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Development and Validation of HPLC-methods for the official control of Coccidiostatics and Antibiotics used as Feed AdditiveS (SMT4-CT98-2216)

Co-ordinator: Dr. J. de Jong

FINAL REPORT

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CANFAS - 2nd Collaborative study for the determination of olaquindox in feedingstuffs by HPLC

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CONTENTS page

SUMMARY

This report describes the results of the 2nd collaborative study of an HPLC method for the growth promoter olaquindox in two piglet feeds. The collaborative study forms part of the EU-project "Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (CANFAS, SMT4-CT98-2216).
In the first collaborative study many laboratories reported difficulties with the practicability of the method due to the low ratio between the volume of extraction solvent (50 ml) and the weight of feed (25 g). For this reason the CANFAS-method was modified in such a way that the ratio between the extraction volume and the sample weight was increased to 5. A second round of collaborative studies for final validation of the method was organised.
The principle of the method is as follows: The sample is extracted by a mixture of water - methanol. The content of olaquindox is determined by reversed-phase high-performance liquid chromatography (HPLC) with UV-detection at 380 nm.
The samples that were tested in the collaborative study were 2 piglet feeds with declared olaquindox contents of 2,5 mg/kg and 10 mg/kg respectively. The feed samples were sent to the participants as blind duplicates. The participants were asked to do duplicate determinations per sample.
Results were reported by 22 laboratories. Statistical evaluation was performed according to ISO 5725. The results show that with the modified method acceptable results are obtained for repeatability (rsd, < 10 %) and reproducibility (Horrat ratios < 2).
During the first collaborative study blank samples were analysed: no interfering substances were detected, so the results obtained for the blank feed were acceptable.
Acceptable results were obtained for recovery, reported values ranged between 52 and 107%.
The final method can be recommended for adoption as an official method and together with the results of the collaborative study it will be sent to the European Commission (CEMA), CEN and ISO.

1 INTRODUCTION

Within the framework of the EU-project "Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (CANFAS-SMT4-CT98-2216), the official EC-method for olaquindox (Directive 98/64/EC) has been validated for low contents in feeds. Olaquindox is a growth promoter that was registered for use in feeds for piglets with contents ranging from 15 - 50 mg/kg (50 - 100 mg/kg for milk replacers). Since September 1999, the use of olaquindox as a feed additive is banned in the EU. In order to allow adequate control of possible illegal use, the objective was to validate the official EC-method (an HPLC method with UV-detection) for contents 5 - 10 times lower than the lowest content formerly permitted, viz. down to 1,5 mg/kg.

The method was validated by LUFA - Augustenberg, Karlsruhe, Germany. Compared with the original method, the ratio between the extraction volume and the sample weight was modified: in the original method this ratio was 10; in order to increase the limit of detection, in the modified method this ratio was decreased to 2 (see report K. Michels, Final report on evaluation of method validation for olaquindox and carbadox in feeds at low contents, 01-11-1999).

Subsequently, the method was subjected to between-lab validation by the State Laboratory, Dublin, Ireland (see report P. Shearan, January 2000) and Istituto Superiore di Sanita (I.S.S.), Roma, Italy (see report G. Brambilla, January 2000). In general, the criteria as described in the amended Project Plan are fulfilled. The recoveries are often lower than 80 % (down to 60 %) but, while the use of olaquindox has been forbidden, this is not regarded as a major shortcoming (see Second Annual Report CANFAS, J. de Jong, 12-08-2000).

Prior to the first collaborative study, a kick-off meeting was organised (Brussels, 13-14/6/2000) and participating laboratories were given the opportunity to familiarise themselves with the method, using feed samples with stated contents of olaquindox. Also prior to the production of the materials for the collaborative study, separate batches of the materials had been produced for homogeneity and stability testing. The between- and within-sample homogeneity was satisfactory and the results showed that olaquindox is stable in the feeds at room temperature during a period of 4 months (see Second Annual Report CANFAS, J. de Jong, 12-08-2000).

In the first collaborative study many laboratories reported difficulties with the practicability of the method due to the low ratio between the volume of extraction solvent (50 ml) and the weight of feed (25 g).

During the evaluation meeting organised after the first collaborative study, it was decided to modify the CANFAS method in such a way that the ratio between the extraction volume and the sample weight was increased to 5. A second round of collaborative studies for final validation of the method was organised.

The samples that were prepared for the collaborative study were two piglet feeds with declared olaquindox contents of 2,5 and 10 mg/kg respectively. The feed samples were sent to the participants as blind duplicates. Before these samples were shipped, the between sample homogeneity of the feed samples containing olaquindox was checked with satisfactory results (see par. 3.1.2).

Together with the samples, a letter with instructions, reporting forms, etc. was sent to the participants (see Appendix 1).

This report describes the results of the 2nd collaborative study.

2 PARTICIPANTS

The following laboratories/persons participated in the collaborative study.

- Administration des Services Technique de l'Agriculture Division des Laboratoires, Ettelbruck, Luxemburg; C. Strottner
- Bundesambt und Forschungszentrum für Landwirtschaft (BFL), Wien, Austria; B. Stoisser, M. Wieshaider
- INETI/DTIA, Lisbon, Portugal; I. Felgueiras, C. Saldanha
- Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy; G. Brambilla, C. Cartoni, M. Fiori.
- Istituto Zooprofilattico Sperimentale della Lombardia e dellémilia Ronagna, Reparto Chimico, Brescia, Italy; E. Faggionato, A. Baiguera.
- Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy; C. Testa, N. Rubattu, A. Serra
- Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy; G. Biancotto, B. Allegretta
- Istituto Zooprofillatico Sperimentale delle regioni Lazio e Toscana, Roma, Italy; A. Ubaldi, A. di Lullo.
- Laboratoire Inter Régional DGCCRF, Rennes, France; C. Genouel, M.C. Rues, M. Joubert.
- Laboratorio Arbitral Agroalimentario, Madrid, Spain; D.A. Pons, J. Muñoz
- Laboratorio Nacional de Sanidad y Produccion Animal - M.A.P.A., Santa Fe, Spain; R. Checa-Moreno, A. Ariza-Avidad.
- Laboratory of the Government Chemist, Teddington, United Kingdom; J. Cowles
- LNIV, Lisbon, Portugal; J.M. Nunes da Costa, M.B. Casqueira.
- LUFA – Augustenburg, Karslruhe, Germany; K. Michels, S. Witzemann.
- LUFA-ITL Kiel, Kiel, Germany; H. Wehage, H. Graepel
- Masterlab, Putten, The Netherlands; K. van Schalm, A. Schaaf.
- National Veterinary Institute, Uppsala, Sweden; E. Nordkvist, A. Stepinska
- Plant Production Inspection Centre Agricultural Chemistry Department, Vantaa, Finland; R. Muhonen, Y. Hyvönen
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Lekens, R. van Sandt
- RIKILT, Wageningen, The Netherlands; H. Kleijnen, H. van der Kamp
- State Laboratory Dublin, Ireland, P. Shearan
- Universität Hohenheim, Landesanstalt für Landwirtschaftliche Chemie, Stuttgart, Germany; K. Schwadorf, A. Eschle

3 MATERIALS

3.1 Samples for collaborative study

3.1.1 Sample composition

Specifications of the samples, which were produced for the collaborative study, are given in Table 1.

Table 1: Specifications of the samples

Type of feed	Declared content	Units	Subcontractor	Date of production
Piglet feed	2,5	mg/kg	IPC – Dier, Barneveld (NL)	25-09-2001
Piglet feed	10	mg/kg	IPC – Dier, Barneveld (NL)	25-09-2001

The complete composition of the feeds is given in Appendix 2 (in Dutch). The main composition of the two feeds is given in Table 2.

Table 2: Main composition of the two feeds

Product: Piglet feed	
Parameter	Content (%)
Crude protein	18,1
Crude fat	4,3
Starch	39,3
Crude fibre	4,4
Crude ash	5,9
Moisture	12,3

The composition of the feed, with regards to the ingredients, was the same as of the feeds that were produced by IPC-Dier in September 1999 for stability testing (see Report on homogeneity and stability studies of samples for the collaborative studies for olaquindox, K. Michels, LUFA Augustenberg, Germany, 05/05/2000) and in September 2001 for the first collaborative study (see report of first collaborative study see RIKILT report 2002.014). The composition of the feeds, in terms of crude protein, fat, etc, was nearly the same. In the produced feeds for the second round of collaborative study the crude ash content is somewhat higher (5,9% - 4,7%). The feed products have been prepared in a quantity of 500 kg each. To achieve a maximum degree of homogeneity halfway through the production 54 kg of feed are withdrawn from the stream for subsampling activities and put into three sacks of 18 kg. After discarding the top layer (ca. 2 kg) about 30 - 50 subsamples of approx. 250 grams have been taken (manual

distribution with a shovel) from each of these sacks. The subsamples were stored in double paper sacks.
All subsamples have been stored at room temperature (ca. 20 °C).

3.1.2 Sample homogeneity

The homogeneity of the samples was studied by LUFA Augustenberg by random selection of 10 subsamples, applying the HPLC-method developed in CANFAS (see Annex 1 of Appendix 1).
The results of the homogeneity determinations of the individual feeds are attached in Appendix 3. Table 3 gives a summary of these results.

Table 3: Results of homogeneity tests for olaquindox in piglet feeds

Results Product	Declared content (mg/kg)	Measured content (mg/kg)	Homogeneity results	
			Between sample CV (%)	Within sample CV (%)
Piglet feed	2,5	2,90	7,5	Not determined
Piglet feed	10	9,90	9,5	Not determined

According to the Project Plan the CV's for homogeneity should not exceed 2 times the CV's for repeatability ($CV_{hom} \leq 2\ CV_r$). Based on previous results of within-lab validation (see Second Annual Report CANFAS, J. de Jong, 12-08-2000) the maximum limit for CV_{hom} was set to 16 %. Both between sample CV's fulfil these requirements. Thus, it is concluded that the samples are sufficiently homogeneous.

3.1.3 Sample logistics

The samples were sent as blind duplicates. The codes are given in Appendix 4. The samples were sent to the participants by courier service on 2 November 2001 together with a letter with instructions (Appendix 1). During transport no special precautions were taken with regards to the temperature of the samples.

3.2 Reference standard

The reference standard was supplied by Dr. A. Plöger, Danish Plant Directorate, Lyngby (DK). According to the specifications (see Report 2002.014), the purity of the reference standard (Lot Nr. 890416) is 99,46 %.
The expiration date of the reference standard was April 2001. The identity and content was checked by RIKILT. The identity could be confirmed by UV, ^{1}H-NMR as well as mass spectrometry. The purity was determined by ^{1}H-NMR and UV spectroscopy and was shown to be approx. 100 % (see Report 2002.014).
The participants were instructed to set the purity of the reference standard to 100 % (see Appendix 1).

4 METHODS

4.1 Method of analysis

The method of analysis is included as annex 1 to Appendix 1. The participants were instructed that this method has to be used without any modifications.

4.1.1 HPLC-conditions

Various types of HPLC-columns were used. The following columns were recommended in the method:

- Hypersil ODS 5 µm, 200 x 4,6 mm;
- Spherisorb ODS-2 5 µm, 250x4,6 mm;
- LUNA C18(2) 250 x 4,6 mm.

The mobile phase described in the method is a mixture of water and methanol 900:100 (v/v). Three laboratories used a different mobile phase.
The HPLC conditions (Column and mobile phase) used by the participants are shown in Table 4.

4.2 Method for statistical evaluation

Statistical evaluation was performed according to ISO 5725 Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method (First edition, 1994-12-15).
The scrutinity of results for consistency and outliers was checked by
Graphical consistency techniques: Mandel's h plot for between-laboratory variability, Mandel's k plot for within-laboratory variability
Numerical outlier techniques: Cochran's test of the within-laboratory variability, Grubbs' test (single and double) for between-laboratory variability
Whenever necessary and appropriate, laboratories which showed consistently high within-cell variation and/or extreme cell means across many levels and/or Cochran or Grubbs' outliers were contacted to try to ascertain the cause of the discrepant behaviour.
The Horwitz equation and the HORRAT ratios form the basis for the evaluation of the reproducibility of the method. The HORRAT ratios are given in Table 5. The HORRAT ratios should be lower than 2 (see W. Horwitz and R. Albert, J.A.O.A.C. 74 (1991) 718-744).

Table 4: HPLC-conditions

Partner	Column	Mobile phase
12	Tracer extrasil ODS 5x25x0,46	As described in the method
15	Inertsil ODS-2; 5 µm; 250 x2,6 mm	As described in the method
16	Phenomenex LUNA C18 (2); 5 µm; 150 x 4,6 mm	As described in the method
17	Sperisorb S10 ODS-1; 10 µ	As described in the method
18	Sperisorb ODS-2; 5 µm; 150 x 4,6 mm;	As described in the method
20	ODS Hypersil C18; 5 µm; 200 x 4,6 mm	As described in the method
21	Supelcosil LC18; 25 cm x 4,6 mm+ supelguard LC18; 2 cm x 4,6 mm	Acetonitril: acetate buffer (0,01M; pH 4,6) Gradient elution
22	Hypersil C18 ODS BDS; 5 µm; 250 x 4,6 mm	As described in the method
23	Not reported	Not reported
24	Waters C18; 5 µm; 250 x 4,6 mm	As described in the method
25	RP C18 Lichrocart; 5 µm; 250 mm x 4 mm (Merck)	Phosphate buffer(0,0? M; pH 2,8): Acetonitrile Gradient elution
26	Spherisorb ODS 2; 5 µm; 250 mm x 4,6 mm	As described in the method
29	Nova-Pak C18; 4 µm; 4,6 x 250 mm	As described in the method
31	As described in the method	As described in the method
32	Lichrospher RP-Select B; 5 RP-Select B; 5 µm; 250x4 mm	As described in the method
33	As described in the method	As described in the method
34	Not reported	Not reported
35	As described in the method	As described in the method
37	Lichrosper RP18-5 endcapped ; 25 x 4 mm	As described in the method
38	Symetry C-18; 3,5 µm; 150 x 2,1 mm	Isocratic methanol/water 5:95
40	C18 sperical; 5 µm; 3,9 x 150 mm Waters	As described in the method
41	As described in the method	As described in the method

5 RESULTS

For each participant the reported results for the samples, the completed questionnaire and representative chromatograms are annexed in Appendix 5.

5.1 Statistical evaluation

Originally laboratory 12 reported results that were not in agreement with the results of the other participants and that deviated much from the theoretical olaquindox concentrations. The reported results from lab 12 were 0.50, 0.53, 0.53, 0.53 mg of olaquindox/kg for the sample with a declared content of 2.5 mg/kg and 1.99, 2.04, 1.99, 2.03 mg of olaquindox/kg for the sample with a declared content of 10 mg/kg. Due to the magnitude of the deviations it was most likely that the results would cause outliers on both levels. Lab 12 was contacted to try to ascertain the cause of the discrepant behaviour. According to the explanation this lab had met problems with the solubility of the reference standard, because they had prepared a stock standard solution more concentrated than the one indicated in the method. After repetition of the analysis by following exactly the procedure as described lab 12 reported new values. Based on the findings mentioned above it was decided to accept the new results.
The results reported by the participants are given in Table 6.
Statistical analysis shows that the results of the laboratories do not contain Cochran or Grubbs' outliers or stragglers. The values for the statistical parameters (mean, relative standard deviations for repeatability and reproducibility) are given in Table 6. According to the Project Plan, the rsd_r-values should be ≤ 10 %. For both samples this criterion is met and consequently it can be concluded that the repeatability is satisfactory.
The Horwitz equation and the HORRAT ratios form the basis for the evaluation of the reproducibility (see W. Horwitz and R. Albert, J.A.O.A.C. 74 (1991) 718-744). The HORRAT ratios are given in Table 5. The HORRAT ratios should be lower than 2. For both samples this criterion is met and established rsd_R-values are in line with values predicted by the Horwitz equation. Consequently it can be concluded that the reproducibility of the changed method is satisfactory.

Table 5: Horrat ratios of the olaquindox collaborative study

Mean	Predicted rsd_R	Established rsd_R	Horrat[1]	Conclusion
2,47	14,0	18,5	1,33	Reproducibility OK
8,79	11,5	13,1	1,13	Reproducibility OK

[1] = Horrat is the ratio between the established rsd_R and the predicted rsd_R

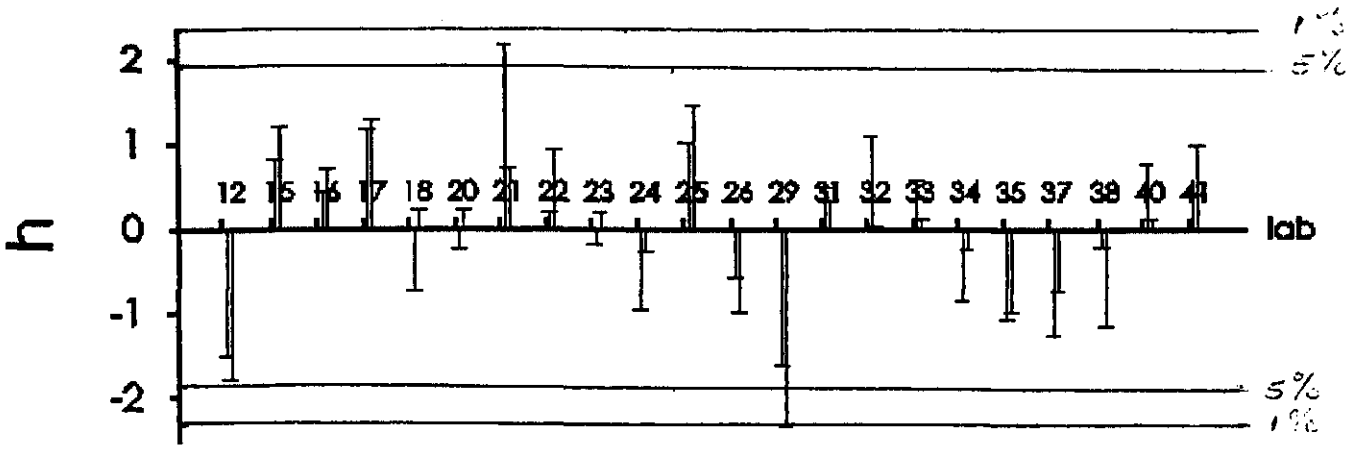
The Mandel h and k plots are shown in Figure 1.

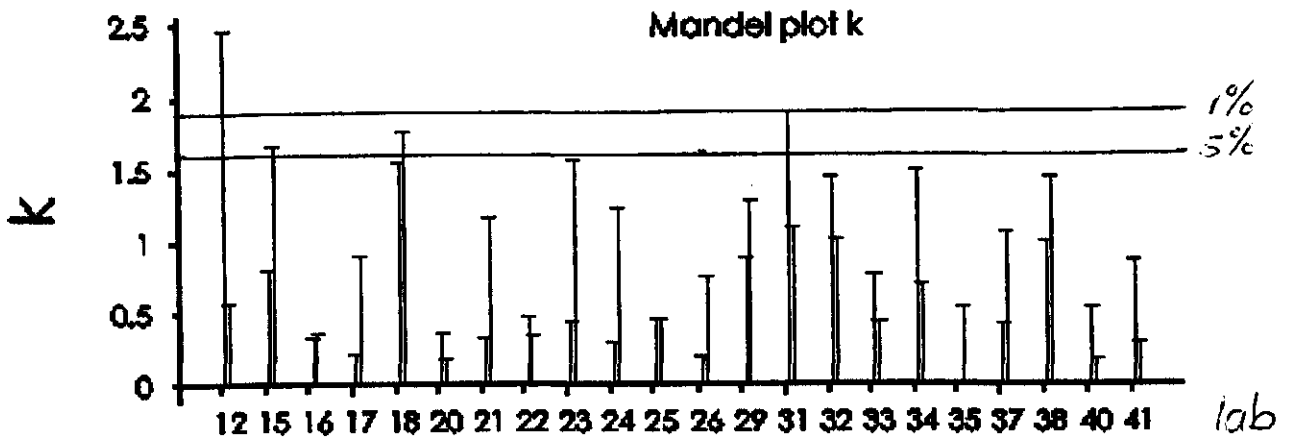
Table 6: Results reported by the participants.

		Result (mg/kg)							
	Sample	OLA2 2.5 ppm	OLA2 2.5 ppm	OLA2 2.5 ppm	OLA2 2.5 ppm	OLA2 10 ppm	OLA2 10 ppm	OLA2 10 ppm	OLA2 10 ppm
Lab									
12		1,90	2,29	1,71	1,34	6,88	6,99	6,57	6,74
15		2,85	2,63	2,89	2,92	10,68	10,41	9,91	9,50
16		2,70	2,63	2,60	2,71	9,73	9,52	9,65	9,49
17		2,97	3,03	3,00	2,95	10,30	10,60	10,05	9,97
18		2,10	1,90	2,10	2,50	8,60	8,50	9,40	9,60
20		2,31	2,33	2,44	2,38	9,09	9,10	9,00	8,99
21		3,40	3,43	3,35	3,48	9,97	9,41	9,80	9,16
22		2,45	2,55	2,60	2,63	9,98	9,73	9,80	9,88
23		2,43	2,30	2,35	2,46	8,55	8,60	9,34	9,50
24		2,10	2,01	2,09	2,01	8,89	7,99	8,40	8,69
25		2,88	2,86	2,88	3,02	10,25	10,33	10,50	10,56
26		2,20	2,19	2,25	2,25	7,32	7,76	7,77	7,85
29		1,66	1,61	1,90	1,86	6,49	6,53	5,70	5,97
31		2,70	3,10	2,37	2,57	8,71	9,09	9,47	9,40
32		3,16	2,81	3,14	2,69	8,77	8,37	9,05	9,05
33		2,70	2,60	2,90	2,70	9,10	8,80	8,90	8,80
34		2,40	2,20	1,90	1,90	8,60	8,30	8,80	8,40
35		2,00	2,00	2,00	2,00	7,90	7,50	7,60	7,70
37		2,02	1,92	1,87	1,88	7,54	8,09	8,34	7,91
38		2,30	2,36	2,60	2,22	7,82	6,86	7,50	7,83
40		2,67	2,82	2,87	2,84	8,84	8,87	8,93	8,96
41		2,67	2,82	2,48	2,60	9,85	9,93	9,77	9,98

number of labs	22	22
m (mg/kg)	2,47	8,79
rsd_r (%)	6,56	3,56
rsd_R (%)	18,5	13,1

Figure 1: Mandel h and k plots of results reported by the participants.





5.2 Recoveries

Table 7: Recoveries

Partner	Spiking level (mg/kg)	Recovery 1 in %	Recovery 2 in %	Average recovery in (%)
12	25	95		95
	50	91		91
15	2,5	89	93	91
16	3,65	75	74	75
17	3,75	98		98
	10	88		88
18	2,5	62	72	67
20	2,5	88	88	88
21	2,5	98	101	100
22	3,2/6,3	105	97	101
23	Not reported	Not reported	Not reported	Not reported
24	2,5	83	80	82
25	2,5	91	90	91
26	2,5	68		68
29	2,5	104	107	106
31	2,5	88	91	90
32	2,5	84	83	84
33	5	102		102
34	Not reported	95		95
35	3,1	106	100	103
37	2,5	79	76	78
38	2	52	53	53
40	2,5	96	98	97
41	2,5	101	99	100

Recoveries range from 52 - 107 %. This range is broader than the range (60 - 90 %) that was measured in the between-lab validation of the method (see Second Annual Report CANFAS, J. de Jong, 12-08-2000).
Although the mean recovery value reported by lab 38 (53 %) is low, it is not a Grubbs outlier or a straggler.

5.4 Remarks

Table 8: Remarks made by the partners

Partner	Remarks
12	No remarks
15	No remarks
16	ad 3.5.1.: Concentration of Olaquindox stock standard solution was 36,5 µg/ml; ad 3.5.2.: Concentration of the corresponding calibration solutions: 0,365; 0,730; 1,825; 3,650 and 7,300 µg/ml ad 5.3.2.: Linear regression calculated: 0,99999 ad 5.2: Extraction: the following parameters were used: - Centrifugation 10 minutes with 7000 rpm (instead of filtration step) - The supernatant liquids were additionally filtered by using membrane filters (Machery&Nagel, Chromafil Type A-45/25, 0,45 µm)
17	No remarks
18	HPLC equipment: pump, autosampler, column oven = HP1050; DAD = HP1100 Slight modifications: - ad 3.5.1.: Stock standard solution = 50 mg, weigh to the nearest 1 mg, in 2000 ml water. - ad 3.5.2.: 1,25 µg/ml standard solution = 5 ml diluted to 100 ml (instead 2,5 ml to 50 ml) - Samples stored at refrigerated temperature until analysis (<8 °C). - Filtration and centrifugation of the extracts.
20	No remarks
21	We centrifuged 10 ml of the final extract instead of filtering the whole extract on a paper filter. Then we filtered 2 ml of the centrifuged extract on an Acrodisc filter (0,45 µm) before HPLC analysis.
22	The Olaquindox content was calculated from the peak area by reference to the calibrations graph.
23	Not reported
24	The extraction step is improved if compared to the first edition of this method. Still, centrifugation is necessary as well as filtration through 0,45 µm just before HPLC injection.
25	Column overpressure recorded after repeated injections with RT not constant. It is suggested to reduce flow rate to 1 ml/min with a slight increase of the organic phase. As alternative we suggest a gradient elution able to clean the column.

Partner	Remarks
26	The procedure was well documented and straight forward to follow. We have had one major problem with retention time stability of olaquindox. Initial injections of all standards and pre-injections (to verify system stability) all gave excellent response, Rt was 9,9 min. The blank sample was then injected and gave a zero response at the Rt of olaquindox. However, after this time the Rt of olaquindox reduced to between 9,0 and 9,2 minutes, but the signal response did not change. Initially we thought that this may have been a temperature effect as we run the samples overnight and we know that the laboratory temperature rises when the air conditioning is switched off. We therefore re-extracted the samples and put the LC-column in an oven at 35 °C and reduced the flow rate to minimise these effects. However, it made no difference.
29	No remarks
31	No remarks
32	No remarks
33	No remarks
34	No remarks
35	No remarks
37	The method is now easier to manipulate using the modifications in Annex I. We carried out the entire method in glass centrifuge tubes. i) flat bed shaker used: these tubes were put horizontal on bed - effective shaking/mixing noted. ii) after shaking the tubes were placed in centrifuge for 5 min. Therefore no need to use GFA filters. Extracts were filtered prior to LC. LC-conditions: working at high psi: 1 ml/min ~ 2800 psi
38	We have used two different feed samples from our collection as blank feed for recovery purposes. They do not belong to the other CANFAS Collaborative feed samples because we spent all of them. So, one sample is lamb feed and the other one is a piglet feed. Both of them had got a similar aspect to the CANFAS Collaborative II feed samples. We have observed that recovery and blank samples make spherical clusters (lump) after addition of olaquindox standard solution in water. These lumps were not broken after addition of methanol.
40	No remarks
41	No remarks

6 CONCLUSIONS

From the results it can be concluded that with the modified method acceptable results are obtained for repeatability (rsd$_r$ < 10 %) and reproducibility (Horrat ratios < 2).
During the first collaborative study blank samples were analysed: no interfering substances were detected, so the results obtained for the blank feed were acceptable.
Reported values for recovery ranged between 52 and 107%. The recoveries are sometimes lower than 80 % (down to 52 %) but, while the use of olaquindox has been forbidden, this is not regarded as a major shortcoming (see Second Annual Report CANFAS, J. de Jong, 12-08-2000).
The remarks made by the participants indicate that no difficulties were encountered. Some laboratories applied centrifugation of the samples instead of filtration. According to the method description this alternative may be applied.
The final method can be recommended for adoption as an official method and together with the results of the collaborative study it will be sent to the European Commission (CEMA), CEN and ISO.

