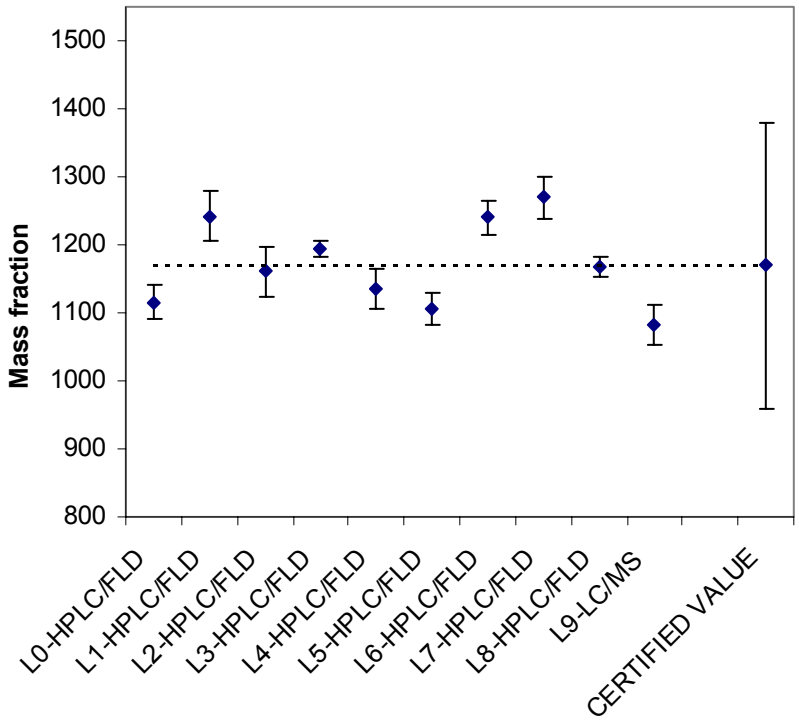

Figure 5 – Mean values and CIs (95%) of flumequine, expressed as mass fraction (µg/kg), obtained by the different participant laboratories. Certified value and uncertainty are also shown.

Table 22 - Mass fraction of oxolinic acid - BCR - 725 (µg/kg).

Lab. code	*Mean*	*STDev*	*H.W. CI (95%)*	*Sample #1*	*Sample #2*	*Sample #3*	*Sample #4*	*Sample #5*	*Sample #6*	*Sample #7*	*Sample #8*
0	**554.3**	14.9	**12.4**	578.0	554.0	554.0	533.0	535.0	565.0	561.0	554.0
1	**611.8**	18.4	**15.4**	634.9	609.9	636.4	612.8	583.1	597.9	599.7	619.7
2	**588.3**	23.4	**19.5**	553.0	575.0	569.0	572.0	610.0	610.0	610.0	607.0
3	**581.4**	7.2	**6.0**	576.3	572.4	584.8	576.0	581.4	581.4	595.9	582.9
4	**563.6**	22.7	**19.0**	565.7	594.1	568.4	558.9	552.1	594.3	547.6	527.5
5	**597.7**	12.2	**10.2**	586.8	610.7	612.6	606.5	596.1	579.7	587.1	602.5
6	**610.0**	17.4	**14.6**	630.0	622.0	634.0	613.0	586.0	597.0	601.0	597.0
7	**648.5**	20.0	**16.8**	673.0	646.0	654.0	645.0	641.0	678.0	616.0	635.0
8	**578.8**	19.1	**16.0**	591.9	582.2	564.2	598.4	603.7	546.9	567.5	575.5
9	**564.3**	13.5	**11.3**	574.0	574.0	544.0	579.0	545.0	572.0	559.0	567.0
10	**667.3**	22.9	**19.1**	705.0	665.0	698.0	666.0	640.0	654.0	661.0	649.0
95% H.W. Confidence Interval	*23,9*										
95% H.W. Tolerance Interval	*115,9*										

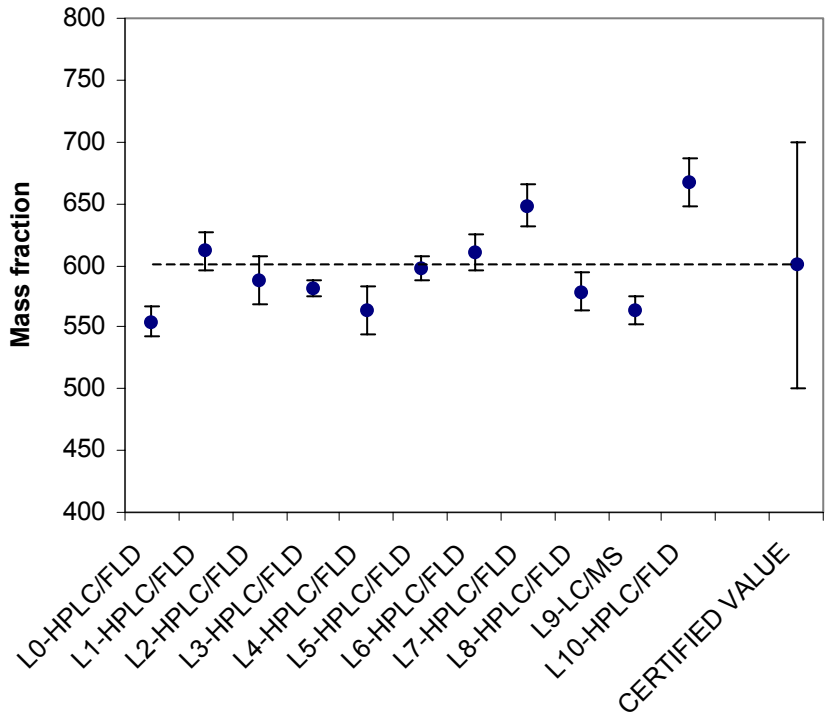

Figure 6 – Mean values and CIs (95%) of oxolinic acid, expressed as mass fraction (µg/kg), obtained by the different participant laboratories. Certified value and uncertainty are also shown.

Figures

The length of the horizontal dotted bar corresponds to the 95% confidence interval. The vertical dotted line features the certified value (average of laboratory averages).

The laboratory code is followed by an indication of the detector used for analyte determination (for details, see list of abbreviations).

European Commission

EUR 21181 – DG Joint Research Centre, Institute for Reference Materials and Measurements –
The certification of the contents (mass fractions) of flumequine and oxolinic acid in freeze-dried salmon tissue, BCR-725
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Abstract
This report describes the preparation, homogeneity, stability and certification studies of a freeze-dried salmon tissue (BCR-725) certified for its contents of flumequine and oxolinic acid. A description of the analytical procedures used in the homogeneity and stability studies as well as in the certification study is included. All individual results of the certification study are reported. All relevant data from the homogeneity and stability studies and certification measurements are presented.

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