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Dr. A. Plöger, Danish Plant Directorate is thanked for supplying the olaquindox reference standard.
Dr. H. van de Voet, Biometris, Wageningen University and Research Centre is thanked for statistical advice.

APPENDIX 1

Letter with instructions, sent with the samples (with four annexes)

Participants CANFAS collaborative study Olaquindox

DATE
2 November 2001

SUBJECT
CANFAS collaborative study olaquindox (71316.24)

ENCLOSURE(S)
4

OUR REFERENCE
01/0026880

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09098104 te Arnhem

THE INTERNET
www.rikilt.wageningen-ur.nl

Dear colleague,

As agreed at the CANFAS evaluation meeting June 19th, 2001 at Tervuren a second round of collaborative study for olaquindox has to be organised. We appreciate your willingness to participate very much. Together with this letter you will find:

- 2 feed samples labeled with the text "additive: OLAQUINDOX" and with a sample code. The samples contain olaquindox in the range between 1 and 15 mg/kg.
- the modified method of analysis (annex 1). By participation you agree with application of this method!
- the reporting form (annex 2). This form will also be send to you by E-mail as an Excel 5.0 file. We strongly prefer to get the results back in electronic form by E-mail; you are asked to use the e-mail address mentioned in the right margin of this letter.
- instructions for handling (milling, storage) of the samples (annex 3).
- a questionnaire (annex 4). We kindly ask you to give us information about the experimental conditions, recoveries, etc.. On this form you can also give your remarks about the method.

The samples must be analysed in *duplicate*.
For recovery purposes we ask you to select a blank piglet feed from your own collection. The reference standard of olaquindox that has to be used (980416) was already sent to you with our letter of 31 May 2000. In the calculations this reference standard can be regarded as 100 % pure.

The **deadline** for reporting the results is **December 14, 2001.**

We wish you and your colleagues the best with the collaborative study. If you have any questions, do not hesitate to contact us.

Kind regards,

dr. Jacob de Jong
CANFAS co-ordinator

ing. J.J.M. Driessen
co-ordinator CANFAS collaborative studies

QUALIFIED BY STERLAB

cc. mrs. D. Bennink, European Commission, DG Research, CII/3, Brussels

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 1 – Modified method of analysis.

Determination of low level contents of Olaquindox in Feedingstuffs

1. Purpose and scope

The method is for the determination of olaquindox in feedingstuffs. The limit of determination (=quantification) is 1,5 mg/kg. The limit of detection (=qualification) is 0,1 mg/kg

2. Principle

The sample is extracted by a water methanol mixture. The content of olaquindox is determined by reversed-phase high-performance liquid chromatography (HPLC) using an UV detector.

3. Reagents

3.1. Methanol

3.2. Methanol, HPLC grade

3.3 Water, HPLC grade

3.4. Mobile phase for HPLC

Water (3.3)-methanol (3.2) mixture, 900+100 (V + V)

3.5. Standard substance: pure olaquindox 2-[N-2'-(hydroxyethyl)carbamoyl]-3-methylquinoxaline-N^1 ,N^4-dioxide, E 851

3.5.1. Olaquindox stock standard solution, 25 µg/ml

Weigh to the nearest 0,1 mg 5 mg of olaquindox (3.5) in a 200 ml graduated flask and add ca. 190 ml water. Then place the flask for 10 min in a ultrasonic bath (4.1). After ultrasonic treatment, bring the solution to room temperature, make up to the mark with water and mix. Wrap the flask with aluminium foil and store in a refrigerator. At this temperature of ≤ 4°C the solution is stable for 1 month.

3.5.2. Calibration solutions

Into a series of 50 ml graduated flasks transfer 0.5, 1.0, 2.5, 5.0 and 10.0 ml of the standard stock solution (3.5.1). Make up to the mark with water (3.3) and mix. These solutions correspond to 0.25, 0.5, 1.25, 2.5 and 5.0 µg of olaquindox per ml respectively.

These solutions must be prepared fresh each day.

4. Apparatus

4.1. Ultrasonic bath

4.2. Mechanical shaker

4.3. Membrane filter, 0.45 µm

4.4. HPLC equipment with variable wavelength ultraviolet detector

4.4.1. Liquid chromatographic column, 250 mmx4mm, C 18, 5 µm packing, or equivalent. See remark 7.2.

5. Procedure

Note: *Olaquindox is light sensitive. Carry out all procedures under subdued light or use amber glass ware.*

5.1. General

5.1.1. Blank feed

For the perfomance of the recovery test (5.1.2) a blank feed should be analysed to check that neither olaquindox nor interfering substances are present. The blank feed should be similar in type to that of the sample and on analysis olaquindox or interfering substances should not be detected.

5.1.2. Recovery test

A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of olaquindox, similar to that present in the sample. To fortify at a level of 2.5 mg/kg, transfer 1 ml of the stock standard solution (3.5.1) to a 250 ml conical flask, add 10 g of the blank feed, mix thoroughly and leave for 10 min mixing again several times before proceeding with the extraction step (5.2). In stead of 40 ml water, 39 ml water should be added in the extraction step.
Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, prepare two independent laboratory sample aliquots (A and B) of the feed to be examined. Spike one of them (A), before extraction with a quantity of olaquindox, similar to that already present in the sample. Both samples are analysed. Calculate the analyte content in sample A and B and calculate the recovery by subtraction.

5.2. Extraction

Weigh to the nearest 0.01 g, approximately 10 g of the sample. Transfer to a 250 ml conical flask, add 10 ml of methanol (3.1) and place the flask for 5 min in the ultra-

sonic bath (4.1). Add 40 ml water and leave in the ultrasonic bath for further 15 min. Remove the flask from the ultrasonic bath, shake it for 30 min on the shaker (4.2) and filter through a folded filter or a glass fibre filter (GFA, Whatman) (see remark 7.1). It is highly recommended to filter the clear samples by using a membrane filter (4.3) additionally. Proceed to the HPLC determination (5.3).

5.3. HPLC determination

5.3.1. Parameters:

The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.

Analytical column (4.4.1). See remark 7.2.

Mobile Phase (3.4): water (3.3) - methanol (3.1.) mixture, 900 + 100 (V+ V)

Flow rate: 1.5 - 2 ml/min

Detection wavelength: 380 nm

Injection volume: 50 µl -100 µl

Check the stability of the chromatographic system injecting several times the calibration solution (3.5.3) containing 1.25 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. Calibration graph

Inject each calibration solution (3.5.3) several times and determine the mean peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights (areas) of the calibration solutions as the ordinates and the corresponding concentrations in pglml as the abscissae.

5.3.3. Sample solution

Inject the sample extract (5.2) and determine the peak height (area) of the olaquindox peaks.

6. Calculation of the results

From the height (area) of the olaquindox peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.3.2).

The olaquindox content w (mg/kg) of the sample is given by the following formular:

$$w = \frac{c * 50}{m}$$

in which:

c = olaquindox concentration of the sample extract (5.2) in µg/ml

m = mass of the test portion in g

7 Remarks

7.1 Instead of filtration through a folded filter a centrifugation step could be carried out. If plastic vials are used for centrifugation, a recovery study should be carried out to validate this application.

7.2 The following columns could be recommended: Hypersil ODS 5 µm 200 x 4.6 mm, Spherisorb ODS 2 5 µm 250 x 4.6 mm, LUNA C18(2) 5 µm 250 x 4.6 mm.

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis:

Analyte: OLAQUINDOX

Sample code / Unit	Result 1 (mg/kg)	Result 2 (mg/kg)

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 3 - Instructions for handling of the samples

1. Storage
Store the samples at room temperature until analysis. Protect the material from direct light.

2. Milling
Grind the feed samples with a mill equipped with a 1 mm screen

3. Mixing of the test samples before weighing
Mix the entire sample thoroughly

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: .....

Chromatographic conditions:

- Column:
  - □ As described in the method
  - □ Other: .....
- Mobile phase:
  - □ As described in the method
  - □ Other: .....
- Flow-rate: ..... ml/min
- Injection volume: .....µl
- Retention time of olaquindox: ..... min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: □ single □ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ..... mg/kg

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001.

ADDITIVE: OLAQUINDOX

Remarks /Comments (if necessary, continue on another page) : .....

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Please complete this questionnaire and return it together with representative chromatograms to:

Ing. J.J.M. Driessen
RIKILT
P.O. Box 230
6700 AE Wageningen
The Netherlands
Fax +31-317-417717

Thank you for your co-operation !

APPENDIX 2

Composition of the feed samples

CANFAS 2nd coll. study

BESTMIX - Afdruk mengopdracht - 26/09/01 - IPC DIER BARNEVELD

Piglet feed with Laquindox

2 250.00 Biggen opfok korrel
biggenvoer van 12 tot 25/30 kg

Grondstof	Silo	%	Gewicht kg	Tol. +/-Afw.	Cumul Gew. kg	10ppm Charge 24	2.5 ppm Charge 25
Weegschaal DW 1							
113 Zonbl.schr.290re	(2)	2.00	[illegible]	0.30	10.00	✓	✓
460 Tapioca65%zetmeel	(4)	7.50	[illegible]	1.13	47.50	✓	✓
77 Soja 45/46(arg/braz)	(9)	13.00	[illegible]	1.95	112.50	✓	✓
Weegschaal DW 2							
145 Tarwe (voer)	(9)	10.00	[illegible]	1.50	50.00	✓	✓
14 Gerst	(11)	37.10	[illegible]	5.57	235.50	✓	✓
40 Mais	(12)	12.00	[illegible]	1.80	295.50	✓	✓
Bijstort SP4							
34 Lynzaad	(0)	5.00	[illegible]	0.75	25.00	✓	✓
105 Vismeel 65.9% re	(0)	4.40	[illegible]	0.66	47.00	✓	✓
Bijstort SP6							
476 Powerfood Twil melkv	(0)	4.00	[illegible]	0.60	20.00	✓	✓
Bijstort SP7							
21 Fumaarzuur	(0)	0.25	[illegible]	0.01	1.25	✓	✓
78 L-lysine HCl	(0)	0.17	[illegible]	0.01	2.10	✓	✓
79 DL-Methio-nine	(0)	0.03	[illegible]	0.00	2.25	✓	✓
117 Krijt/kalksteen	(0)	0.45	[illegible]	0.02	4.50	✓	✓
228 Monocal Belgie	(0)	0.50	[illegible]	0.03	7.00	✓	✓
485 Zout	(0)	0.10	[illegible]	0.01	7.50	✓	✓
508 Prem biggen Rikilt	(0)	1.00	[illegible]	0.05	12.50	✓	✓
Vloeistoffen							
474 Melasse riet >450s	(3)	2.50	12.50	0.38	12.50	✓	✓
			Totaal :		500.00		

RETOURPRODUKT — — —

INSTELLINGEN

T.R. : aud 50%
V.Z. : grof/(fijn) .. 80.. %
Z.F. : 2½.... mm 2opp
H.M. : (hoog)/laag toeren
kringloop : ja/(nee)
L.M. : voormengen 60. sec
namengen 30. sec
M.D. : ..75.. l/h

Meel temp : .50. °C korrels 77 °C
Matrijs diam. : 2,5 x 35. mm
K.P. : 30.. Amp
Laagdikte Ko : 4.5 cm
Zeef Ko : — mm
Kruimelen : ja/(neen)
Eindvochtgehalte 12,0 %

2 250.00 Biggen opfok korrel
biggenvoer van 12 tot 25/30 kg

Nr	Grondstofnaam	Aandeel	Gewicht
14	Gerst	37.10000	185.500
77	Soja 45/46(arg/braz)	13.00000	65.000
40	Mais	12.00000	60.000
145	Tarwe (voer)	10.00000	50.000
460	Tapioca65%zetmeel	7.50000	37.500
34	Lynzaad	5.00000	25.000
105	Vismeel 65.9% re	4.40000	22.000
476	Powerfood Twil melkv	4.00000	20.000
474	Melasse riet >450s	2.50000	12.500
113	Zonbl.schr.290re	2.00000	10.000
508	Prem biggen Rikilt	1.00000	5.000
228	Monocal Belgie	0.50000	2.500
117	Krijt/kalksteen	0.45000	2.250
21	Fumaarzuur	0.25000	1.250
78	L-lysine HCl	0.17000	0.850
485	Zout	0.10000	0.500
79	DL-Methio-nine	0.03000	0.150
	Totaal	100.00000	500.000kg

Nr	Nutrient	Berekend		Verschil	Minimum	Maximum
1	Re	181.11	g		180.00	200.00
2	Rvet	42.70	g			65.00
3	Rc	43.83	g			45.00
4	Vocht	123.47	g			130.00
5	Ras	58.90	g		1.00	
6	Zetmeel	393.49	g	-26.51	420.00	
8	Ca	7.76	g		7.00	9.00
9	P	6.36	g		1.00	
11	Lysine	11.37	g			
12	Methion	3.84	g			
13	Meth+cys	6.97	g			
14	Trypt.	2.21	g			
15	Threon.	6.81	g			
16	Isoleuc	6.78	g			
19	Linolz.	10.07	g		10.00	
30	EW*100	106.96		-0.04	107.00	107.00
32	P-vert	3.58	g		3.00	
34	dvLys v	9.53	g		9.00	
35	dvmet	3.19	g		3.00	
36	dvM+C	5.41	g		5.40	
37	dvtryp v	1.72	g		1.70	
38	dvtreo v	5.01	g	-0.09	5.10	
50	Cu	73.96	mg			
51	Na	1.78	g		1.50	3.00
53	K	8.74	g			12.00
54	Cl	3.98	g		1.50	
59	Gewicht	100.00			100.00	100.00
100	vit. A	4000.00	i.e.			
101	vit.D3	800.00	i.e.			
102	vit.E	734.68	mg			
103	BHA/ethy	0.02	mg			
106	ethopab	13.00	mg			

APPENDIX 3

Homogeneity of samples

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Homogeneity test 2nd collaborative study

Additive : **Olaquindox**
Product : **Piglet feed, 10 ppm**

Date of determination : October 29th, 2001

Sample	Content ppm
345312	10,5
345314	10,2
345327	10,6
345328	10,3
345344	8,7
345345	8,7
345363	8,3
345372	10,5
345393	10,9
345425	10,4

Homogeneity Crtiterion : $CV_{between}$ = < 15%	**OK**	
Average (ppm)	**9,90**	
SD (between samples)	**0,940**	
CV (between samples)	**9,5**	Result Grubb's test
Grubb's test, single lower	**1,696**	**no outlier**
Grubb's test, single upper	**1,016**	**no outlier**
Grubb's test, double lower	**0,5574**	**no outliers**
Grubb's test, double upper	**0,5574**	**no outliers**

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Homogeneity test 2nd collaborative study

Additive : **Olaquindox**
Product : **Piglet feed, 2.5 ppm**

Date of determination : October 29th, 2001

Sample	Content ppm
345301	2,7
345318	2,7
345323	2,8
345334	3,2
345339	3,1
345371	3,1
345392	2,6
346402	3,2
345412	2,9
345432	2,9

Homogeneity Crtiterion : $CV_{between}$ = < 15%	**OK**	
Average (ppm)	**2,90**	
SD (between samples)	**0,219**	
CV (between samples)	**7,5**	Result Grubb's test
Grubb's test, single lower	**1,372**	**no outlier**
Grubb's test, single upper	**1,372**	**no outlier**
Grubb's test, double lower	**0,5574**	**no outliers**
Grubb's test, double upper	**0,5574**	**no outliers**

APPENDIX 4

Sample codes

Sample codes supplied to the participants in the olaquindox collaborative study, 2nd round

OLAQUINDOX number of participants 22	OLA2 piglet 2.5 ppm OLA 1a	OLA2 piglet 2.5 ppm OLA 1b	OLA2 piglet 10 ppm OLA 2a	OLA2 piglet 10 ppm OLA 2b
Participant code				
12	125397	125419	125309	125421
15	155332	155407	155316	155400
16	165403	165423	165427	165389
17	175313	175431	175331	175352
18	185350	185353	185368	185395
20	205377	205428	205367	205417
21	215341	215401	215351	215422
22	225373	225416	225361	225424
23	235310	235325	235342	235365
24	245326	245410	245307	245426
25	255349	255383	255306	255411
26	265404	265413	265390	265398
29	295354	295356	295319	295418
31	315414	315429	315359	315399
32	325364	325409	325305	325375
33	335304	335347	335308	335362
34	345366	345386	345321	345379
35	355317	355322	355357	355406
37	375311	375387	375343	375405
38	385355	385369	385336	385374
40	405315	405391	405381	405385
41	415320	415330	415396	415430

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 12

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 14-01-2002

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
125309	6,88	6,99
125397	1,90	2,29
125419	1,71	1,34
125421	6,57	6,74

CANFAS COLLABORATIVE STUDIES - 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Date (s) of analysis: 21-dec-01

Chromatografic conditions:

Column:

1- As described in the method:
2- Other: **Tracer extrasil ODS 5x25x0,46**

Mobil phase

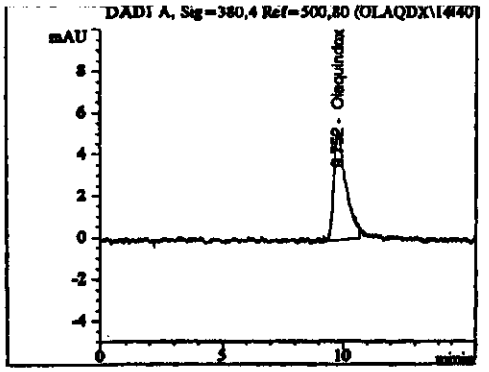
1- As described in the method: **yes**
2- Other:

Flow rate: **1,3** mlxmin^{-1}
Injection volume: **150** ul
Retention time of olaquindox: **9,7** min.

Cromatograms: ¡In the file word annex!

Recovery results:

1- Percentaje recovery: **94,7% and 91,4%**
2- Single / duplicate determinations: **no**
3- If duplicte, please give bth percentages:
4- Spiking level: **25 and 50 ug**



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 11/15/02 8:07:47 AM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,4 Ref=500,80

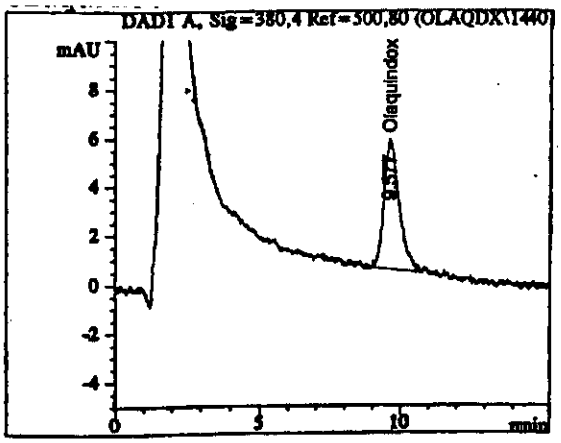
RetTime [min]	Type	Area [mAU*s]	Amnt/Area	Amount [ug/ml]	Grp	Name
9.752	PB	189.33951	1.75131e-3	3.31593e-1		Olaquindox

Totals : 3.31593e-1

Results obtained with enhancced integrator!

==========

**** End of Report ***



External Standard Report

Sorted By : Signal
Calib. Data Modified : 1/15/02 8:07:47 AM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,4 Ref=500,80

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ug/ml]	Grp	Name
9.577	PB	197.40469	1.75131e-3	3.45718e-1		Olaquindox

Totals : 3.45718e-1

Results obtained with enhanced integrator!

*** End of Report ***

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 15

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 30,11,2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
155316	10,68	10,41
155332	2,85	2,63
155400	9,91	9,50
155407	2,89	2,92

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: ..

Contact person: . .....

Date(s) of analysis: .....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Inertisil ODS-2 5µm x 250 mm x 4.6 mm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.0 ml/min
- Injection volume: 25 µl
- Retention time of olaquindox: 11 min

Chromatograms: Please include representative chromatograms of:

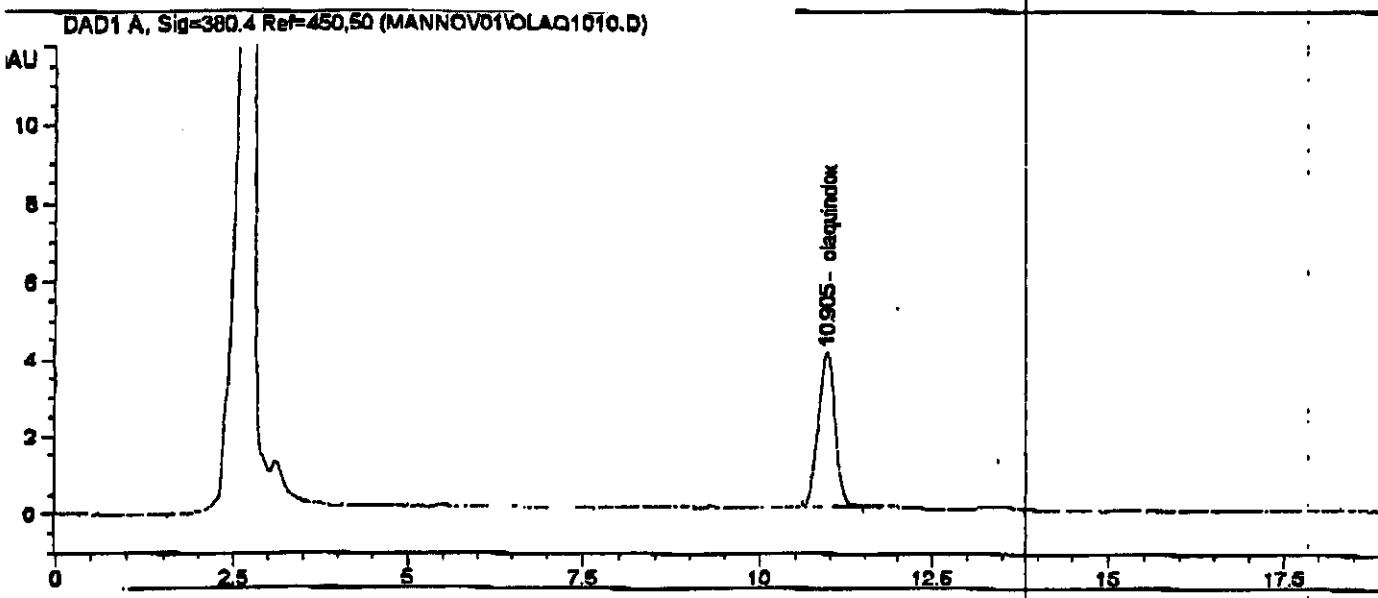
- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 88,8 % and 92,8% →
- Spiking level: 2.5 mg/kg

sample:
155400



External Standard Report

l By : Signal
Data Modified : Monday, December 03, 2001 10:07:00 AM
lier : 1.0000
n : 5.0000

1: DAD1 A, Sig=380,4 Ref=450,50

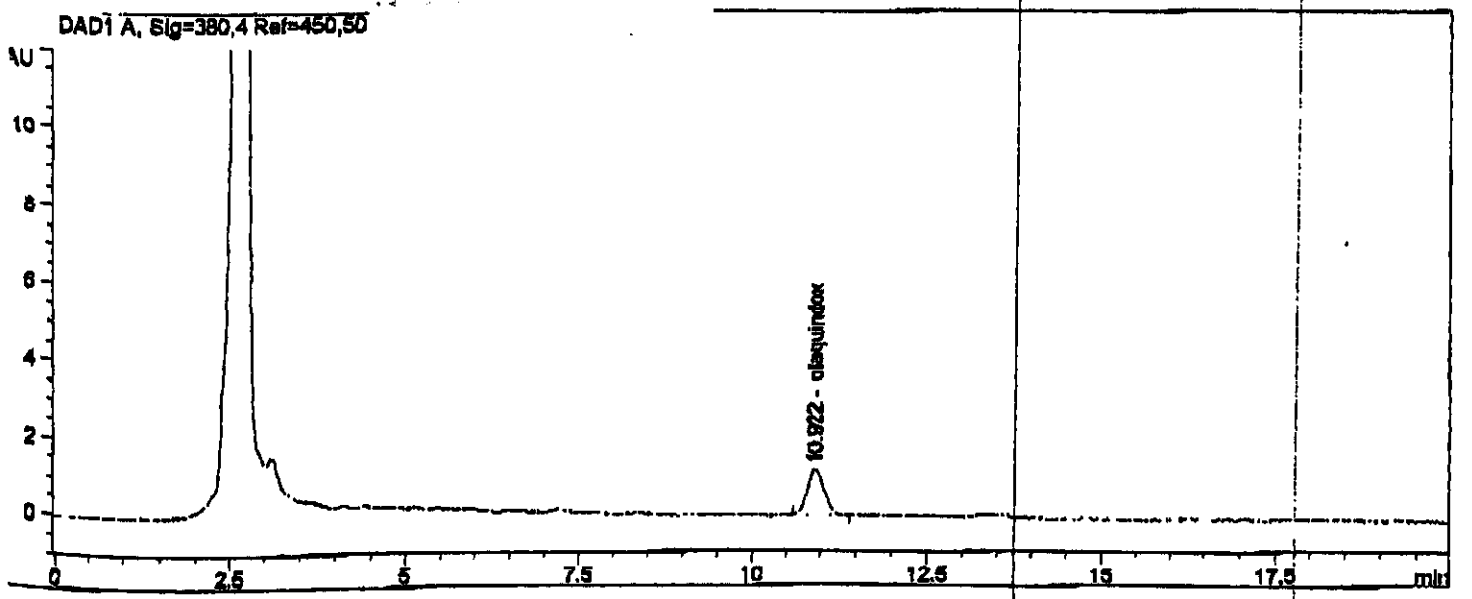
ıe	Type	Area [mAU*s]	Amt/Area [ng/ul]	Amount	Grp	Name
5	BB	66.08059	2.91140e-2	9.91049		olaquindox

: 9.91049

s obtained with enhanced integrator!

*** End of Report ***

sample 155407



External Standard Report

d By : Signal
Data Modified : Monday, December 03, 2001 10:07:00 AM
ller : 1.0000
on : 5.0000

l 1: DAD1 A, Sig=380,4 Ref=450,50

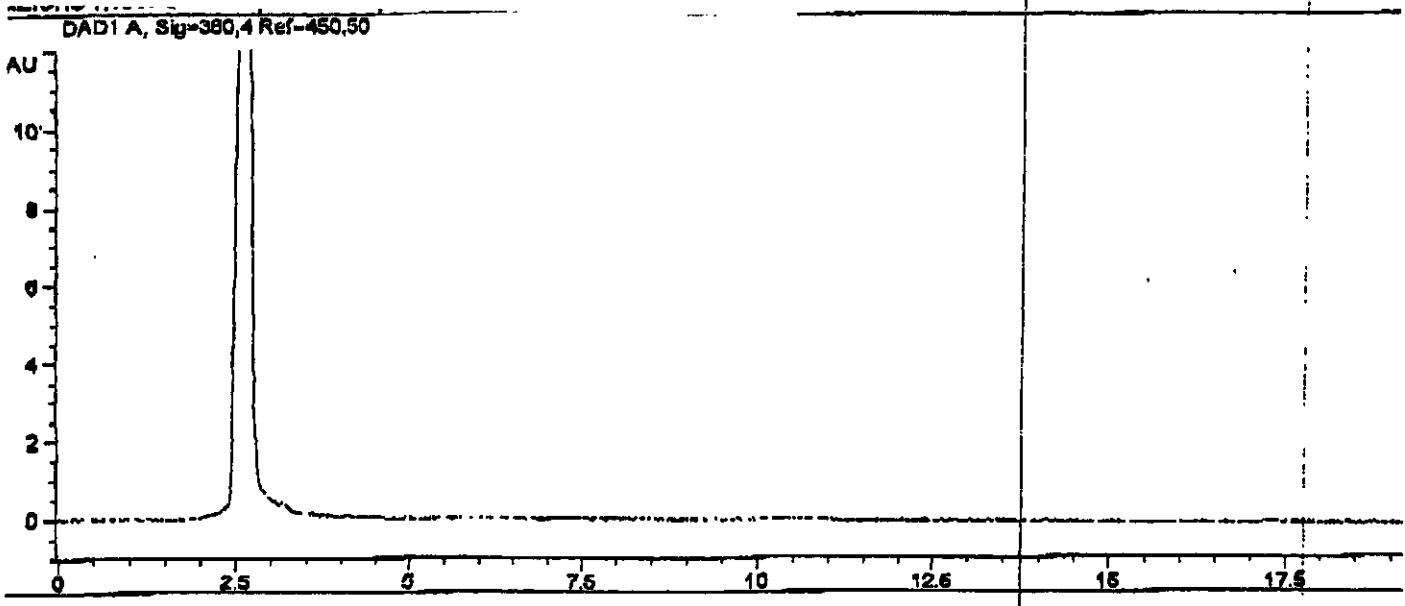
me	Type	Area [mAU*s]	Amt/Area [ng/ul]	Amount	Grp	Name
2	BB	20.10433	2.87603e-2	2.89103		olaquindox

s : 2.89103

lts obtained with enhanced integrator!

*** End of Report ***

blank feed (Pig)



External Standard Report

d By : Signal
. Data Modified : Monday, December 03, 2001 10:07:00 AM
ɔlier : 1.0000
on : 5.0000

ıl 1: DAD1 A, Sig=380,4 Ref=450,50

me	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
10	-	-	-			olaquindox

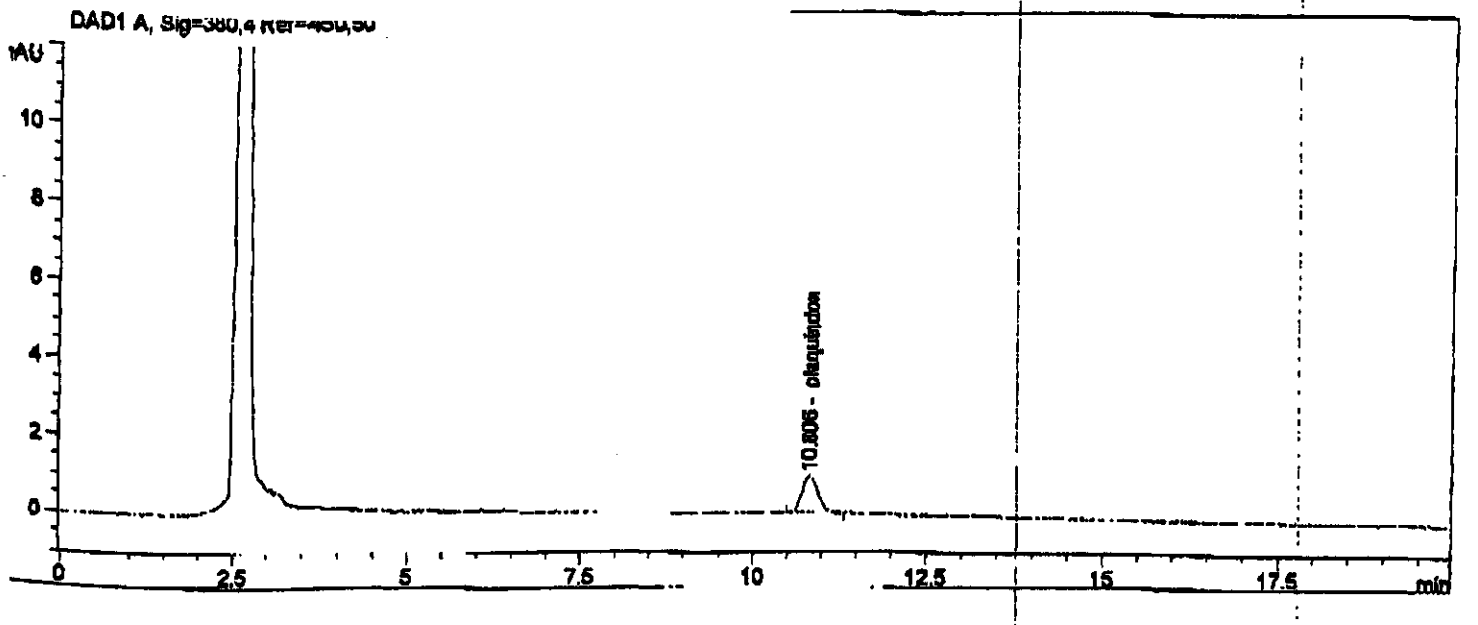
ε : 0.00000

ılts obtained with enhanced integrator!
ımings or Errors :

ıing : Calibrated compound(s) not found

15

Spike = 2.5ug/kg



External Standard Report

d By : Signal
Data Modified : Monday, December 03, 2001 10:07:00 AM
ller : 1.0000
n : 5.0000

l 1: DAD1 A, Sig=380,4 Ref=450,50

ne	Type	Area [mAU*s]	Amt/Area [ng/ul]	Amount	Grp	Name
6	BB	16.21838	2.86401e-2	2.32248		olaquindox
:			2.32248			

Rec = $\frac{2.32}{2.5} \times 100 = 92.8\%$

ts obtained with enhanced integrator!

*** End of Report ***

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 16

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: Nov. 20/21, 2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
165389	9,73	9,52
165403	2,70	2,63
165423	2,60	2,71
165427	9,65	9,49

CANFAS COLLABORATIVE STUDIES - 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory:

Contact person:

Date(s) of analysis: November 20./21., 2001

Chromatographic conditions:

- Column:
  - □ As described in the method
  - x Other: Phenomenex LUNA C18(2), 5 µm, 150 x 4.6 mm
- Mobile phase:
  - x As described in the method
  - □ Other:

  .....
- Flow-rate: 1.3 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: 6.9 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

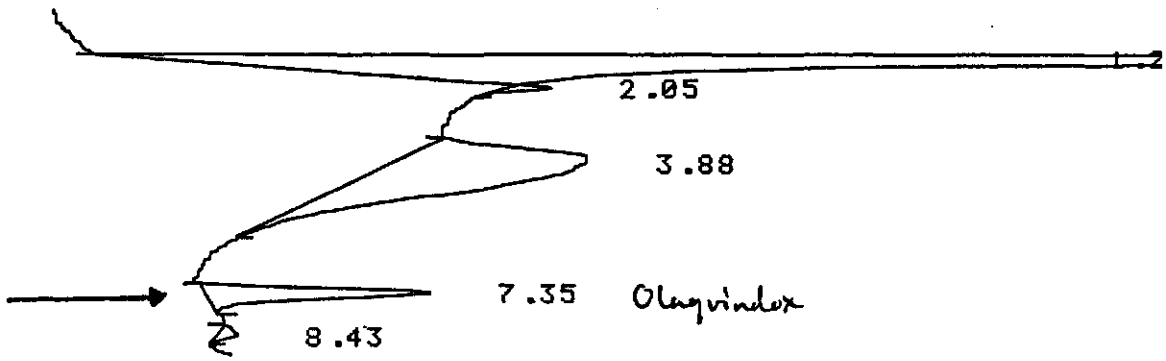
Recovery results:

- Percentage recovery: 74.7 %
- Single / duplicate determinations: □ single x duplicate
- If duplicate, please give both percentages: 75.3 and 74.0 %
- Spiking level: 3.65 mg/kg

CANFAJ ~ Olaquindox

Blind blank feed sample, fort
with Olaquindox 3,65 m

CH. 1 C.S 5.00 ATT 1 OFFS 10 11/20/01 03:03



INJ NO. OF STD : 1 / 1 REP , 1st level

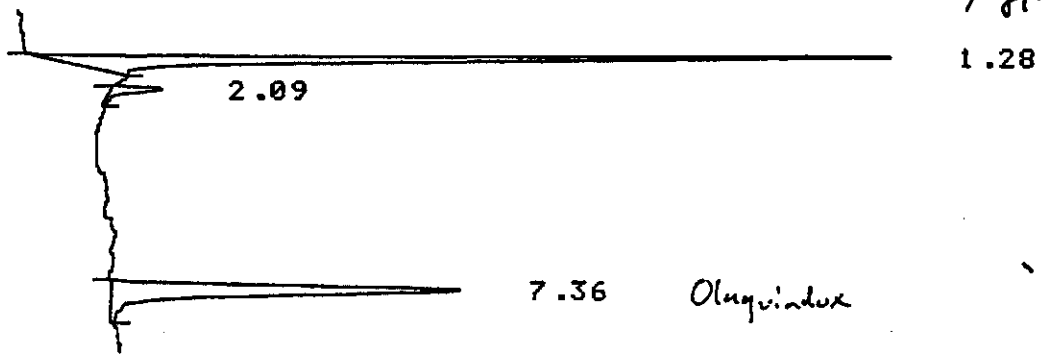
D-2500 11/20/01 03:03

FILE: 3 CALC-METHOD: EXT-STD TABLE: 9 CONC: HEIGHT

NO.	RT	AREA	HEIGHT	UG/ML	NAME
4	7.35	6477	356	0.730	OLA

Olaquindox-Standard-Sol
0,730 µg/ml

CH. 1 C.S 5.00 ATT 1 OFFS 10 11/20/01 03:14

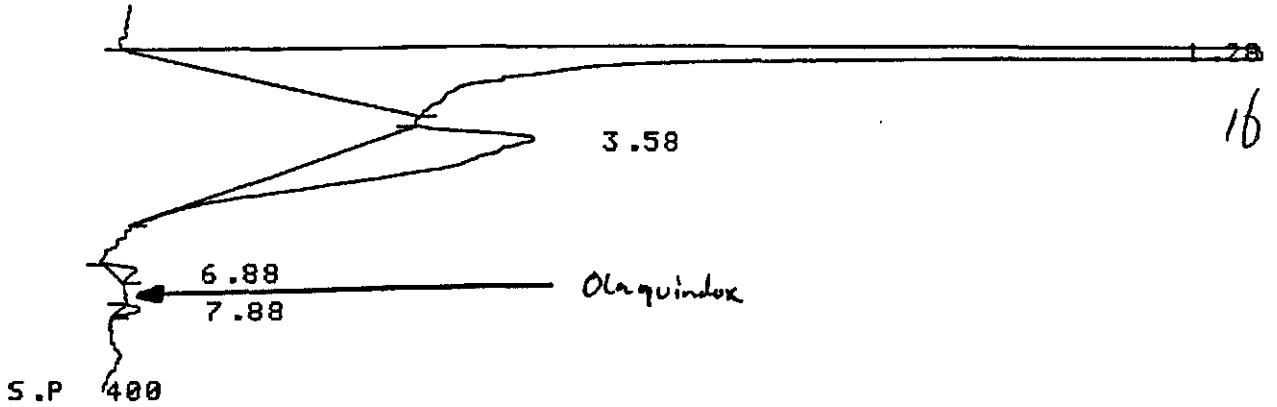


D-2500 11/20/01 03:14

FILE: 3 CALC-METHOD: EXT-STD TABLE: 9 CONC: HEIGHT

NO.	RT	AREA	HEIGHT	UG/ML	NAME
1	1.28	11894	1321	0.001	
3	7.36	9437	550	1.128	OLA
TOTAL					

CH. 1 C.S 5.00 ATT 1 OFFS 10 11/20/01 02:29 "Blind blank feed sample"



16

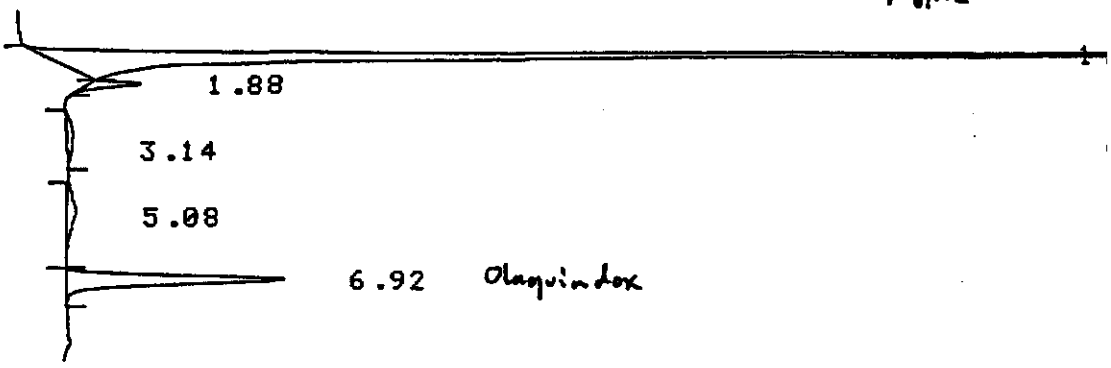
D-2500 11/20/01 02:29

FILE: 3 CALC-METHOD: EXT-STD TABLE: 9 CONC: HEIGHT

NO.	RT	AREA	HEIGHT	UG/ML	NAME
1	1.28	101796	5552	0.006	
2	3.58	20992	263	0.000	
TOTAL		122788	5815	0.006	

PEAK REJ : 100
SF : 1.000
SAMP-AMT : 1.000

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/21/01 18:22 0,730 µg/ml



INJ NO. OF STD : 1 / 1 REP , 1st level

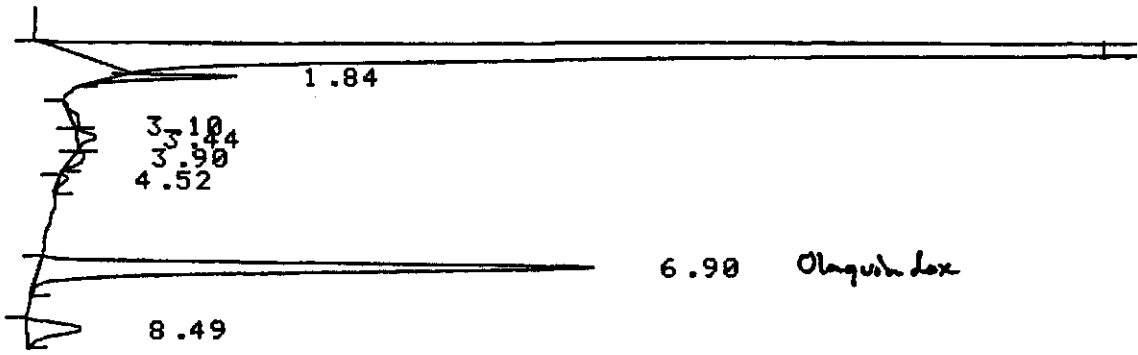
D-2500 11/21/01 18:22

FILE: 3 CALC-METHOD: EXT-STD TABLE: 8 CONC: AREA

NO.	RT	AREA	HEIGHT	UG/ML	NAME
5	6.92	21683	1380	0.730	OLAQUI

Sample code 16542

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/21/01 18:33



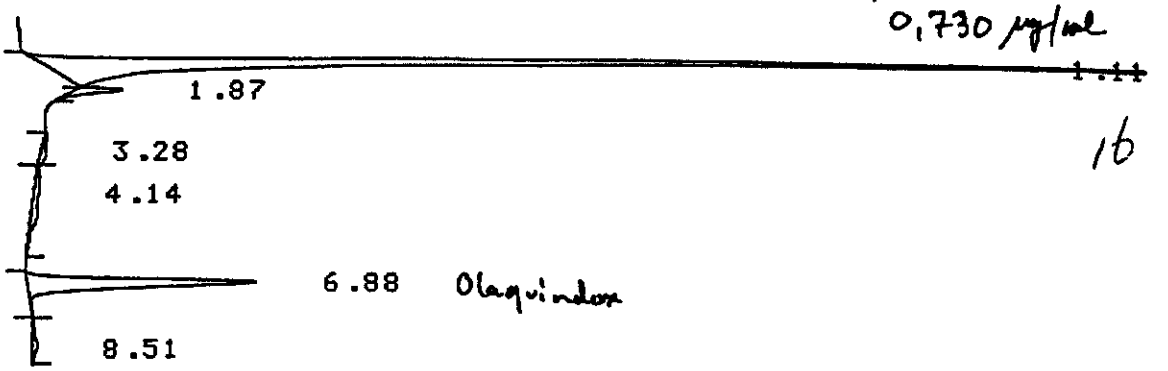
D-2500 11/21/01 18:33

FILE: 3 CALC-METHOD: EXT-STD TABLE: 8 CONC: AREA

NO.	RT	AREA	HEIGHT	UG/ML	NAME
1	1.16	466313	35283	0.466	
2	1.84	5756	763	0.006	
3	3.10	1303	34	0.001	
4	3.44	1792	119	0.002	
5	3.90	1525	64	0.002	
6	4.52	695	56	0.001	
7	6.90	57303	3513	1.929	OLAQUI

CH. 1 C.5 5.00 ATT 3 OFFS 10 11/21/01 17:18 Olaquindox-std-1c.

0,730 µg/ml



16

INJ NO. OF STD : 1 / 1 REP , 1st level

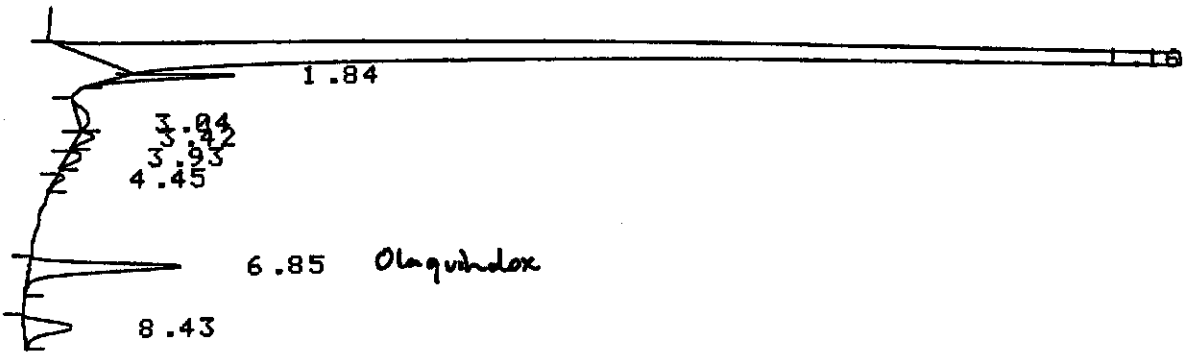
D-2500 11/21/01 17:18

FILE: 3 CALC-METHOD: EXT-STD TABLE: 8 CONC: AREA

NO.	RT	AREA	HEIGHT	UG/ML	NAME
5	6.88	21662	1455	0.730	OLAQUI

Sample code 165423

CH. 1 C.5 5.00 ATT 3 OFFS 10 11/21/01 17:29



D-2500 11/21/01 17:29

FILE: 3 CALC-METHOD: EXT-STD TABLE: 8 CONC: AREA

NO.	RT	AREA	HEIGHT	UG/ML	NAME
1	1.16	454728	34035	0.455	
2	1.84	5416	722	0.005	
3	3.04	2285	64	0.002	
4	3.42	1502	103	0.002	
5	3.93	1568	94	0.002	
6	4.45	488	42	0.000	
7	6.85	15843	971	0.534	OLAQUI
8	8.43	6917	313	0.007	

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 17

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 9.+10.01.2002

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
175313	2,97	3,03
175331	10,30	10,60
175352	10,05	9,97
175431	3,00	2,95

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .

Contact person: .. ..

Date(s) of analysis: 9. u. 10. 1. 2002

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: Spherisorb S10 ODS-1 10µ
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1,0 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: 6,9 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 98 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 3,75 mg/kg

- Percentage recovery : 88 %
- single
- Spiking level : 10 mg/kg

D-7000 HSM: Olaquindox Series: 0226

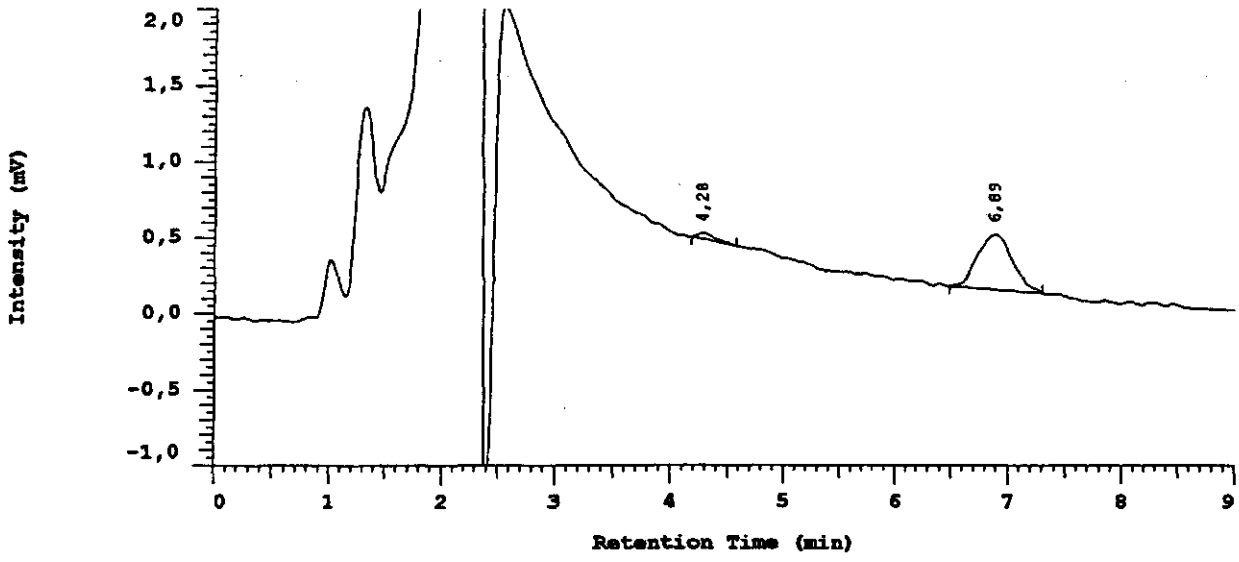
Sample Name: 175313

Analyzed: 09.01.02 13:58
Reported: 15.01.02 10:13
Processed: 15.01.02 10:13

Data Path: C:\Win32App\HSM\OLAQU\DATA\0226\
Application: Olaquindox
Series:0226
Injection from this vial: 1 of 1
Vial Number: 6
Volume: 20,0 ul

Sample Description:

Chrom Type: Fixed WL Chromatogram, 380 nm



Acquisition Method: Olaquindox
Column Type: RP 18
Pump A Type: L-7100
Solvent A: MeOH/H2O
Solvent B: MeOH/H2O
Solvent C: MeOH/H2O
Solvent D: MeOH/H2O
Peak Quantitation: AREA
Sample Amount: 0,200
Calculation Method: EXT-STD
Scale Factor 1: 1,000

Name	RT	Area	Conc 1
	4,28	431	0,000
Olaqu	6,89	7772	2,973
		8203	2,973

Peak rejection level: 0

D-7000 HSM: Olaquindox **Series: 0229**

Sample Name: 175352

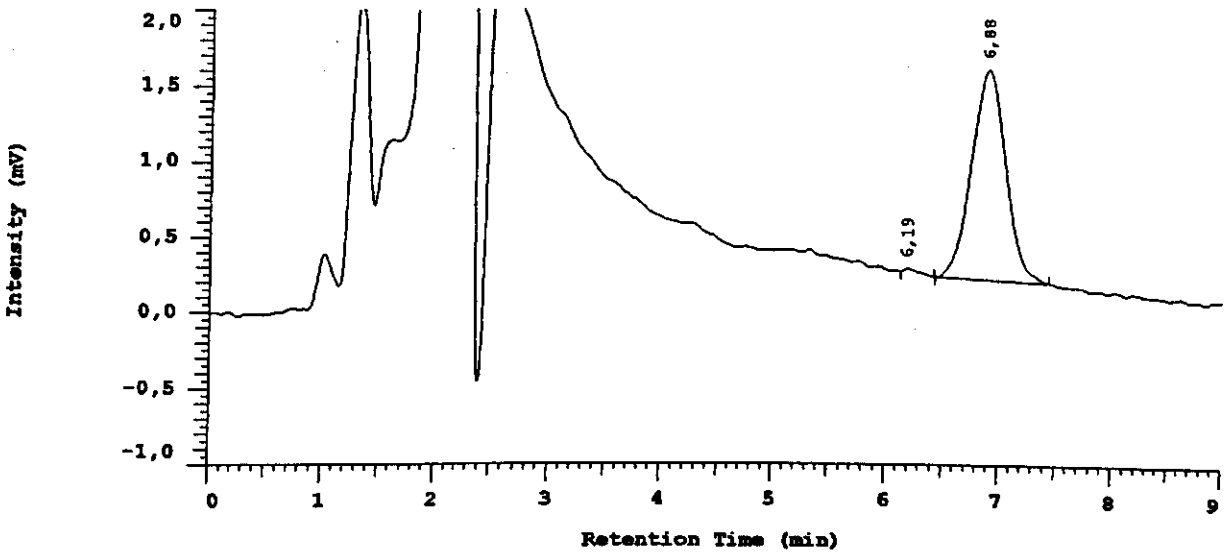
Analyzed: 10.01.02 11:58
Reported: 15.01.02 10:20
Processed: 15.01.02 10:19

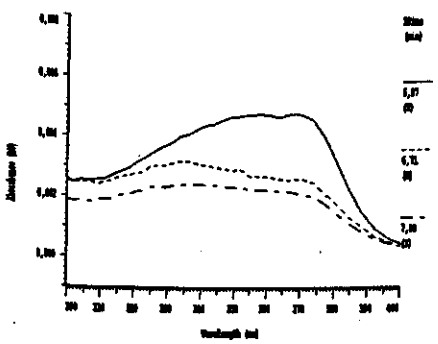
Data Path: C:\Win32App\HSM\OLAQU\DATA\0229\
Application: Olaquindox
Injection from this vial: 1 of 1

Series:0229
Vial Number: 4
Volume: 20,0 ul

Sample Description:

Chrom Type: Fixed WL Chromatogram, 380 nm





Acquisition Method: Olaquindox
Column Type: RP 18
Pump A Type: L-7100
Solvent A: MeOH/H2O
Solvent B: MeOH/H2O
Solvent C: MeOH/H2O
Solvent D: MeOH/H2O
Peak Quantitation: AREA
Sample Amount: 0,200
Calculation Method: EXT-STD
Scale Factor 1: 1,000

Name	RT	Area	Conc 1	BC
	6,19	0	0,000	
Olaqu	6,88	29814	9,967	MC
		29814	9,967	

Peak rejection level: 0

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 18

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 10-12-2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
185350	2,10	1,90
185353	2,10	2,50
185368	8,60	8,50
185395	9,40	9,60

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: ..10../12/..2001.....

Chromatographic conditions:

- Column:
  - As described in the method Spheri-5 ODS2 ; 150 x 4,6 mm ; 5µm
  - [X] Other: .....
- Mobile phase:
  - [X] As described in the method
  - [ ] Other: .....
- Flow-rate: ..1..... ml/min
- Injection volume: ..50...µl
- Retention time of olaquindox: 6,7.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

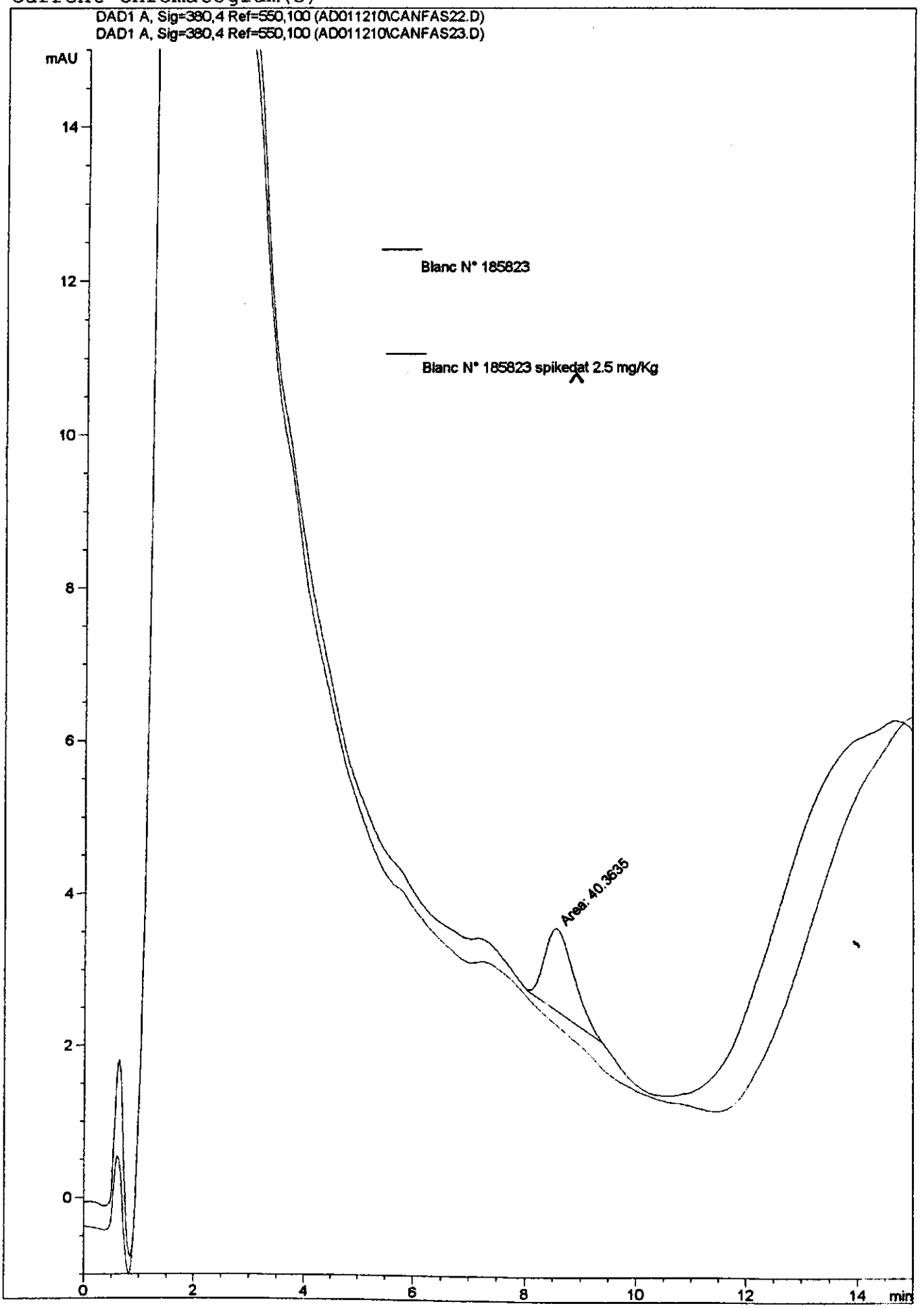
Please indicate the olaquindox peak with an arrow

Recovery results:

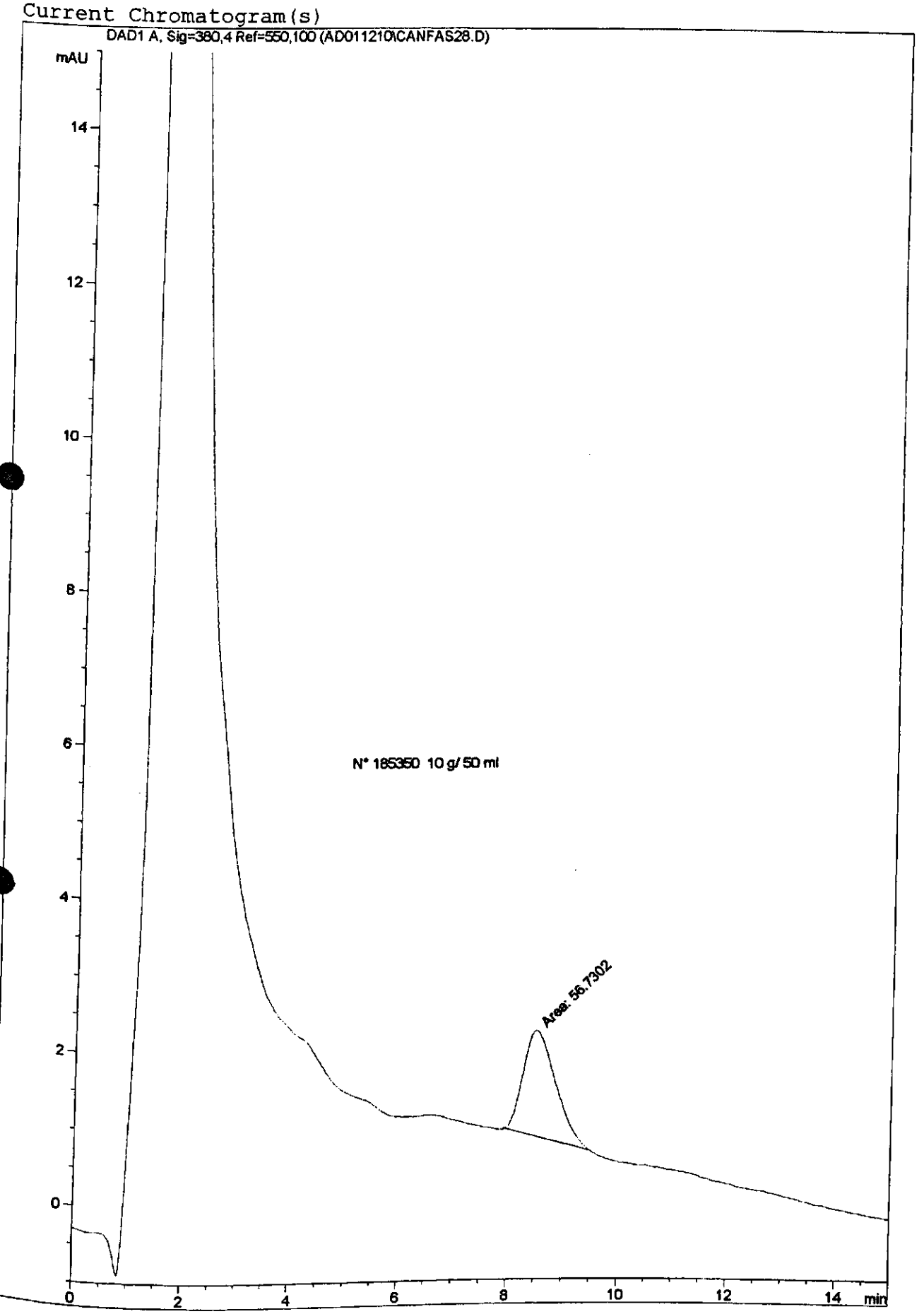
- Percentage recovery: 67.. %
- Single / duplicate determinations: [ ] single [X] duplicate
- If duplicate, please give both percentages: 62. % and 72. %
- Spiking level: ..2,5... mg/kg

of window 38: Current

Current Chromatogram(s)

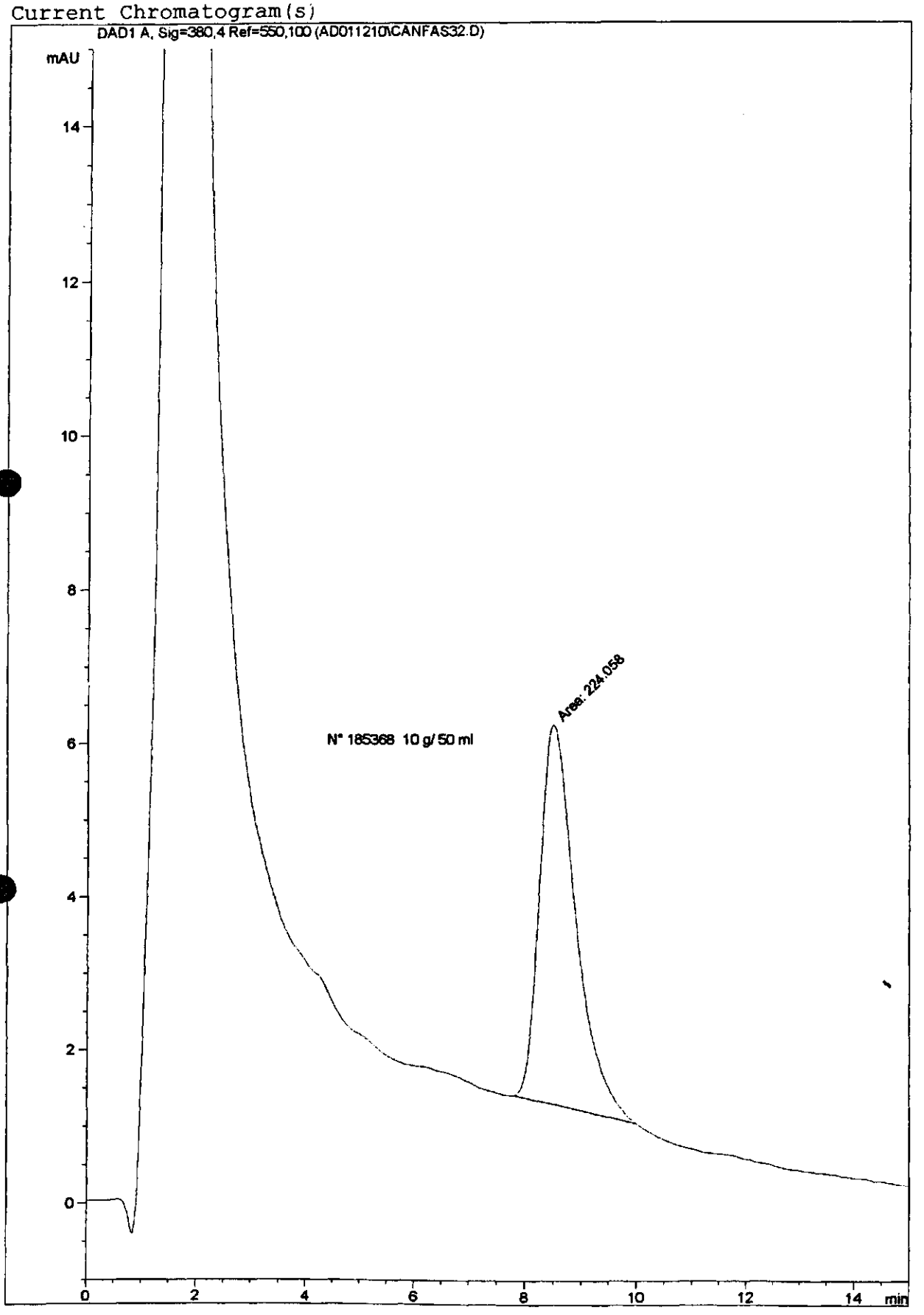


Current Chromatogram(s)

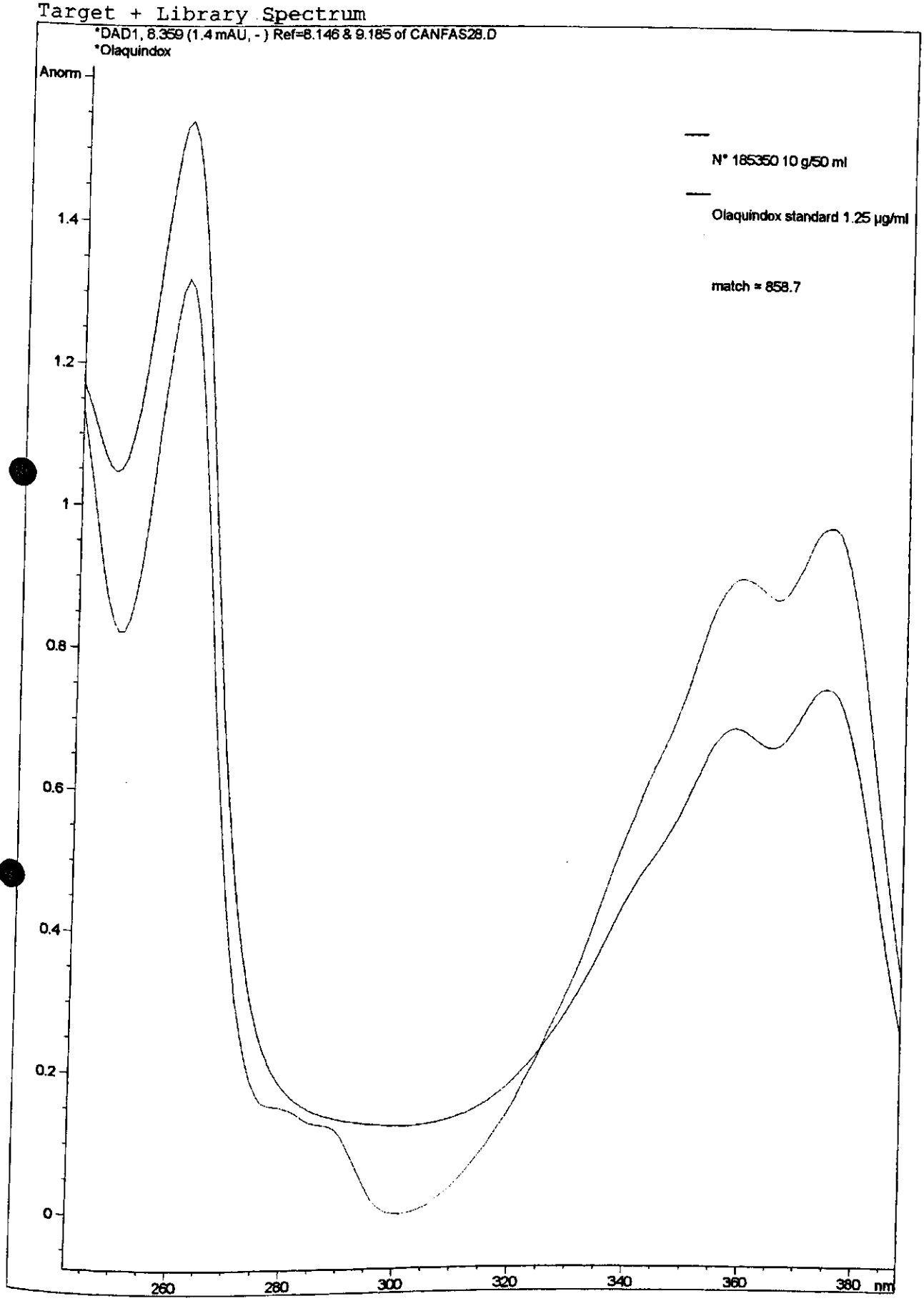


t of Window 38: Current Chromatogram(s)

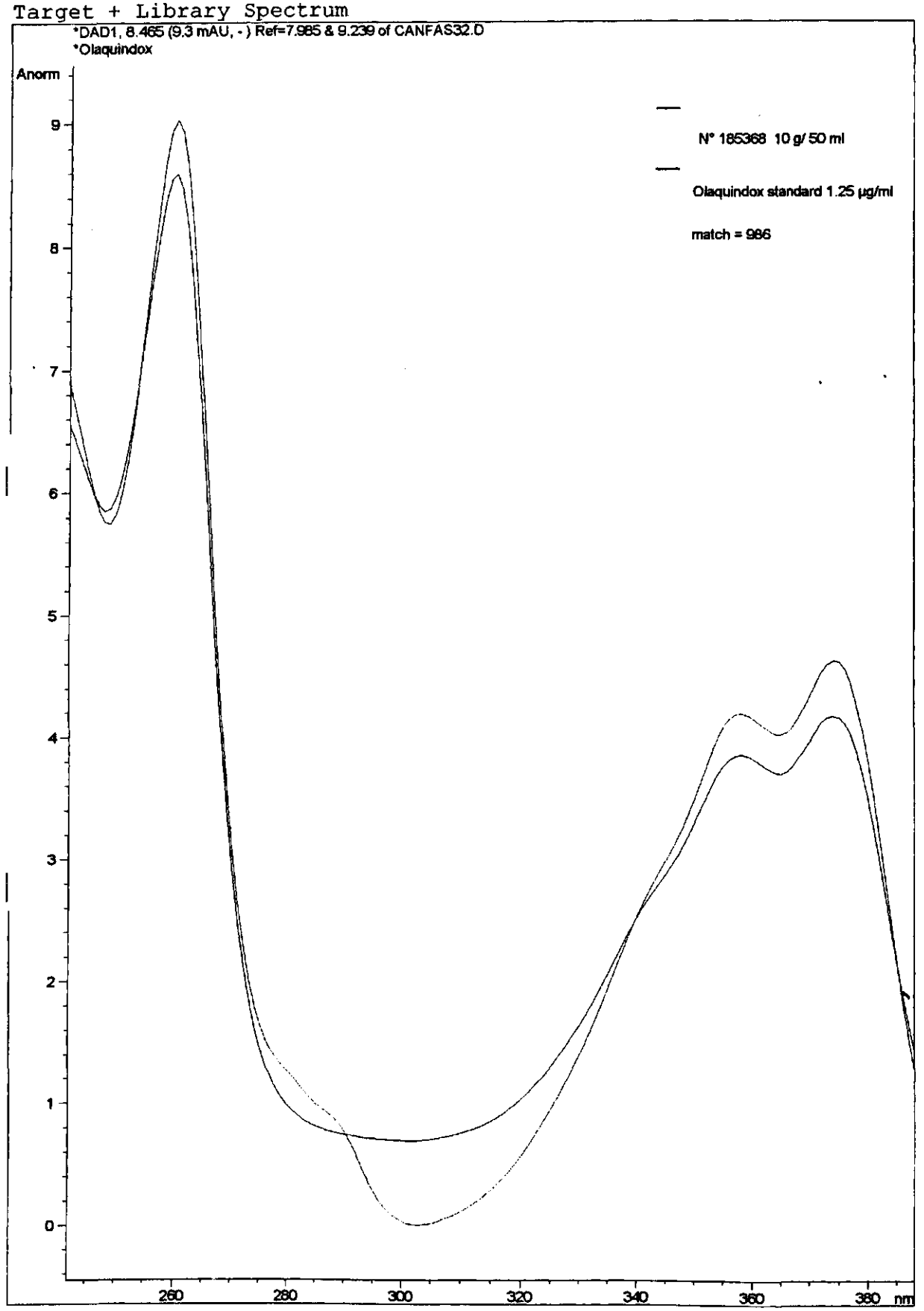
Current Chromatogram(s)



Target + Library Spectrum



Target + Library Spectrum



APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 20

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 24/11-04/12/2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
205367	9,09	9,10
205377	2,31	2,33
205417	9,00	8,99
205428	2,44	2,38

CANFAS COLLABORATIVE STUDIES NOVEMBER 2001 –

ADDITIVE: OLAQUINDOX

Annex 4 – Questionnaire

Laboratory: .

Contact person:

Date of analysis: december 2001

Chromatographic conditions:

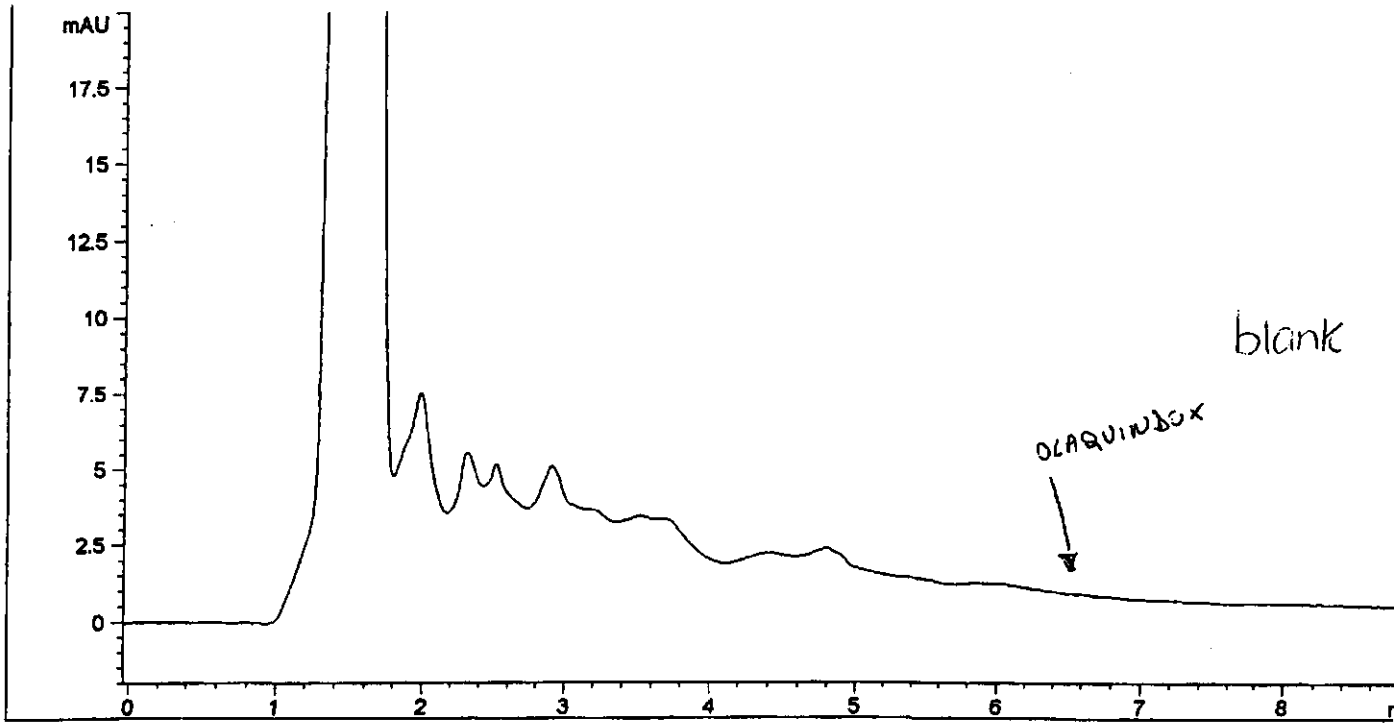
- Column:
  - ☐As described in the method
  - ✓Other: ODS Hypersyl C18 200x4.6 mm, 5 µm
- Mobile phase:
  - ✓As described in the method
  - ☐Other
- Flow rate: 1.5 ml/min
- Injection volume: 100 µl
- Retention time of nicarbazin: 6.8 min

Chromatograms: Please include representative chromatograms of:

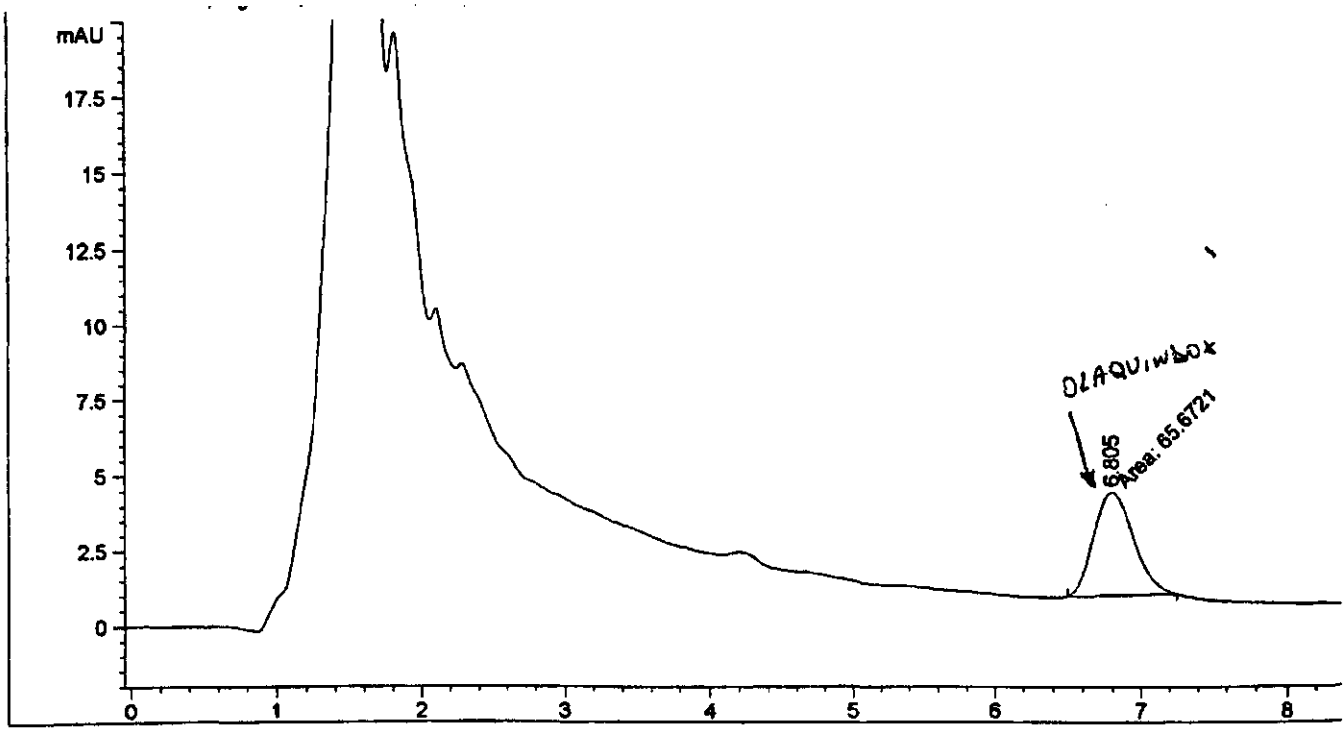
- **Blind positive feed sample**
- **Blind blank feed sample**

Recovery results:

- Percentage recovery: 87.7 %
- Duplicate determination: 87.8 % and 87.6 %
- Spiking level: 2.5 mg/Kg



External Standard Report



External Standard Report

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 21

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 23-11-2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
215341	3,40	3,43
215351	9,97	9,41
215401	3,35	3,48
215422	9,80	9,16

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: 22/11 e 23/11/2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: SUPELCOSIL LC 18 25cm × 4,6 mm + SUPELGUARD LC 18 2cm × 4,6 mm
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: GRADIENT ELUTION (ACETONITRILE – ACETATE BUFFER 0,01 M pH. 4,6)
- Flow-rate: 1,2 ml/min
- Injection volume: 50 µl
- Retention time of olaquindox: 7,65 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

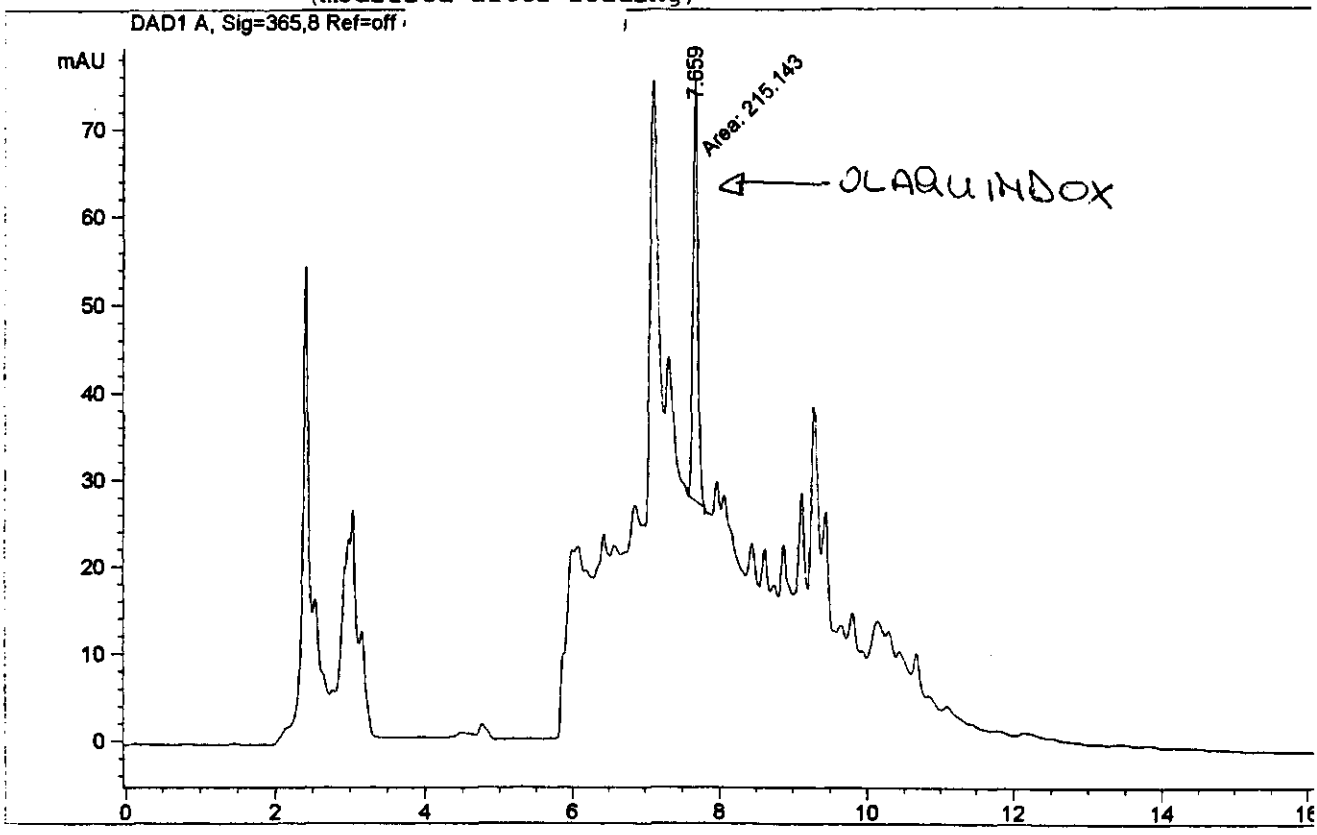
Recovery results:

- Percentage recovery: 99,2 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 98... % and 100,8 %
- Spiking level: 2,5... mg/kg

Data File

Sample Nam

Injection Date	: 22/11/2001 14.27.01	Seq. Line	: 3
Sample Name	: 5422-A	Location	: Vial 2
Acq. Operator	:	Inj	: 1
		Inj Volume	: 50 µl
Acq. Method	: '		
Last changed	: 22/11/2001 13.39.28		
Analysis Method	: C:\H		
Last changed	: 23/11/2001 9.43.43		
	(modified after loading)		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	23/11/2001 9.43.38
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=365,8 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng inj.]	Grp	Name
7.659	MM	215.14258	4.55740e-1	98.04917		OLAQ-olaquindox
Totals :				98.04917		

Results obtained with enhanced integrator!

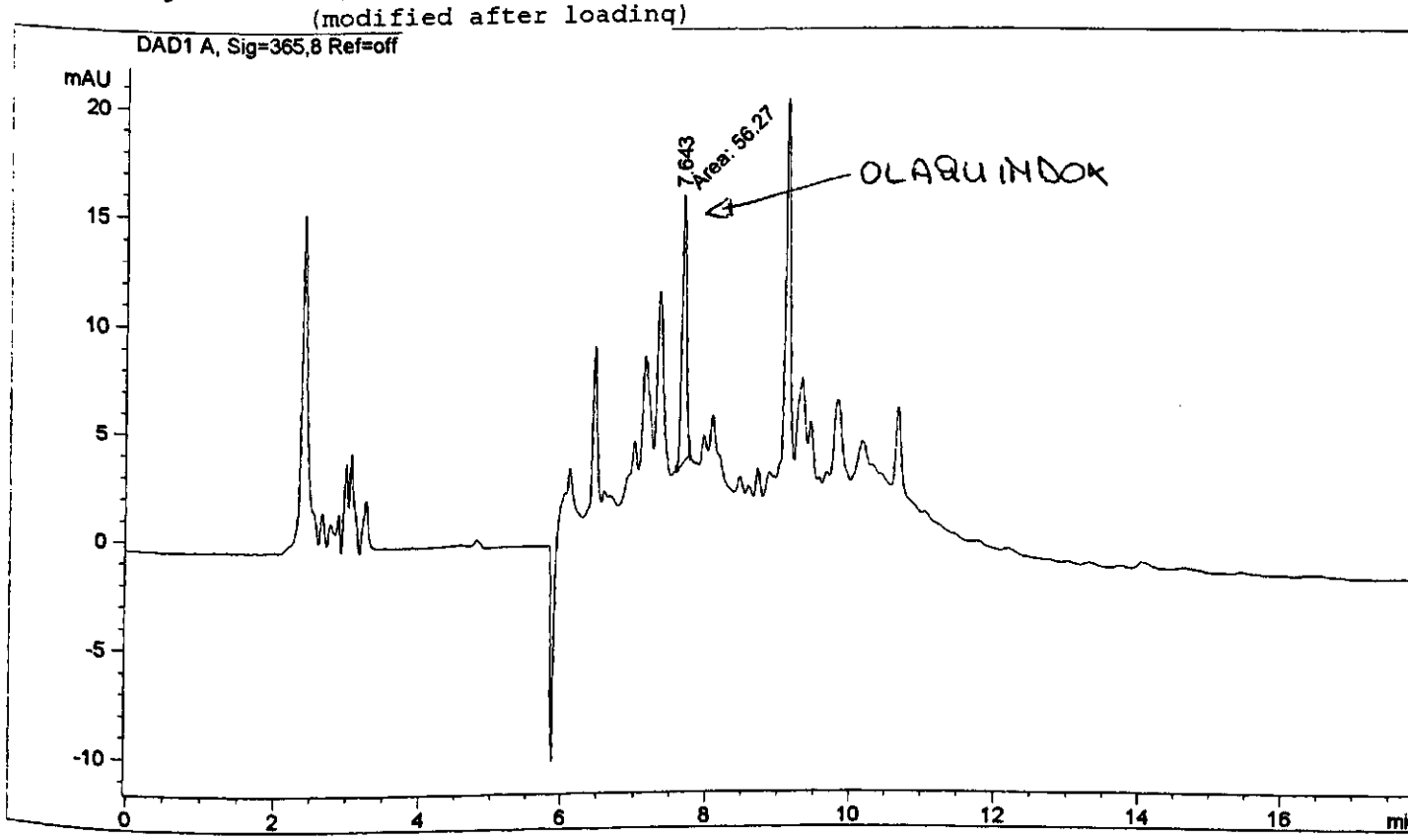
Sample Name:

21

Injection Date : 22/11/2001 16.48.06
Sample Name :
Acq. Operator : SPIKED SAMPLE

Seq. Line : 9
Location : Vial 7
Inj : 1
Inj Volume : 50 µl

Acq. Method :
Last changed : 22/11/2001 13.39.28
Analysis Method :
Last changed : 23/11/2001 10.38.09
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 23/11/2001 9.43.38
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,8 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng inj.]	Grp	Name
7.643	MM	56.26999	4.47699e-1	25.19200		OLAQ-olaquindox
Totals :				25.19200		

Results obtained with enhanced integrator!

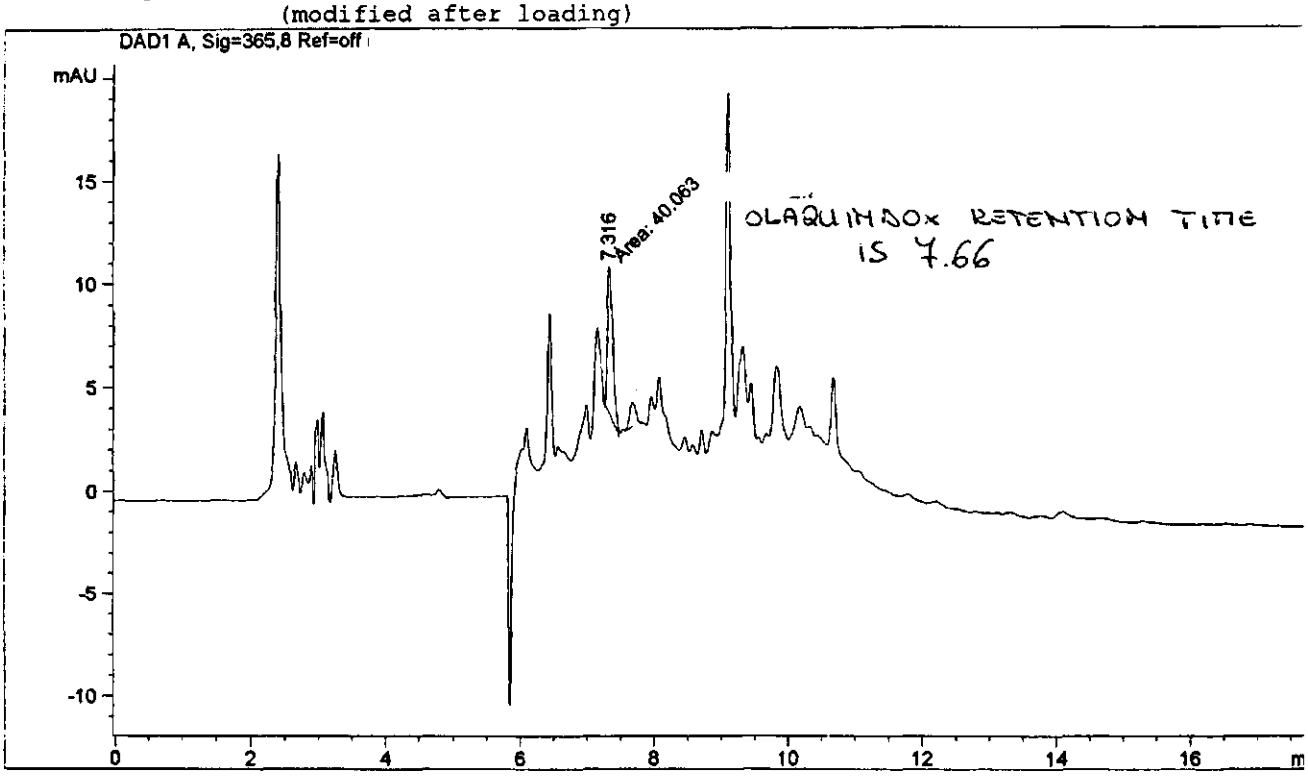
Da

Sample Name: 21

Injection Date : 22/11/2001 16.24.33
Sample Name : BLANKFEED
Acq. Operator :

Seq. Line : 8
Location : Vial 6
Inj : 1
Inj Volume : 50 µl

Acq. Method :
Last changed : 22/11/2001 13.39.28
Analysis Method : C·
Last changed : 23/11/2001 10.38.09
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 23/11/2001 9.43.38
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,8 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng inj.]	Grp	Name
7.652		-	-	-		OLAQ-olaquindox

Totals : 0.00000

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 22

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 01/11/15 01/11/20

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
225361	9,98	9,73
225373	2,45	2,55
225424	9,80	9,88
555416	2,60	2,63

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: 01.11.15 ; 01.11.20

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Hypersil C18 ODS BDS 5µm, 250 x 4.6 mm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.3 ml/min
- Injection volume: 50 µl
- Retention time of olaquindox: 8 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 101 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 105 % and 97 %

Spiking level: 3.2 / 6.3 mg/kg

NEW TIMED EVENTS FROM BAYONOX

******* **EXTERNAL STANDARD TABLE** *******

******************** 11-17-2001 09:23:27 Version 5.1 ********************
* Sample Name: blank Data File: D:bayo07
* Date: 11-11-2001 13:29:09Method: BAYONOX 11-17-2001 08:50:10 # 287
* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#:
* Starting Peak Width: 2 Threshold: 1 Area Threshold: 100
****

Starting Delay: 0.00
Area reject: 1000
Amount injected: 50.00
Sample Weight: 1.00000

Ending retention time: 12.00
One sample per 0.200 sec.
Dilution factor: 1.00

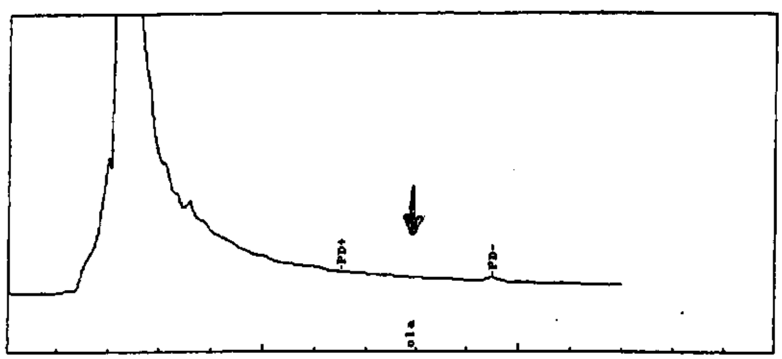
PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in ug/ml	NORMALIZED CONC	AREA	AREA/ HEIGHT	HEIGHT BL	REF PEAK	% DELTA RET TIME	CONC/

TOTAL AMOUNT = 0.0000

PEAKS NOT FOUND IN THIS RUN

NAME	ADJUSTED RET.TIME.	REFERENCE PEAK
ola	7.89	ola

Data File = D:bayo07.PTS Printed on 11-17-2001 at 09:23:31
Start time: 0.00 min. Stop time: 15.00 min. Offset: 0 mv.
Low Value: 0 uv High Value: 222486 uv Scale factor: 8.0



Blank

******* EXTERNAL STANDARD TABLE *******

********** 11-17-2001 09:25:48 Version 5.1 **********

Sample Name: prov nr 2 Data File: D:BAYO028

Date: 11-16-2001 13:21:20Method: D:BAYONOX 11-17-2001 09:25:41 # 292

Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#:

Starting Peak Width: 15 Threshold: 1 Area Threshold: 200

****

Starting Delay: 0.00 Ending retention time: 15.00

Area reject: 200 One sample per 0.200 sec.

Amount injected: 50.00 Dilution factor: 1.00

Sample Weight: 1.00000

PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in ug/ml	NORMALIZED CONC	AREA	HEIGHT	AREA/ HEIGHT	BL	REF PEAK	% DELTA RET TIME	CONC/AREA
1	8.151	ola	0.5100	100.0000%	33455	2147	15.6	1	1	0	1.5244E-05

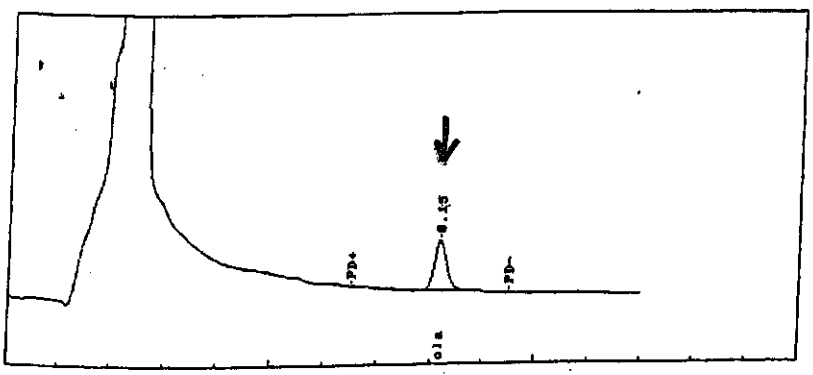
TOTAL AMOUNT = 0.5100

Peaks, times, and heights stored in: D:BAYO028.ATB

Data File = D:BAYO028.PTS Printed on 11-17-2001 at 09:25:50

Start time: 0.00 min. Stop time: 15.00 min. Offset: 0 mv.

Low Value: 0 uv High Value: 99159 uv Scale factor: 8.0



Sample 225373

******* EXTERNAL STANDARD TABLE *******

********** 11-17-2001 09:26:43 Version 5.1 **********

Sample Name: prov nr 4 Data File: D:BAYO030

Date: 11-16-2001 13:48:11Method: D:BAYONOX 11-17-2001 09:25:41 # 29

Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#:

Starting Peak Width: 15 Threshold: 1 Area Threshold: 200

****

tarting Delay: 0.00 Ending retention time: 15.00

rea reject: 200 One sample per 0.200 sec.

nount injected: 50.00 Dilution factor: 1.00

ample Weight: 1.00000

AK UM	RET TIME	PEAK NAME	CONCENTRATION in ug/ml	NORMALIZED CONC	AREA	AREA/ HEIGHT	HEIGHT BL	REF PEAK	% DELTA RET TIME	CONC/
1	8.131	ola	1.9754	100.0000%	129585	8302	15.6 1	1	0	1.524

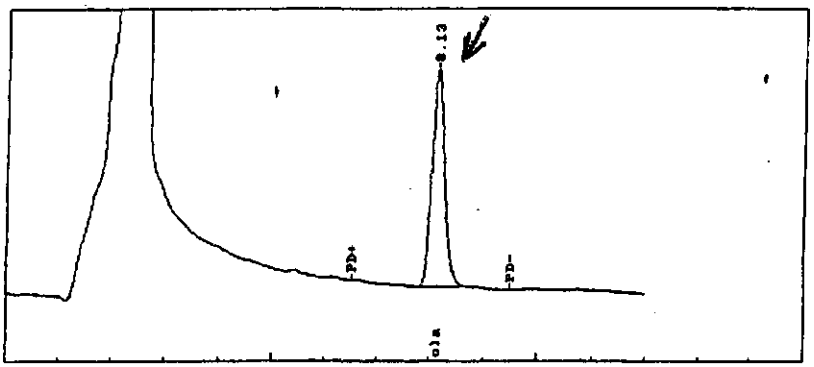
TOTAL AMOUNT = 1.9754

[redacted]s, times, and heights stored in: D:BAYO030.ATB

ata File = D:BAYO030.PTS Printed on 11-17-2001 at 09:26:46

tart time: 0.00 min. Stop time: 15.00 min. Offset: 0 mv.

ow Value: 0 uv High Value: 89418 uv Scale factor: 8.0



Sample 225424

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 23

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 15-12-2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
235325	2,35	2,46
235310	2,43	2,30
235342	8,55	8,60
235365	9,34	9,50

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 24

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 12 dec 2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
245307	8,89	7,99
245326	2,10	2,01
245410	2,09	2,01
245426	8,40	8,69

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: 12th December 2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: C18, 5 µm, 250 x 4.6 mm Waters Xterra
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.1 ml/min
- Injection volume: 70 µl
- Retention time of olaquindox: 9.9 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate

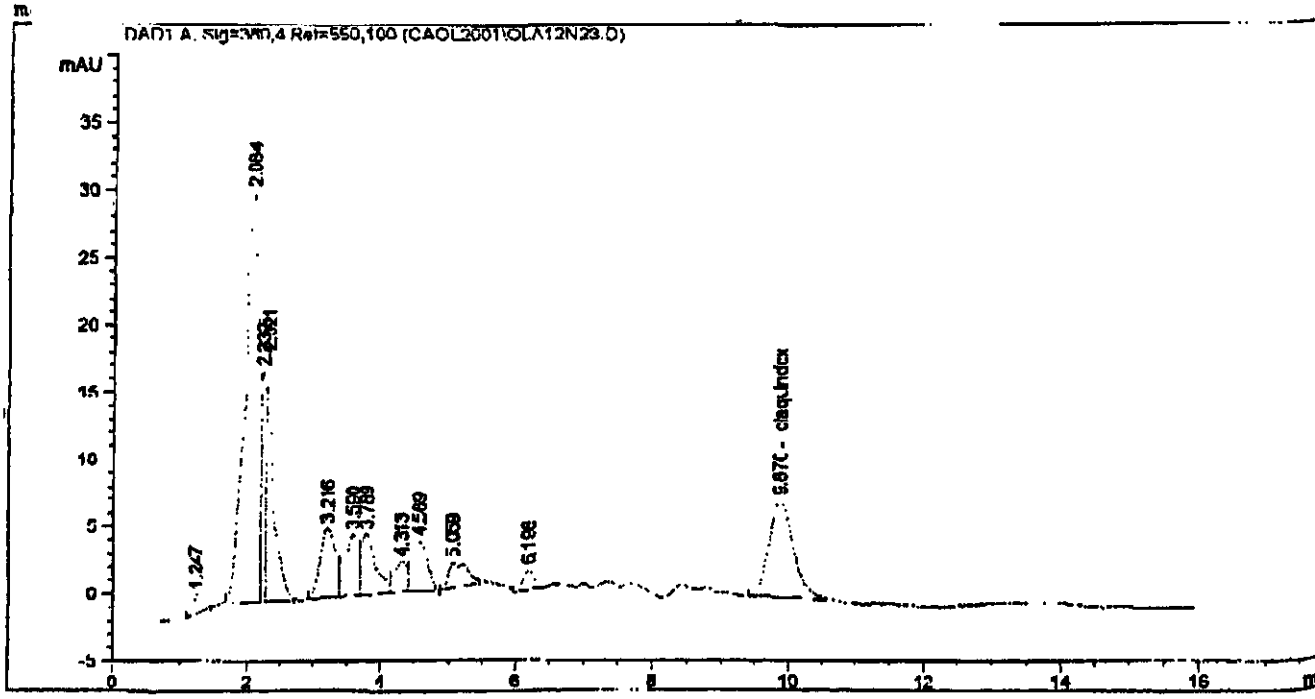
  If duplicate, please give both percentages: 83 % and 80 %

  Spiking level: 2.5 mg/kg

~ple h. 245307 24

RING-TEST CANFAS OLAQUINDOX

Injection Date : 12/12/01 16.56.48
Sample Name : Location : Vial 1
Acq. Operator :
Method : C:
Last changed : 12/12/01 15.23.45 by 1
(modified after loading)



External Standard Report 2A

Sorted By : Signal
Calib. Data Modified : 12/12/01 13.11.35
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,4 Ref=550,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
9.870	PB	177.15282	1.00387e-2	1.77838		olaquindox

Totals : 1.77838

Results obtained with enhanced integrator!

*** End of Report ***

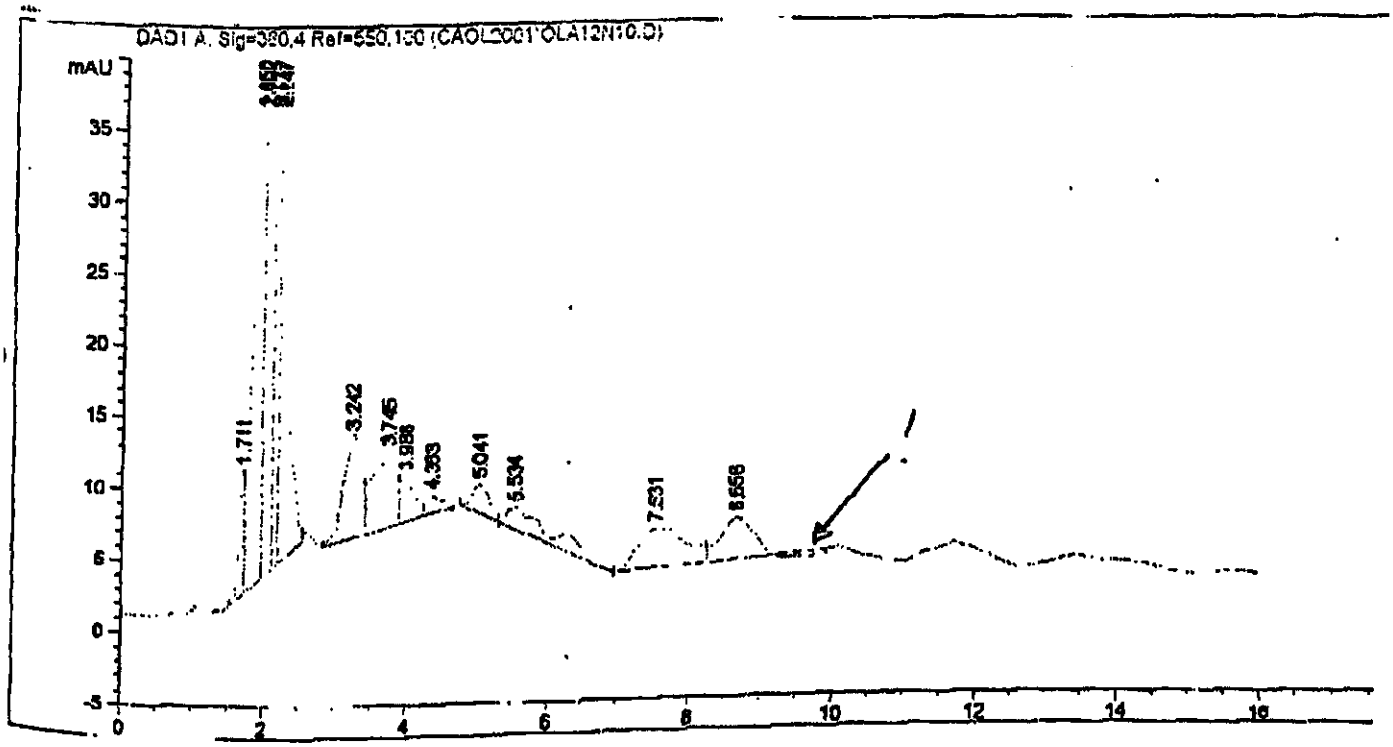
RING-TEST CANFAS OLAQUINDOX

negative sample

24

Injection Date : 12/12/01 12.50.29
Sample Name :
Acq. Operator :
Acq. Method : C:\HPCHEM\1\METHODS\CANFOL.M
Last changed : 12/12/01 12.59.47 b
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\
Last changed : 12/12/01 13.11.24 b
(modified after loading)

Location : Vial 1



External Standard Report negative sample

Sorted By : Signal
Calib. Data Modified : 12/12/01 12.59.44
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,4 Ref=550,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
9.091		-	-	-		olaquindox

Totals : 0.00000

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 25

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis: 06 dec 01

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
255349	2,88	2,86
255306	10,25	10,33
255383	2,88	3,02
255411	10,50	10,56

25

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 – Questionnaire

Laboratory: [redacted]

Contact person: [redacted]

Date(s) of analysis: .....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: RP C18 LICHROCART 250-4 (5µm) MERCK
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: H_3PO_4 0,01M pH = 2,8 (DEA) / ACN 80% GRADIENTE LINEAR
- Flow-rate: 1 ml/min
- Injection volume: 60 µl
- Retention time of olaquindox: 6,4 min

Chromatograms: Please include representative chromatograms of:

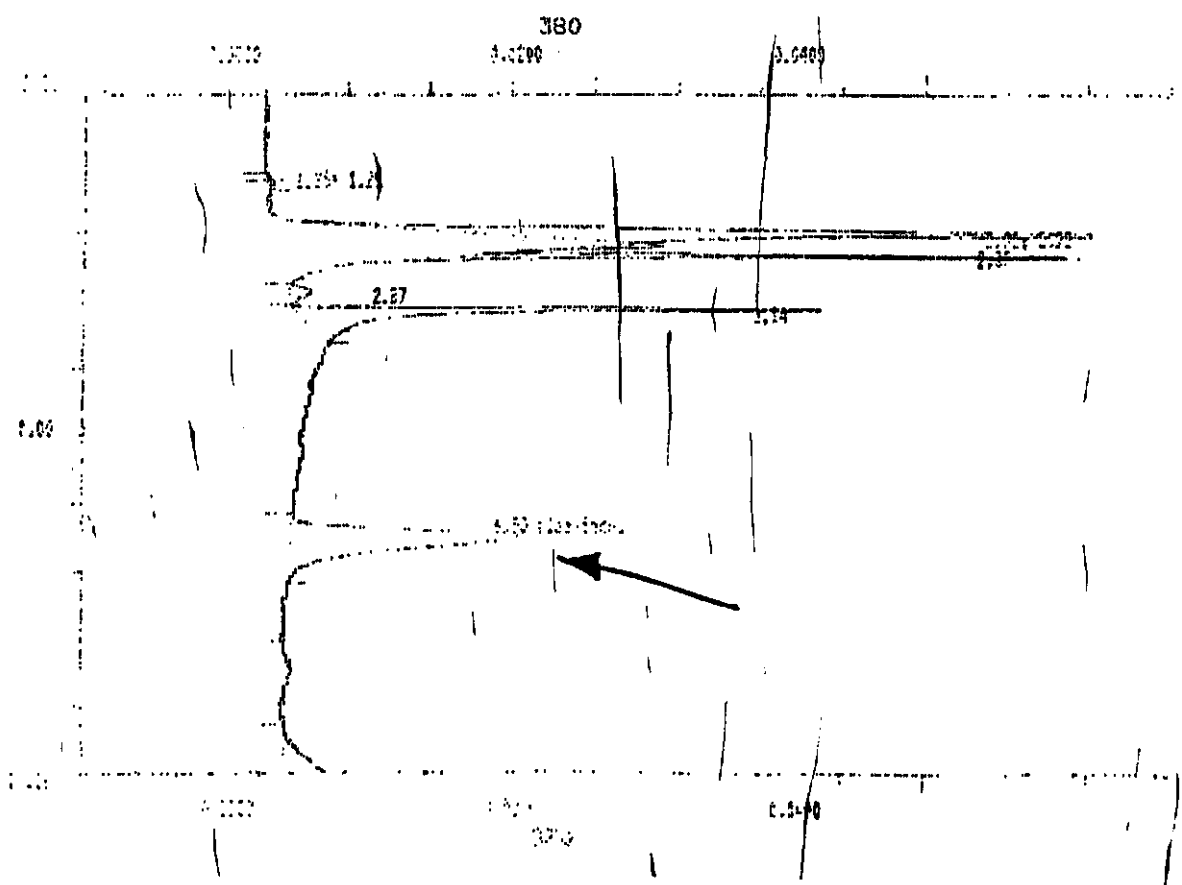
- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 90 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 90,90 % and 90,95 %
- Spiking level: 2,5 mg/kg

25



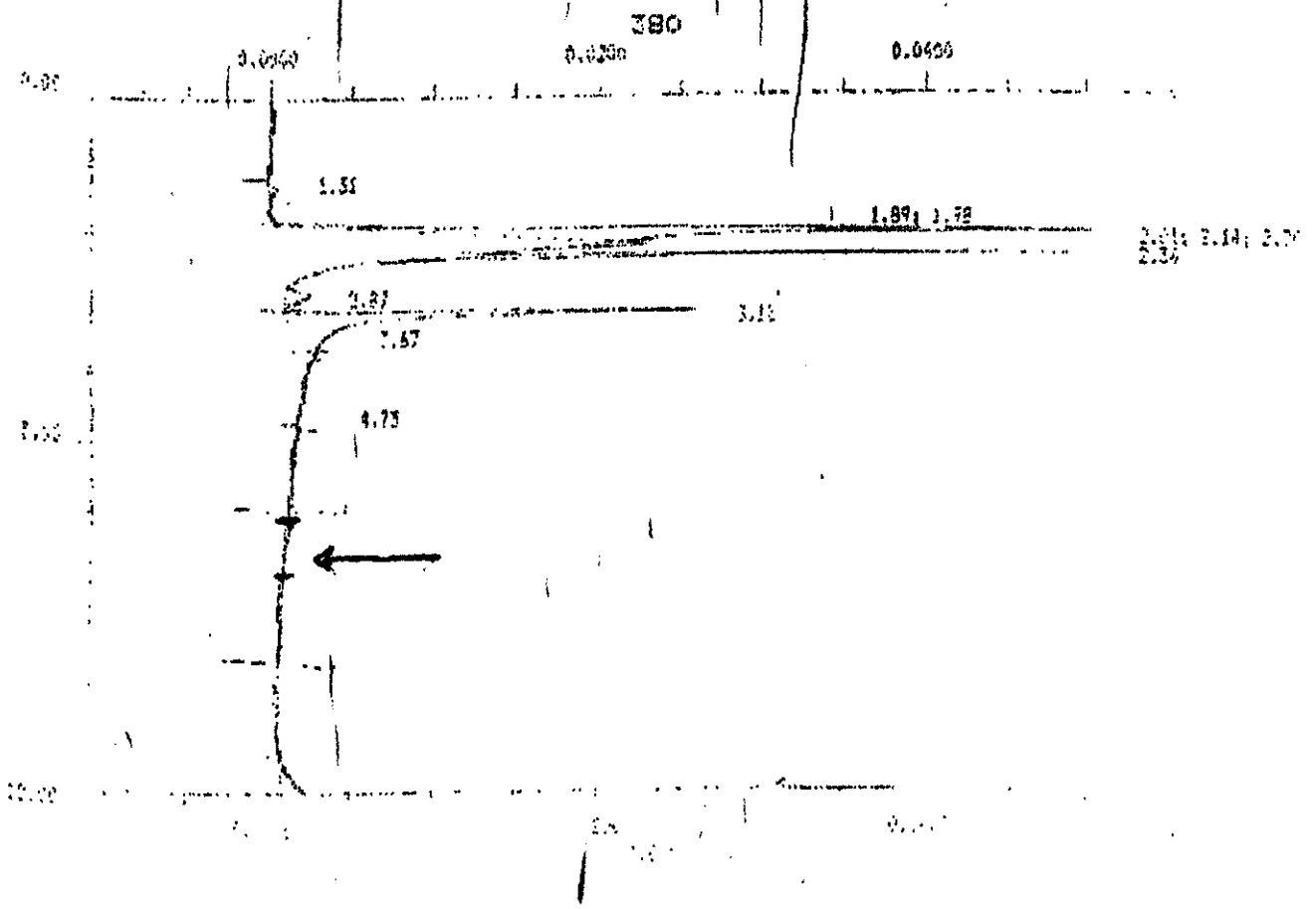
3/4

25

		CHAN	LEV	REP	TYPE	DIRECTORY
SELECTION	DATA	[illegible]	1	1	Data	C:\SOLUTION\DATA\
	METHOD	[illegible]				C:\SOLUTION\SYSTEM\

SAMPLE TABLE [illegible]
SYSTEM 1: SYSTEM1

Chart Speed 1.00 cm/min



41

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 26

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 19-11-2001

Analyte: OLAQUINDOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
265390	7,32	7,76
265398	7,77	7,85
265404	2,20	2,19
265413	2,25	2,25

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: 19/11/2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: SPHERISORB ODS 2 5µm 250 x 4.6mm
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.0 ml/min
- Injection volume: 100 µl
- Retention time of olaquindox: * min See Comments

OVEN @ 35°C

Chromatograms: Please include representative chromatograms of:

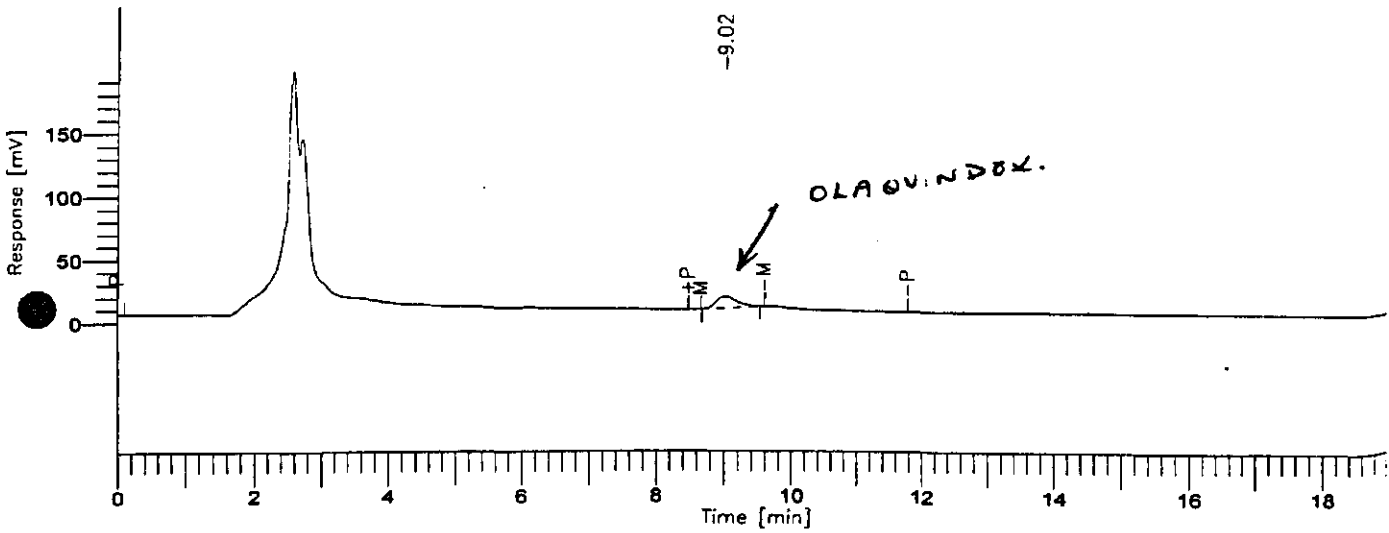
- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 68 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 2.5 mg/kg

Software Version: 4.1<2F12>
Date: 20/11/01 16:24
Sample Name : B3014229B
Data File : C:\TC4\CANFAS\OLAQUI~1\REPEAT~1\DATA024.RAW Date: 19/11/01 23:
Sequence File: C:\TC4\CANFAS\OLAQUI~1\REPEAT~1\RPTTRIAL.SEQ Cycle: 24 Channe
Instrument : BOX_0 Rack/Vial: 0/0 Operator:
Sample Amount : 1.0000 Dilution Factor : 1.00



DEFAULT REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	9.018	203243.24	9421.37	100.00	100.00	*BB	21.57
0	9.862	0.00	0.00	0.00	0.00		------
		203243.24	9421.37	100.00	100.00		

Missing Component Report

Component	Expected Retention (Calibration File)
Olaquindox	9.862

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 29

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 21_11_01

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
295319	6,49	6,53
295354	1,66	1,61
295356	1,90	1,86
295418	5,70	5,97

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .....

Date(s) of analysis: ..... 01.11.22 .....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: ..... Nova-Pak C18 4µm 4.6X250mm .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ..... 1,5 ..... ml/min
- Injection volume: .. 100 .. µl
- Retention time of olaquindox: 7,3 .. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

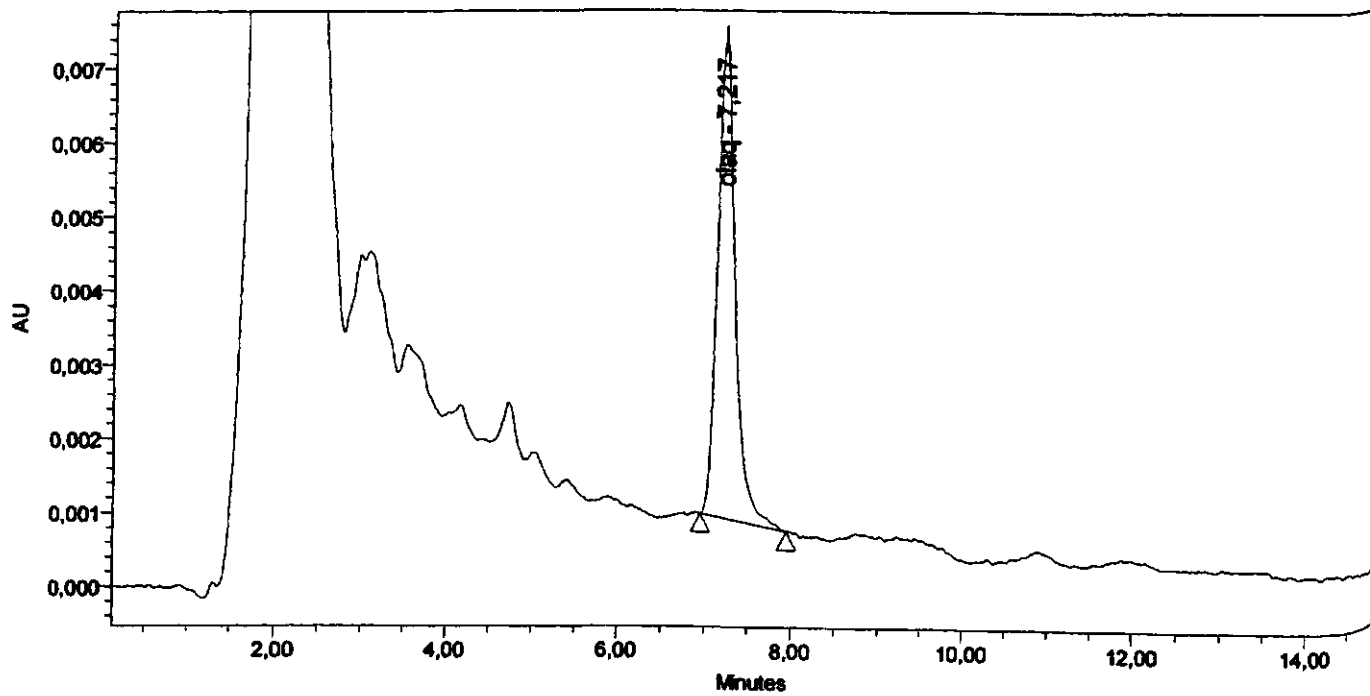
- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 104 % and 107 %
- Spiking level: .. 0,5 .. mg/kg

Olaquindox Report

Reported by User: System

Project Name: Olaquindox

Sample Name:	**Spiked blank II 2,5 mg/kg**	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	21-11-2001 22:04:34
Vial:	35	Acq. Method Set:	Olaquindox
Injection #:	2	Date Processed:	22-11-2001 10:49:10
Injection Volume:	100,00 ul	Processing Method:	
Run Time:	15,0 Minutes		
Sample Set Name:	**OLAQUINDOX Nov 2001**	Proc. Chnl. Descr.:	PDA 380,0 nm



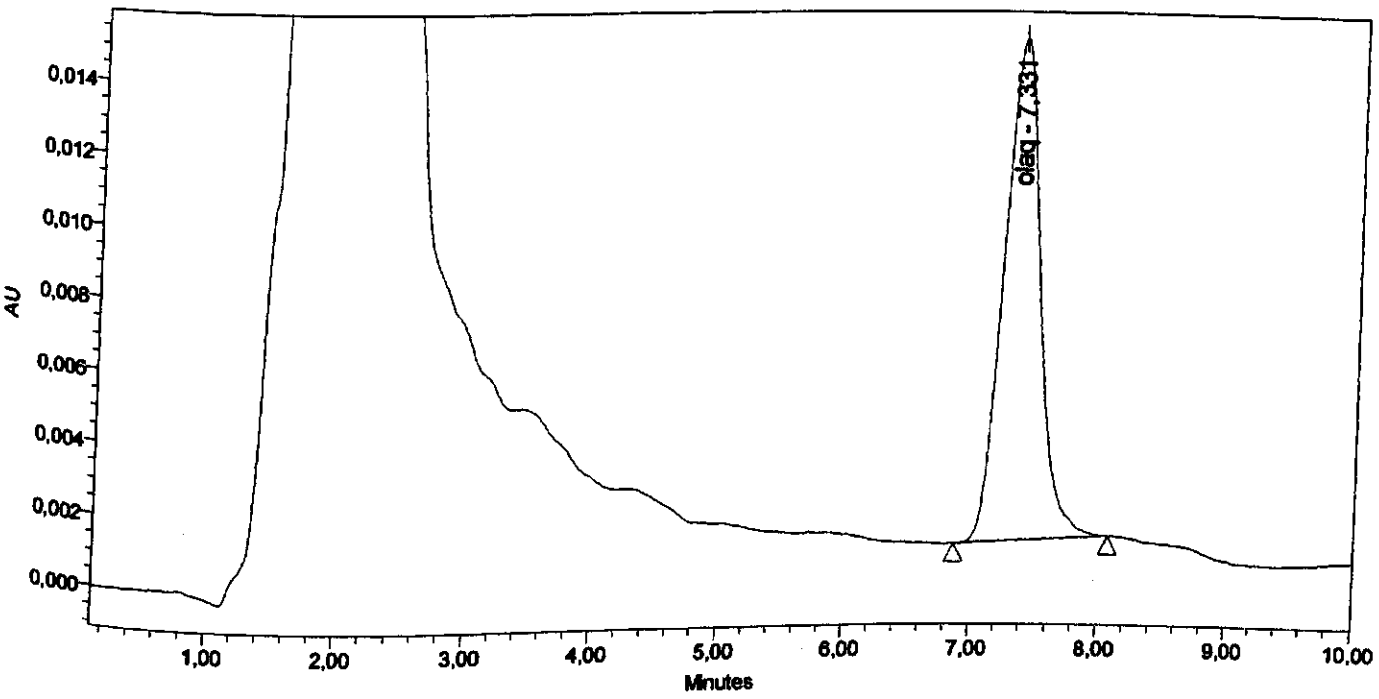
	Name	RT	Area	Height	Amount	Units
1	olaq	7,217	91906	6464	0,521	Uug/ml

Olaquindox Report

Reported by User: System

Project Name: Olaquindox

Sample Name:		Acquired By:	System
Sample Type:	Unknown	Date Acquired:	22-11-2001 15:33:52
Vial:	26	Acq. Method Set:	Olaquindox
Injection #:	1	Date Processed:	23-11-2001 11:55:47
Injection Volume:	100,00 ul	Processing Method:	
Run Time:	10,0 Minutes		
Sample Set Name:	**olaquindox 22_01**	Proc. Chnl. Descr.:	PDA 380,0 nm



	Name	RT	Area	Height	Amount	Units
1	olaq	7,331	275380	13954	1,140	Uug/ml

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 31

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 27-11-2001

Analyte: OLAQUINDOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
315359	8,71	9,09
315399	9,47	9,40
315414	2,70	3,10
315429	2,37	2,57

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .. ..

Contact person: .. ..

Date(s) of analysis: ....27-11-01.....

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: .....1,5..... ml/min
- Injection volume: ..100...µl
- Retention time of olaquindox: .7,27.min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 83,5%
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 82,3 % and 84,7 %

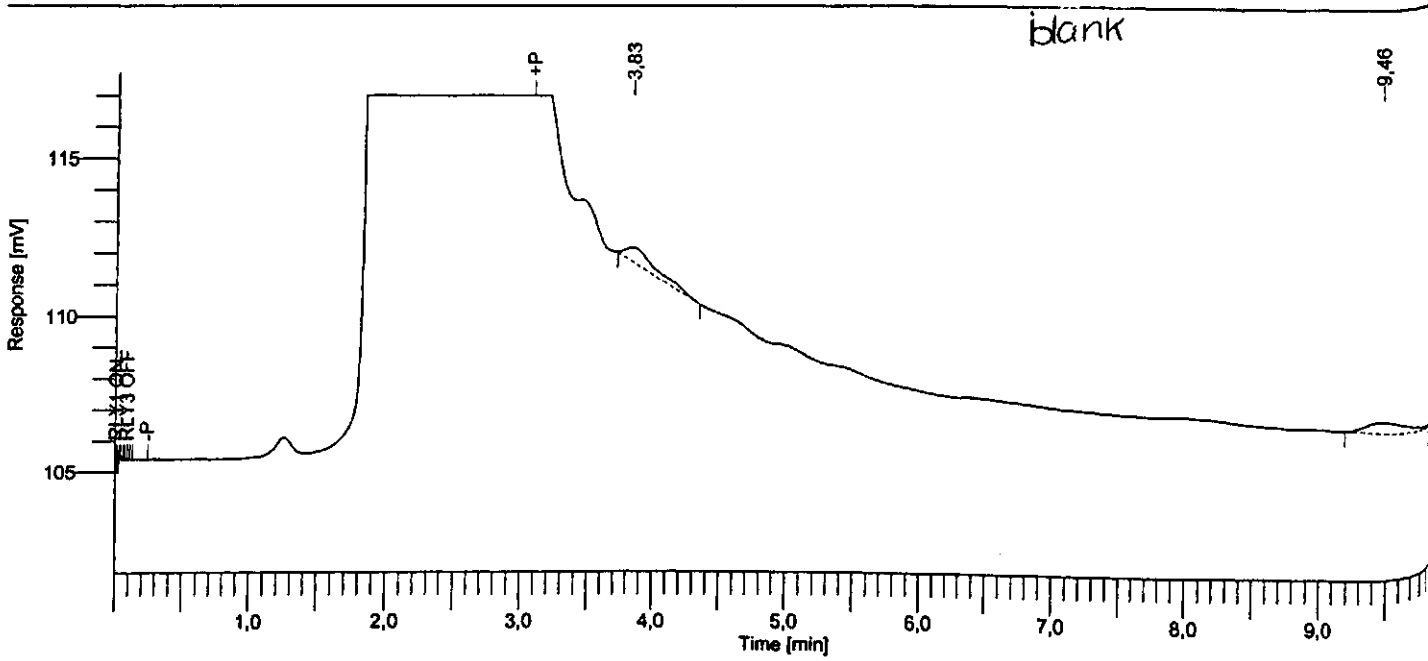
Spiking level: ..2,5... mg/kg

Software Version	: 6.1.2.0.1:D19	Date	: 13-12-01 9:35:33
Sample Name	:	Data Acquisition Time	: 27-11-01 16:40:05
Instrument Name	: HPLC-5	Channel	: A
Rack/Vial	: 0/0	Operator	
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 6		

31

Result File
Sequence Fi

blank



olaquindox

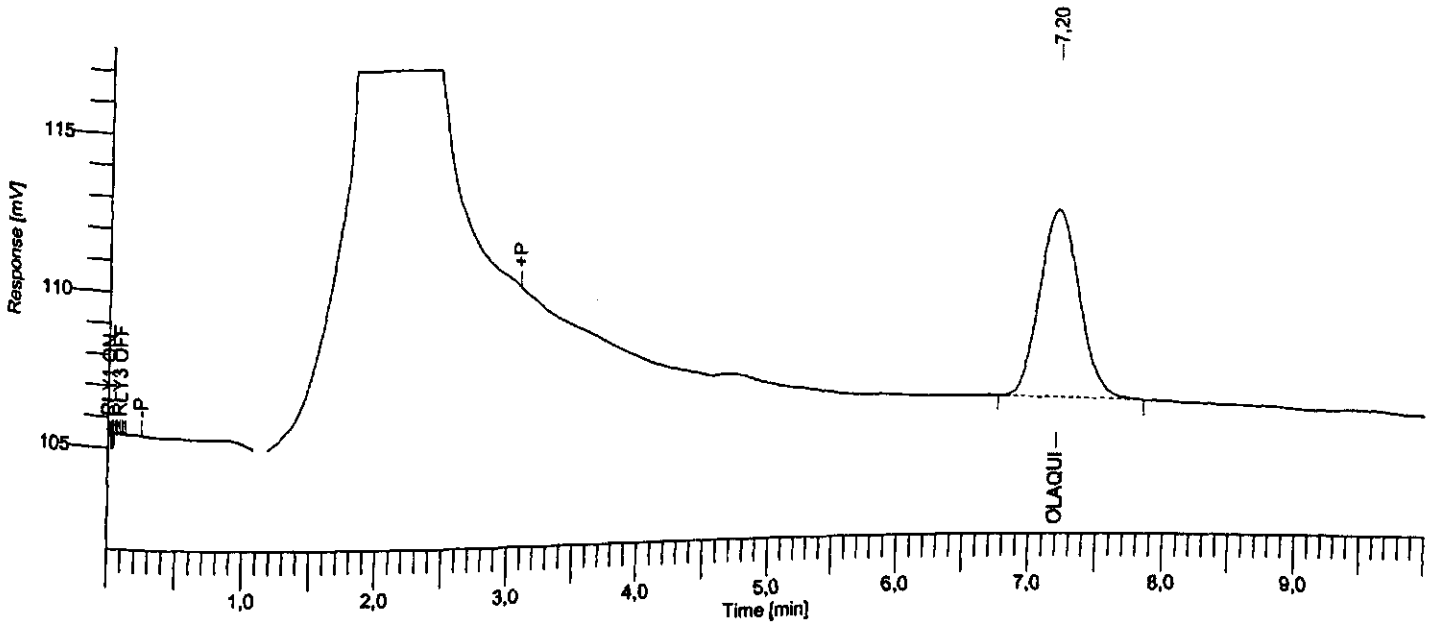
Peak #	Component Name	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
-	olaquindox	7,185	0,00	0,00	----	----		----
			0,00	0,00	0,00	0,00		

Missing Component Report

Component	Expected Retention (Calibration File)
olaquindox	7,185

Code

Software Version	: 6.1.2.0.1:D19	315359	Date	: 13-12-01 9:36:20	31
Sample Name	: 004273-a		Data Acquisition Time	: 27-11-01 17:45:02	
Instrument Name	: HPLC-5		Channel	: A	
Rack/Vial	: 0/0		Operator	:	
Sample Amount	: 1,000000		Dilution Factor	: 1,000000	
Cycle	: 9				



olaquindox

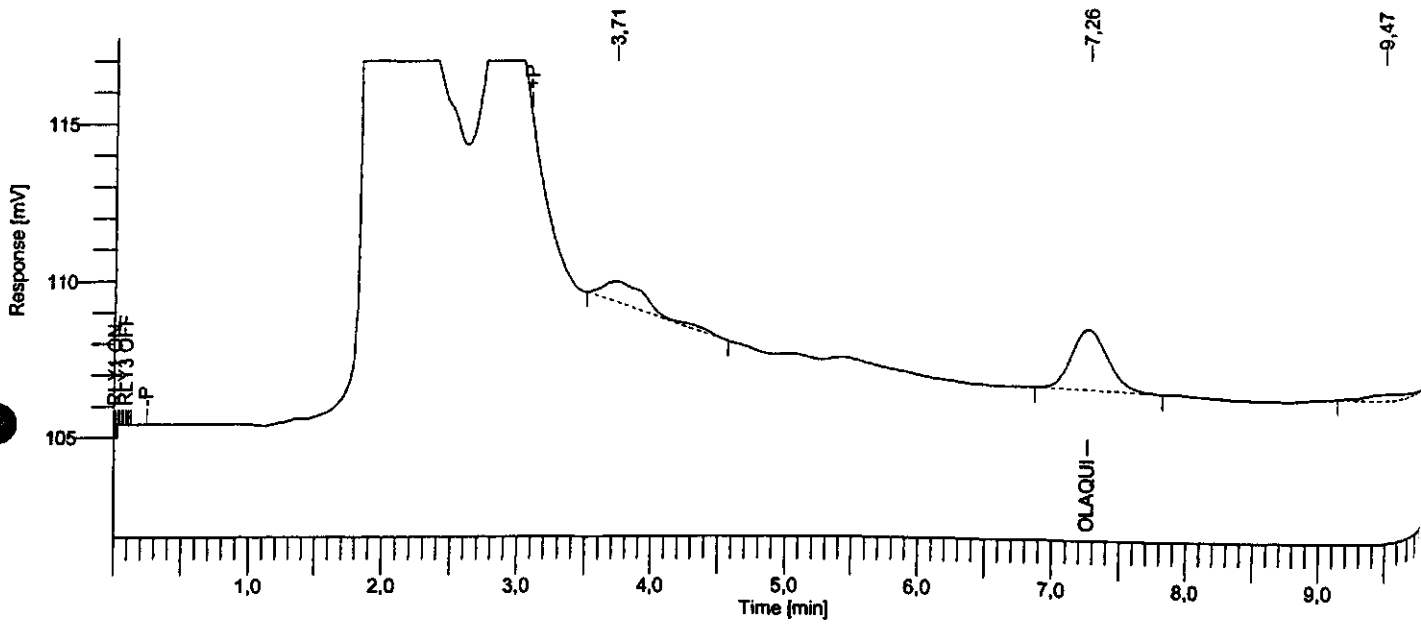
Peak #	Component Name	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	olaquindox	7,201	124104,00	6005,22	100,00	100,00	BB	20,6660
			124104,00	6005,22	100,00	100,00		

Missing Component Report

Component	Expected Retention (Calibration File)

All components were found

Software Version	: 6.1.2.0.1:D19	315 414	Date	: 13-12-01 9:37:42	31
Sample Name	: 004275-a		Data Acquisition Time	: 27-11-01 20:16:38	
Instrument Name	: HPLC-5		Channel	: A	
Rack/Vial	: 0/0		Operator	:	
Sample Amount	: 1,000000		Dilution Factor	: 1,000000	
Cycle	: 16				



olaquindox

Peak #	Component Name	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
2	olaquindox	7,259	36148,25	1917,65	100,00	100,00	BB	18,8503
			36148,25	1917,65	100,00	100,00		

Missing Component Report

Component	Expected Retention (Calibration File)

All components were found

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 32

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 12-11-2001

Analyte: OLAQUINDOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
325305	8,77	8,37
325364	3,16	2,81
325375	9,05	9,05
325409	3,14	2,69

CANFAS COLLABORATIVE STUDIES - 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 12/11/2001

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: LiChrospher ® *RP-select B* (5µm), 250 x 4 mm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1,8 ml/min
- Injection volume: 70 (µL)
- Retention time of olaquindox: 4,14 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

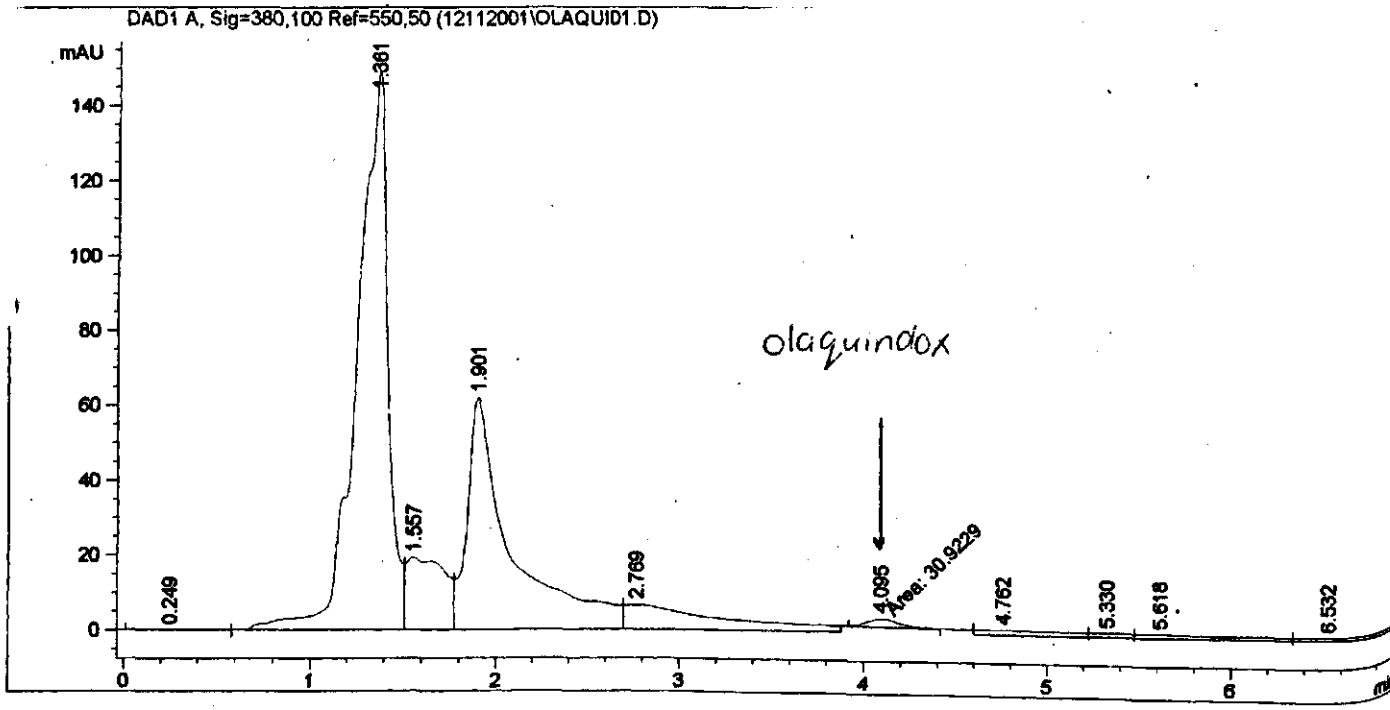
- Percentage recovery: 83.3 %
- Single/duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 83.61% and 82.89%
- Spiking level: 2.5 mg/Kg

File C:\HPCHEM\1\DATA\12112001\OLAQUI01.D

10.0023g

32

Injection Date : 11/12/01 2:29:51 PM Seq. Line : 1
Sample Name : 325364 Vial : 1
Acq. Operator Inj : 1
Inj Volume : 70 µl
Acq. Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed :
Analysis Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed :
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 11/12/01 4:41:01 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,100 Ref=550,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area [ug/ml]	Amount	Grp	Name
4.095	MM	30.92293	2.04513e-2	6.32414e-1		Olaquindox

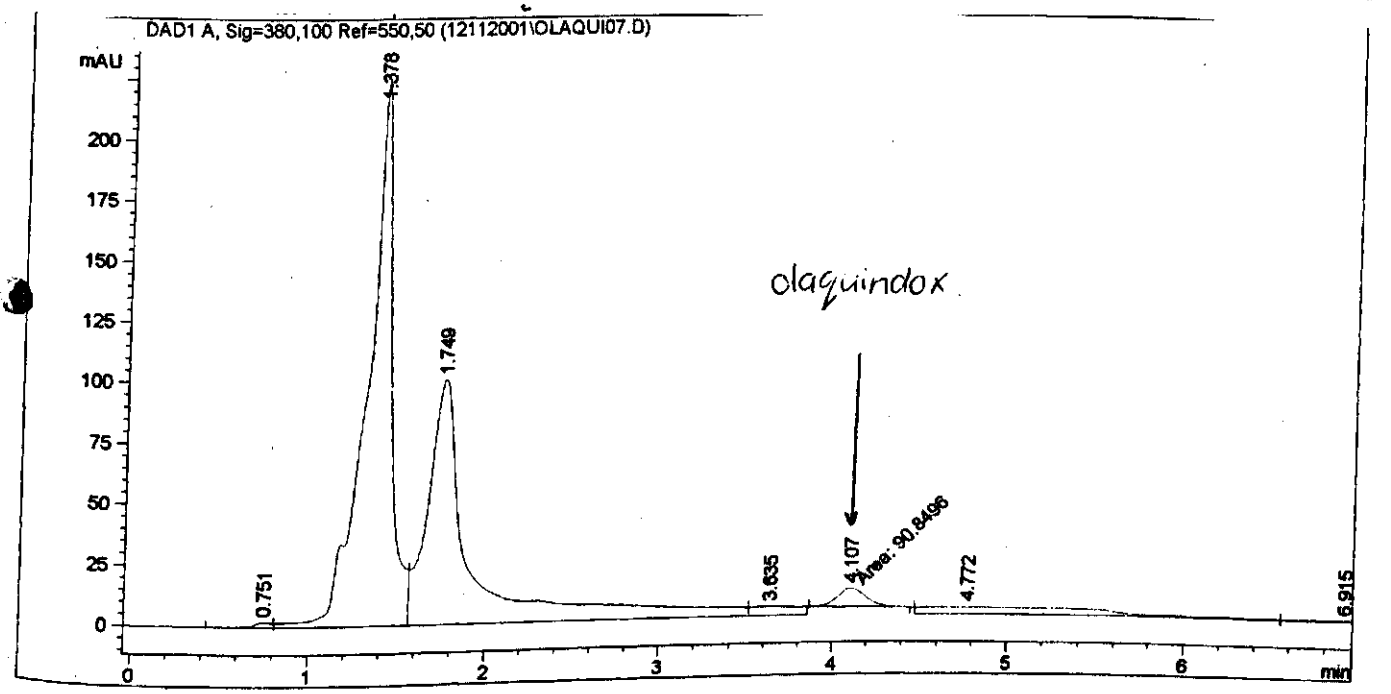
Totals : 6.32414e-1

Results obtained with enhanced integrator!

Data File C:\HPCHEM\1\DATA\12112001\OLAQUI07.D Sample Name: 325375

[redacted] - 10.0029g 32

Injection Date : 11/12/01 3:27:35 PM Seq. Line : 7
Sample Name : 325375 Vial : 7
Acq. Operator Inj : 1
Inj Volume : 70 µl
Acq. Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed
Analysis Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed :
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 11/12/01 4:41:01 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,100 Ref=550,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area [ug/ml]	Amount	Grp	Name
4.107	MM	90.84964	1.99336e-2	1.81096		Olaquindox

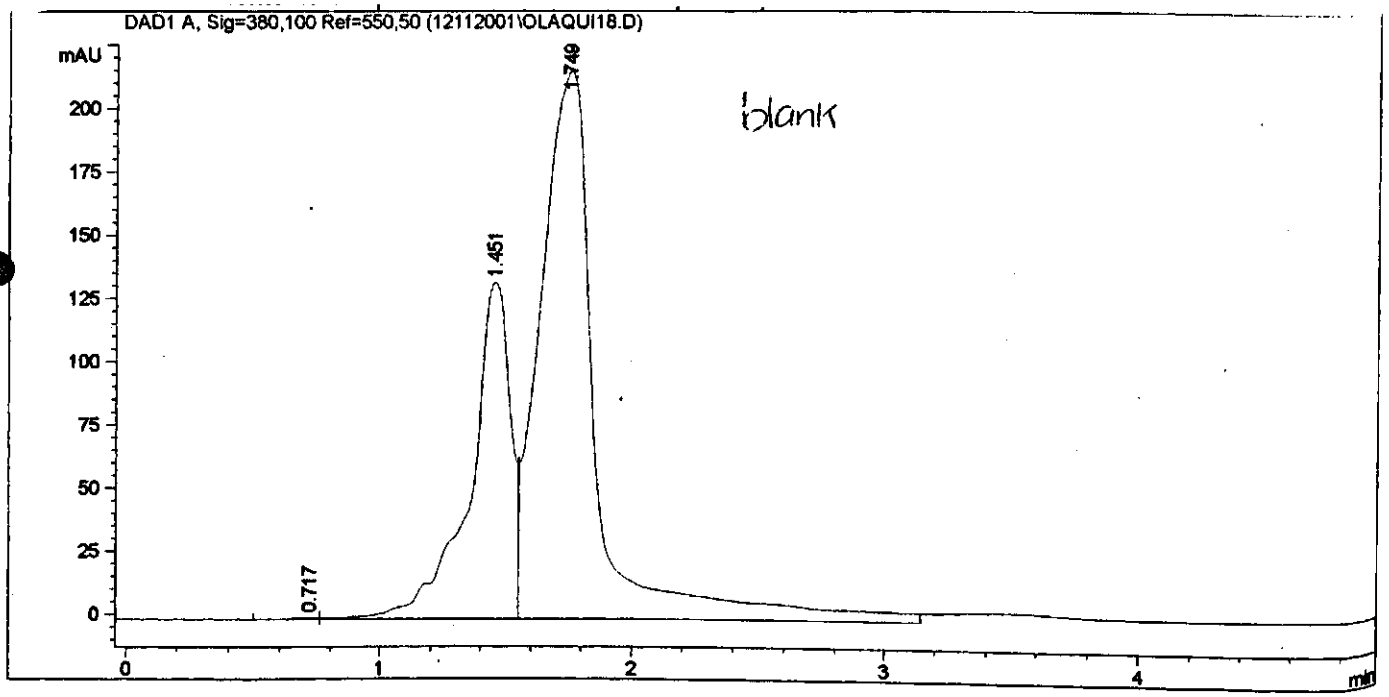
Totals : 1.81096

Results obtained with enhanced integrator!

File C:\HPCHEM\1\DATA\12112001\OLAQUI18.D Sample Name: Blank

32

Injection Date : 11/12/01 4:57:15 PM Seq. Line : 18
Sample Name : Blank Vial : 13
Acq. Operator : Inj : 1
Inj Volume : 70 µl
Acq. Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed :
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed : 11/
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 11/12/01 4:41:01 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,100 Ref=550,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area [ug/ml]	Amount	Grp	Name
4.139	-	-	-			Olaquindox

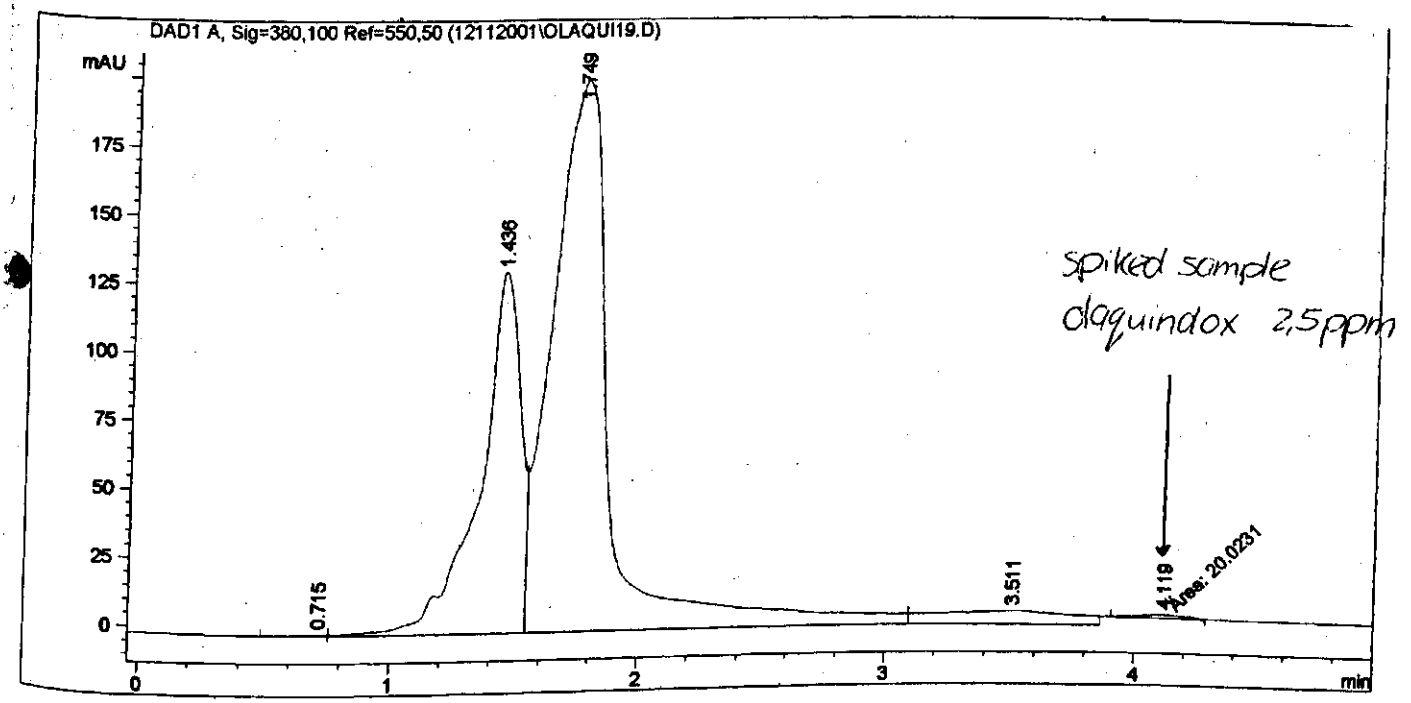
Totals : 0.00000

Results obtained with enhanced integrator!

File C:\HPCHEM\1\DATA\12112001\OLAQUI19.D Sample Name: Spiked 1

32

Injection Date : 11/12/01 5:04:52 PM Seq. Line : 19
Sample Name : Spiked 1 Vial : 14
Acq. Operator : Inj : 1
Inj Volume : 70 µl
Acq. Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed : 2:01
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\OLAQUIN.M
Last changed : 1
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 11/12/01 4:41:01 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,100 Ref=550,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area [ug/ml]	Amount	Grp	Name
4.119	MM	20.02315	2.08786e-2	4.18055e-1		Olaquindox

Totals : 4.18055e-1

Results obtained with enhanced integrator!

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 33

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 11-7-2001

Analyte: OLAQUINDOX

Sample code / Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
335304	2,70	2,60
335308	9,10	8,80
335347	2,90	2,70
335362	8,90	8,80

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Que

Laboratory: ....

Contact persor .....

Date(s) of analysis: .....7./11./01.....

Chromatographic conditions:

- Column:
  - [X] As described in the method
  - [ ] Other: .....
- Mobile phase:
  - [X] As described in the method
  - [ ] Other: .....
- Flow-rate: ..... ml/min
- Injection volume: .....µl
- Retention time of olaquindox: ..9,9.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

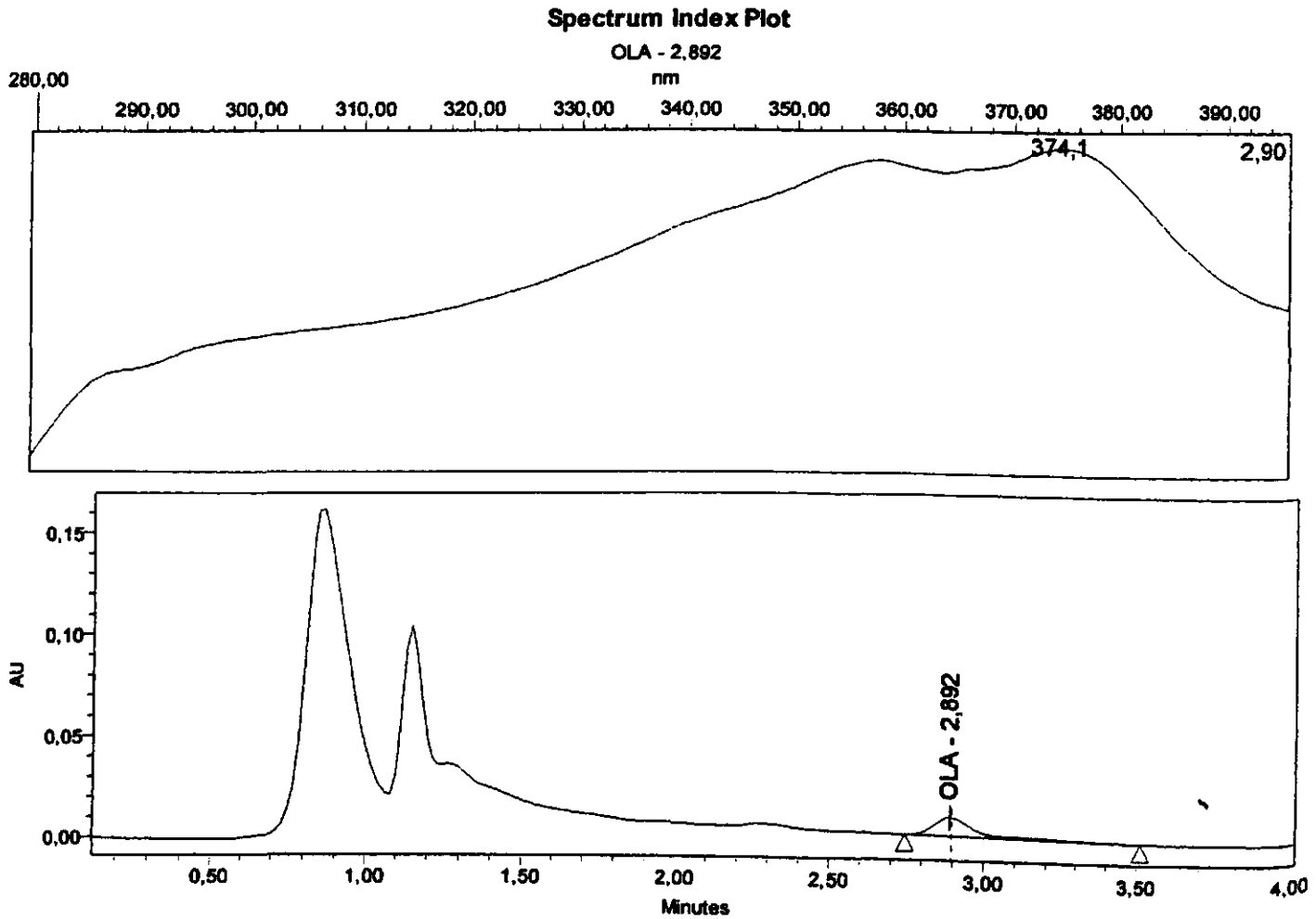
- Percentage recovery: 102 %
- Single / duplicate determinations: [X] single [ ] duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ...5..... mg/kg

Sample Set Name OLA07

User Name RVSA

Current Date 7/11/01

Current Time 02:05:47



Peak Results

	SampleName	Name	RT	Area	Height	Amount	Units
1	6361/01	OLA	2,892	88145	9141	2,877	mg/kg

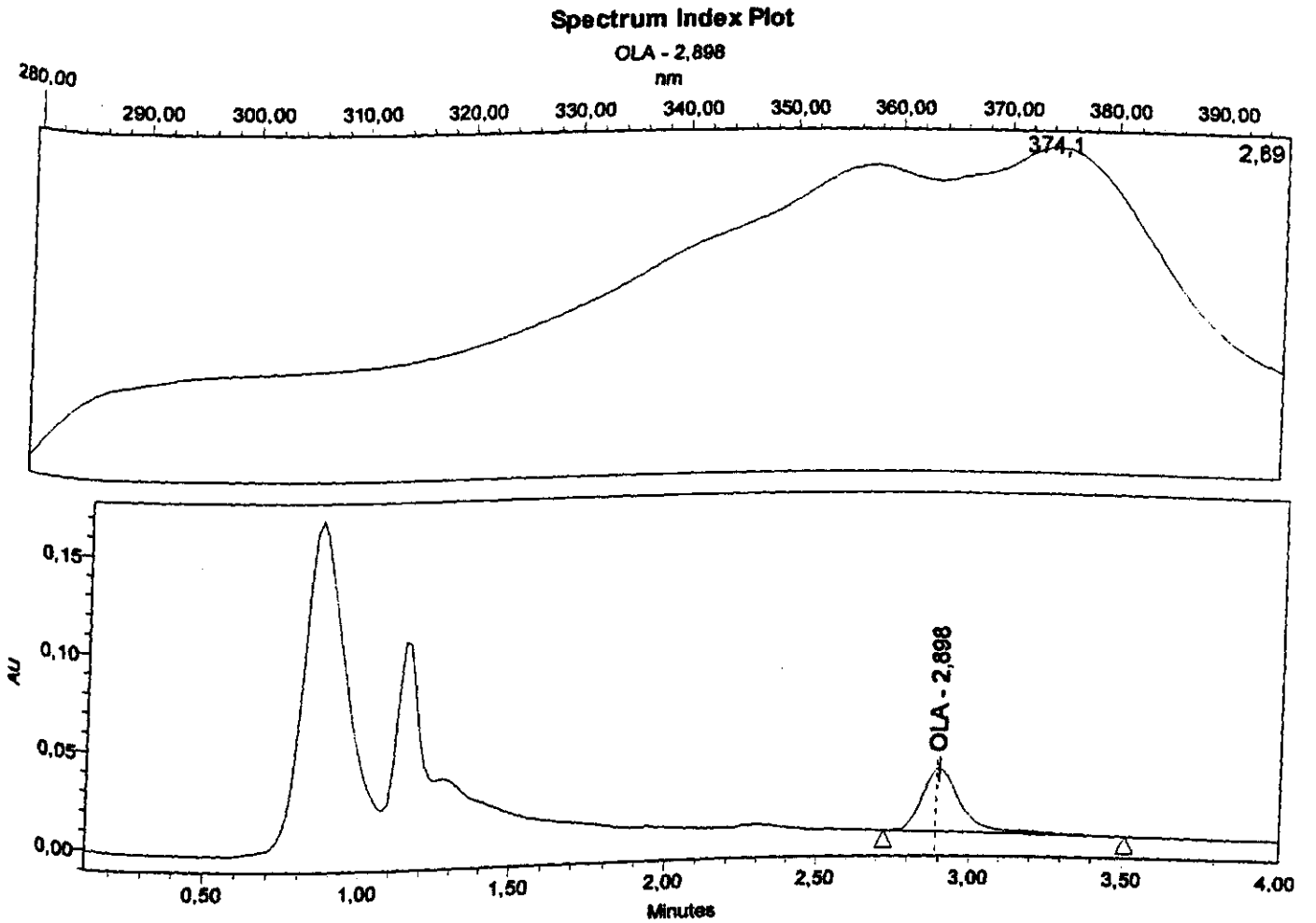
335347

Sample Set Name OLA07

User Name RVSA

Current Date 7/11/01

Current Time 02:05:45



Peak Results

	SampleName	Name	RT	Area	Height	Amount	Units
1	6360/01	OLA	2,898	284980	32412	9,048	mg/kg

355308

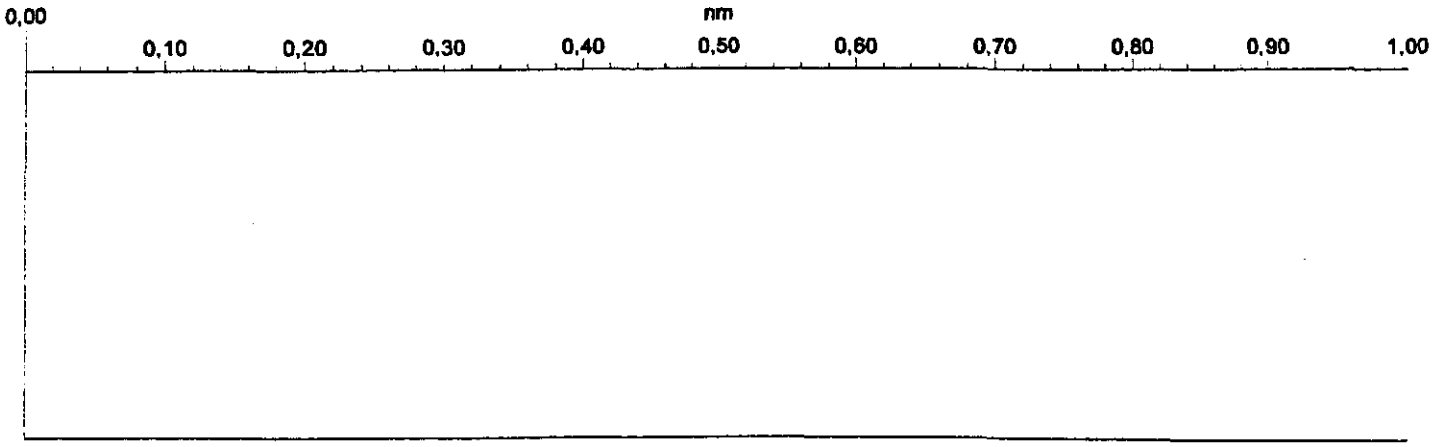
Sample Set Name OLA07

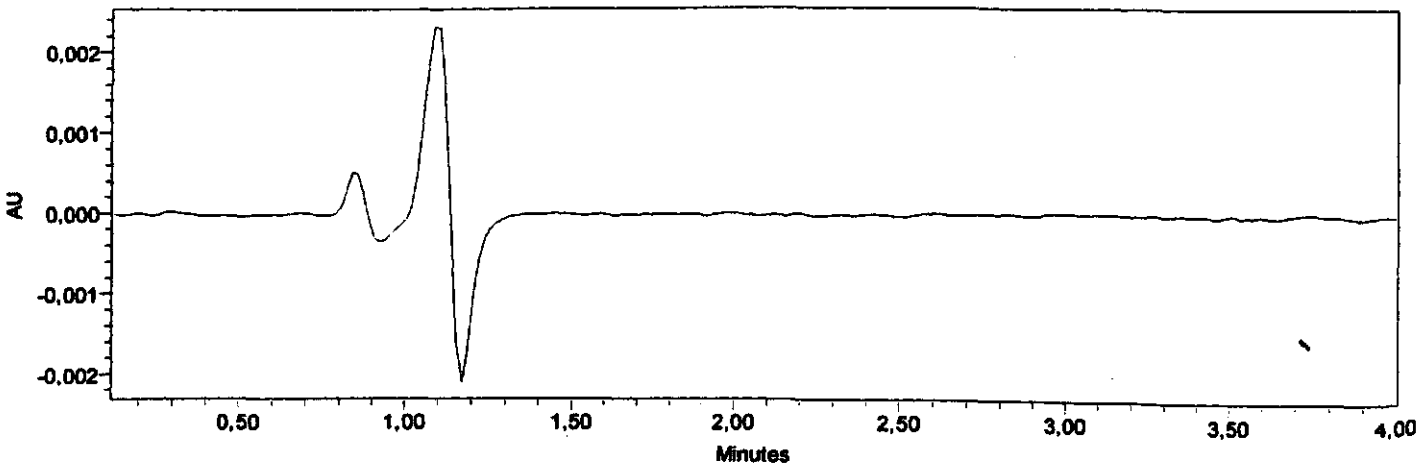
User Name RVSA

Current Date 7/11/01

Current Time 02:05:38

Spectrum Index Plot





Peak Results

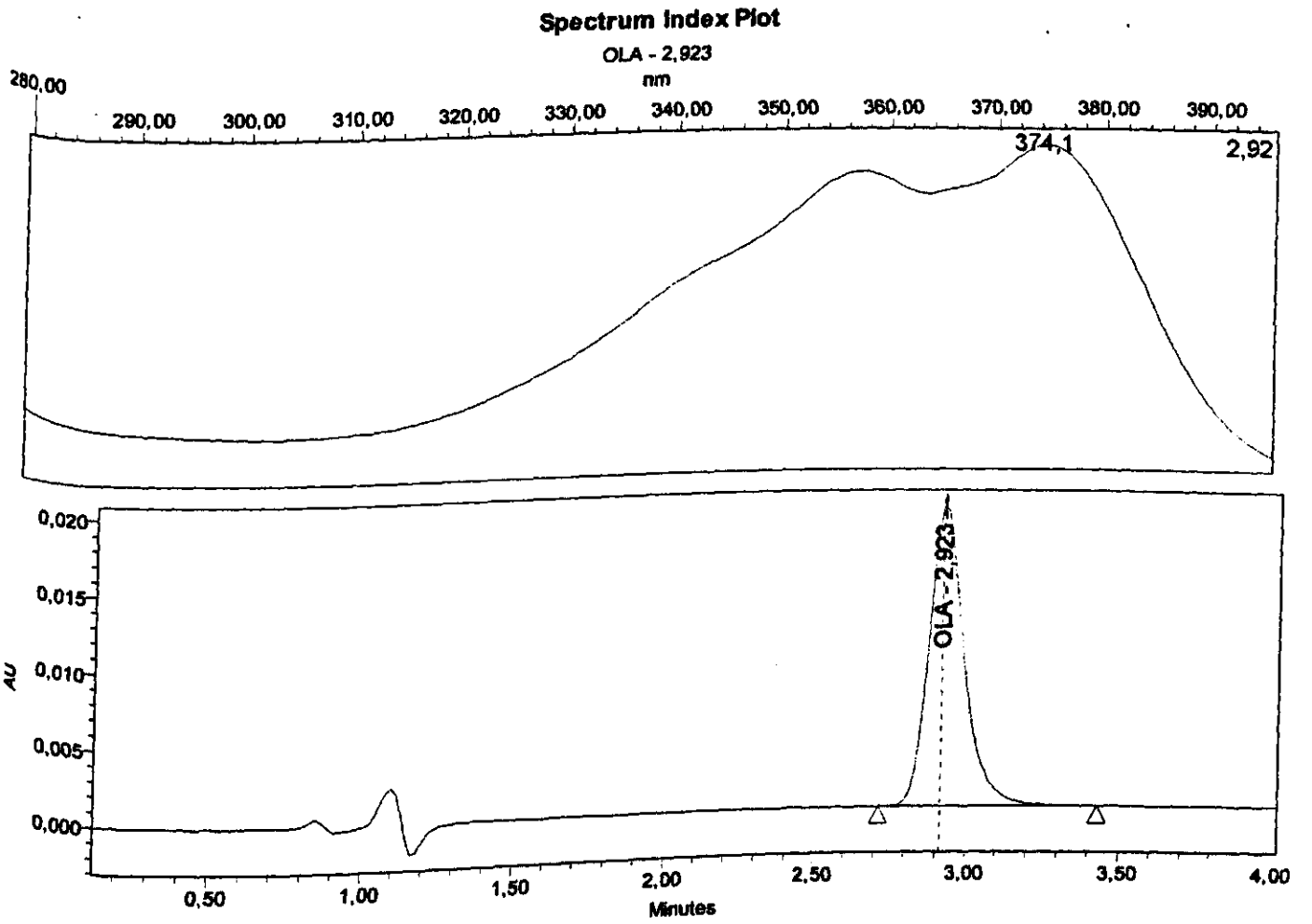
	SampleName	Name	RT	Area	Height	Amount	Units
1	BLK	*OLA*	2,873				

Sample Set Name OLA07

User Name RVSA

Current Date 7/11/01

Current Time 02:05:39



Peak Results

	SampleName	Name	RT	Area	Height	Amount	Units
1	BLK + 5	OLA	2,923	159505	19958	5,114	mg/kg

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 34

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 08-01-2002

Analyte: OLAQUINDOX

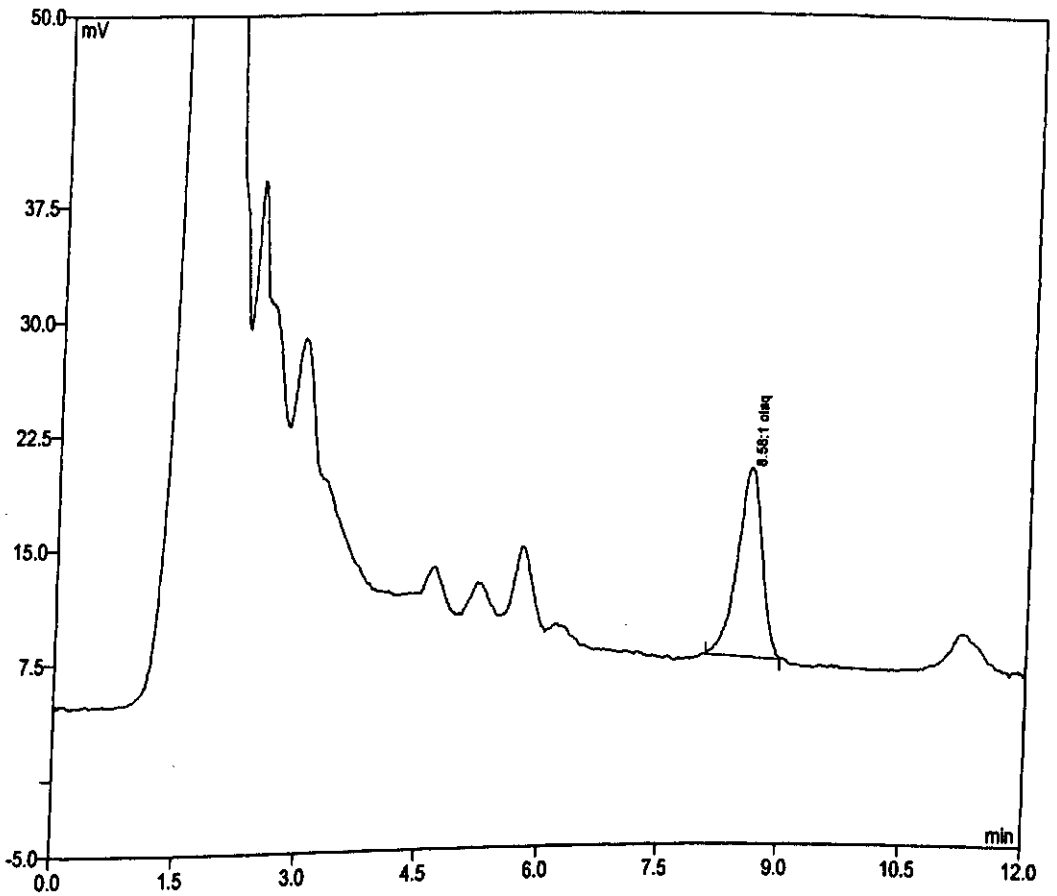
Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
345321	8,60	8,30
345366	2,40	2,20
345379	8,80	8,40
345386	1,90	1,90

Channel 2

KromaSystem 2000 Version 1.83 RESULT REPORT: INTEGRATION

SYS2 - OLAQ37.SMP (modified):
No. 08: 345321 10g/50ml Acquired : 08.01.02 12:25:20
Channel 2: DETECT 332 Processed: 18.03.02 11:51
No Text

Program File ..... OLAQ001 .....
Worksheet ..... OLAQ .....
Peak Table ..... OLAQUIND .....
Parameter Table .. OLAQUIND .....
Report File ....
Document File ....



No.	PNo	Ret.Time min	Type	Name	Area mV*min	Amount	Rel.Ar %
1	1	8.58	MOD	olaquindox	4.4462e+000	8.5929e+000	100.00
					4.4462e+000	8.5929e+000	100.00

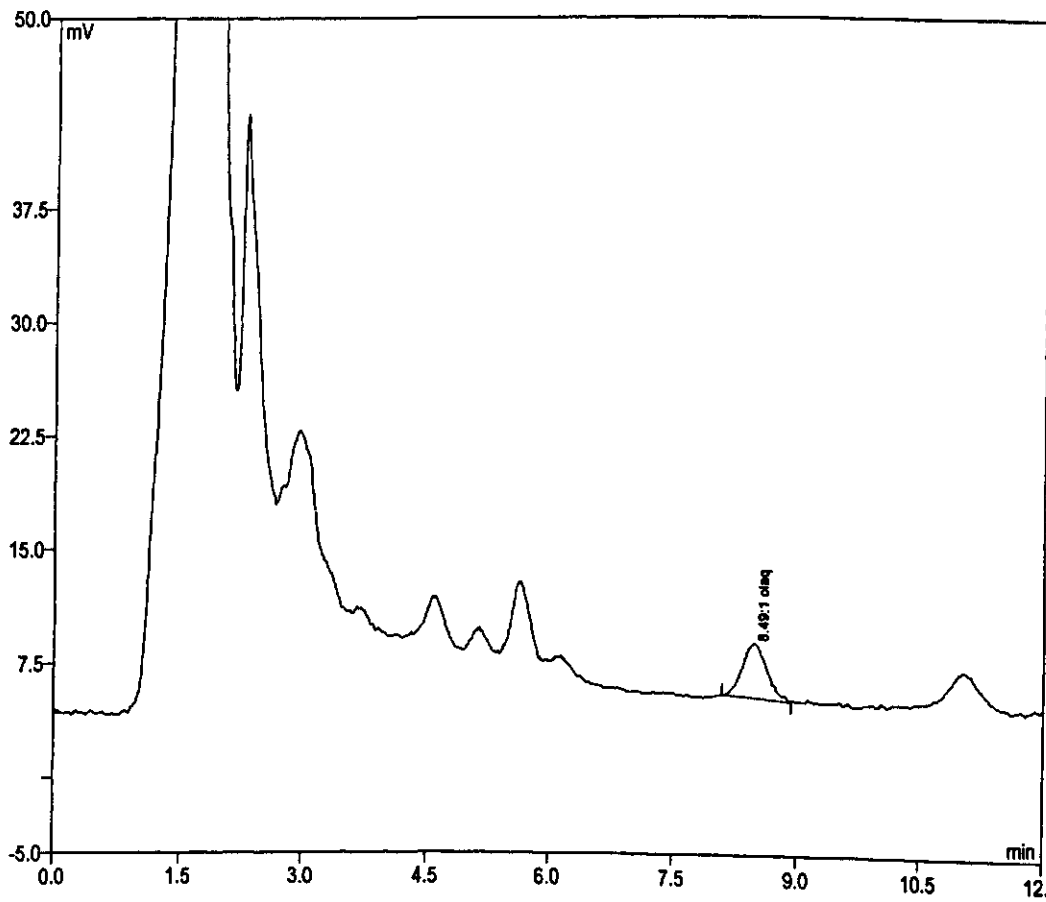
KromaSystem 2000

Channel 2

KromaSystem 2000 Version 1.83 RESULT REPORT: INTEGRATION

SYS2 - OLAQ37.SMP (modified):
No. 10: 345366 10g/50ml Acquired : 08.01.02 12:51:31
Channel 2: DETECT 332 Processed: 18.03.02 11:51
No Text

Program File ..... OLAQ001 .....
Worksheet ..... OLAQ .....
Peak Table ..... OLAQUIND .....
Parameter Table .. OLAQUIND .....
Report File ....
Document File ....



No.	PNo	Ret.Time min	Type	Name	Area mV*min	Amount	Rel.Ar %
1	1	8.49	MLR	olaquindox	1.2242e+000	2.3659e+000	100.00
					1.2242e+000	2.3659e+000	100.00

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 35

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 12/13-11/2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
355317	2,00	2,00
355322	2,00	2,00
355357	7,90	7,50
355406	7,60	7,70

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: .

Date(s) of analysis 12/13 - 11 - 2001

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: .....1,5..... ml/min
- Injection volume: ..50....µl
- Retention time of olaquindox: ..7,4 min

Chromatograms: Please include representative chromatograms of:

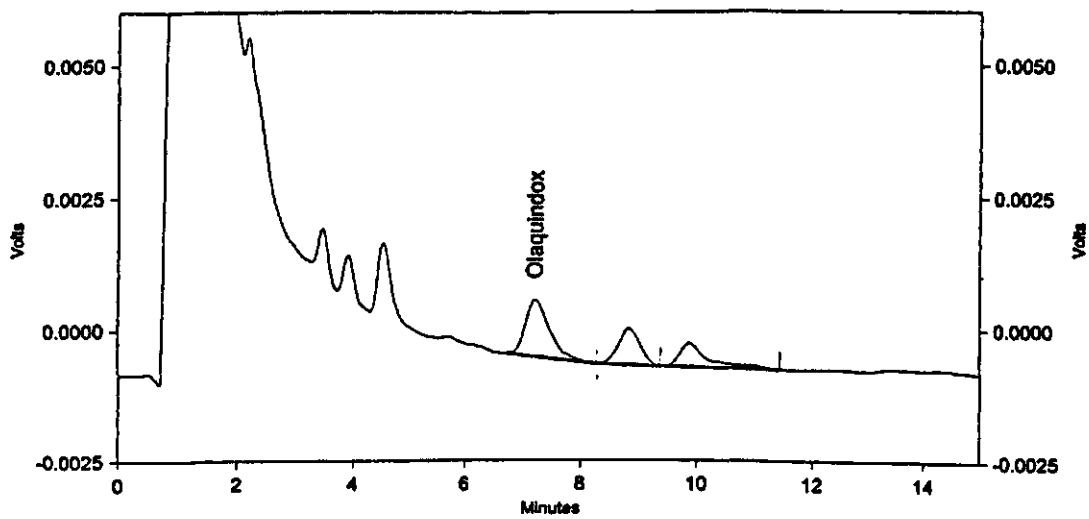
- Blind positive feed samples
- Blind blank feed sample ~~(from your own~~ collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

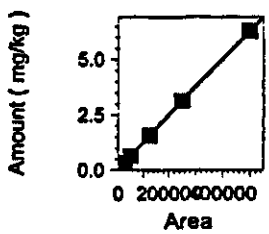
Recovery results:

- Percentage recovery: 102,7 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 105,7 % and 99,7 %
- Spiking level: ..3,1... mg/kg

Olaquindox



ɜak: Olaquindox -- ESTD -- UV-Detect



UV-Detector Results

	Pk #	Retention Time	Area	Height	ESTD concentration	Units
Olaquindox	1	7.20	31052	1070	1.95262	mg/kg

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 37

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: **Task 4 COLLABORATIVE STUDY - 2nd round**

Lab-name:

Contact person: e-mail:
fax:
telephone:

Date of analysis: 11-12-2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
375311	2,02	1,92
375343	7,54	8,09
375387	1,87	1,88
375405	8,34	7,91

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: .....

Contact person: ....

Date(s) of analysis: ...11/12/01.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: ...LICHROSPHER RP18-5 ENDCAPPED (25cm x 4.6mm i.d.)...
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: ...1..... ml/min
- Injection volume: ...50....µl
- Retention time of olaquindox: .11.5. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ...71.5... %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: ...79... % and ...76... %
- Spiking level: ..2.5.... mg/kg

Olaquindox in Feed
Mode: Reprocessed Data
Original Results: C:\TSP\SYSTEM1\Data\111201olaqps.RES
Reprocessed Results: C:\TSP\SYSTEM1\Data\111201olaqps.HMS

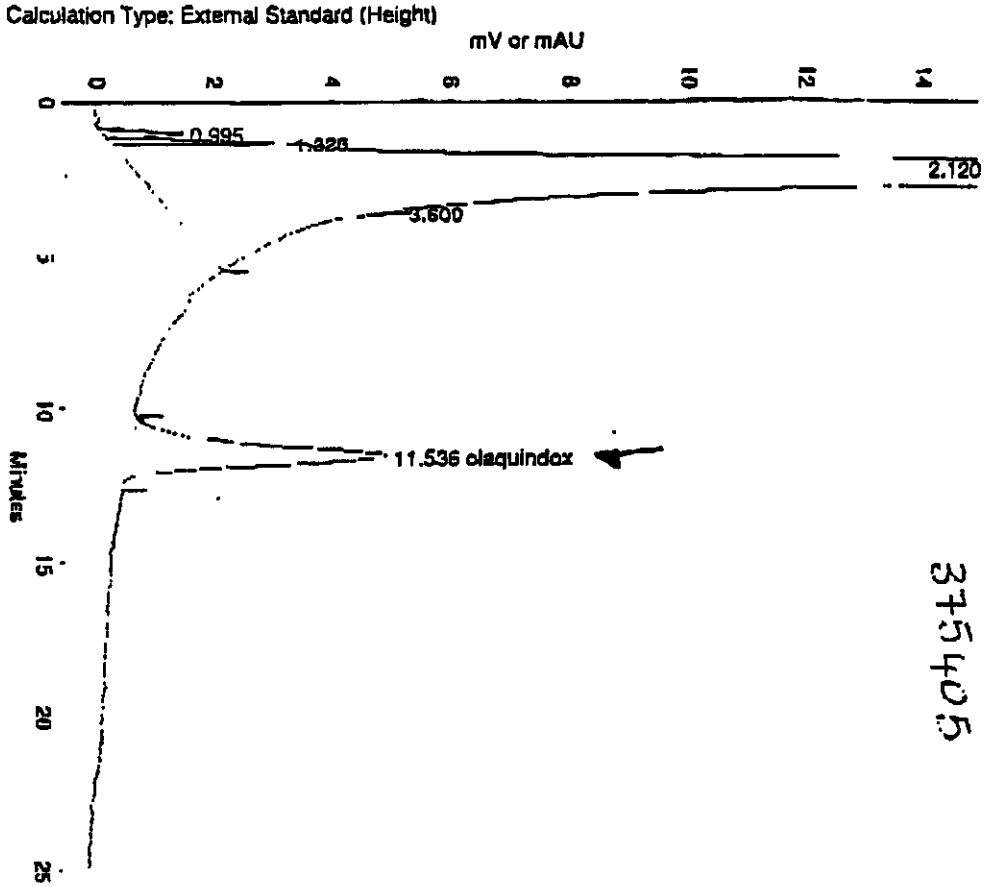
Analysis Report

Name: 881b *Vial: A19* *Injection: 1 of 1*
Description: 881b
Type: Sample
Injection Volume: 50.0 µL

Injected On: 12-12-01 06:01:38

Acquisition Log
Column Pressure (PSI): 2992 Column Temperature (C): N/A Pump Flow Stability: 0.8
Noise (microAU): 6e+01 Drift (microAU/min): -1e+02
Run-Time Messages: None

Signal 1: UV2000 A 380 nm
Calculation Type: External Standard (Height)



881b/rpt1: UV2000 A 380nm

37405

37

Analysis Report

Name: 879b
Description: 079b
Type: Sample
Injection Volume: 50.0 µL

Vial: A14

Injection: 1 of 1

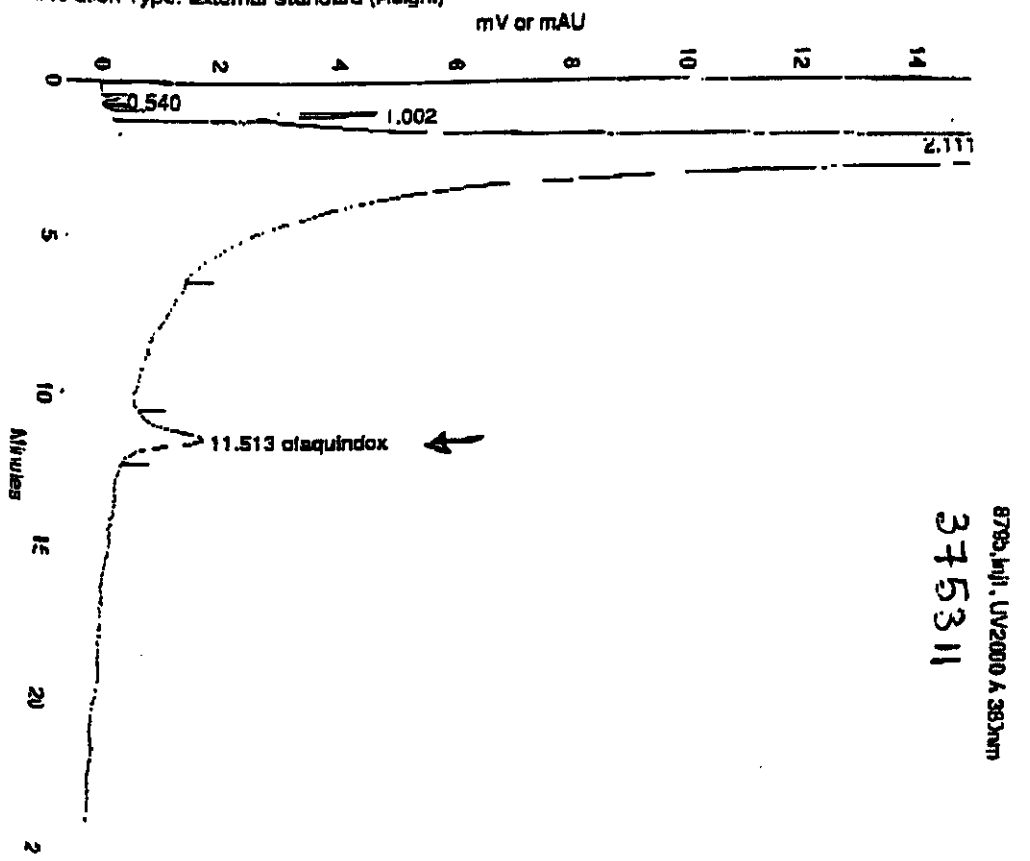
Injected On: 12-12-01 03:25:00

Acquisition Log
Column Pressure (PSI): 2958
Noise (microAU): 9e+01
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): -2e+01

Pump Flow Stability: 1.1

Signal 1: UV2000 A 380 nm
Calculation Type: External Standard (Height)



879b.Inj1, UV2000 A 383nm

375311

1_

Olaquindox In Feed
Mode: Reprocessed Data
Original Results: C:\TSP\SYSTEM1\Data\111201olaqps.RES
Reprocessed Results: C:\TSP\SYSTEM1\Data\111201olaqps.RMS

37

Analysis Report

Name: blk feed
Description: blk feed
Type: Sample
Injection Volume: 50.0 µL

Vial: A08

Injection: 1 of 1

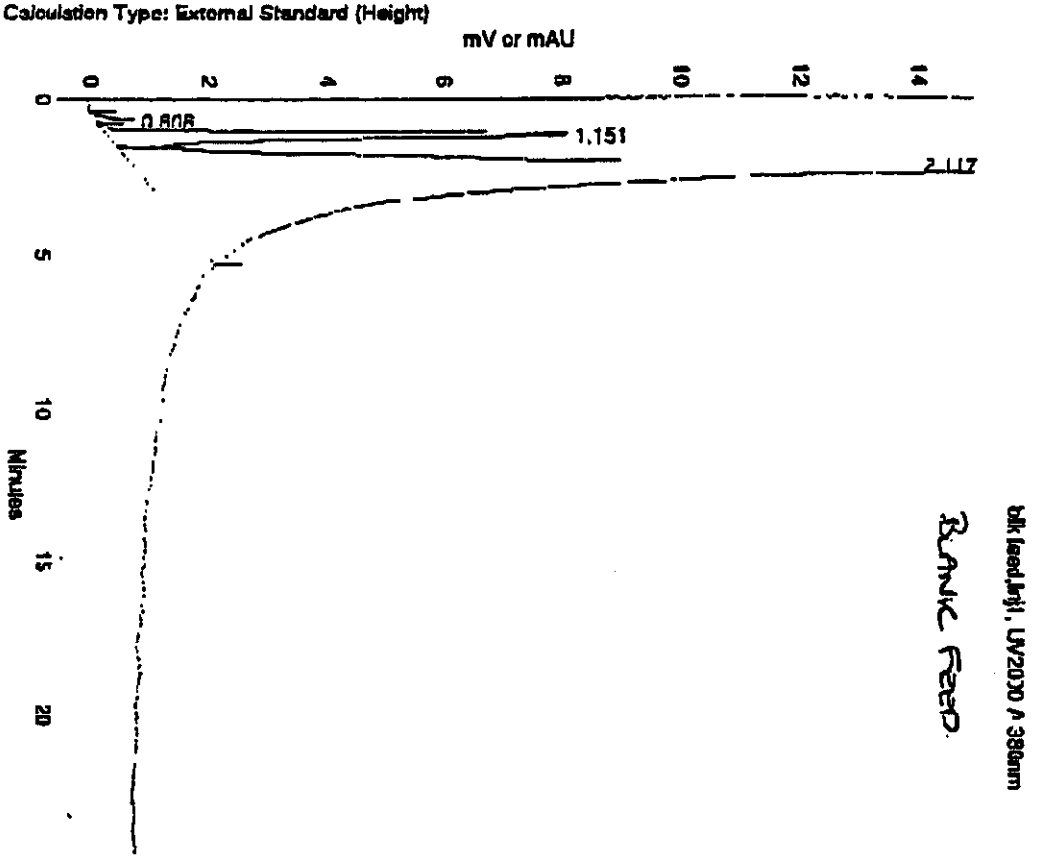
Injected On: 12-12-01 00:17:07

Acquisition Log
Column Pressure (PSI): 2806
Noise (microAU): 3e+01
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): 2e+02

Pump Flow Stability: 1.7

Signal 1: UV2000 A 380 nm
Calculation Type: External Standard (Height)



BLANK FEED

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 38

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 10-12-2001

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
385336	7,82	6,86
385355	2,30	2,36
385369	2,60	2,22
385374	7,50	7,83

CANFAS COLLABORATIVE STUDIES - OLAQUINDOX II

Annex 4 – Questionnaire

Laboratory:

Contact Person:

Date(s) of analysis: **12/10/01**

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - X Other: **Symetry® C-18, 150 x 2,1 mm, 3.5 µm**
- Mobile phase:
  - ☐ As described in the method
  - X Other: **Isocratic MeOH/Water (5:95)**
- Flow-rate: **0.3** ml/min
- Injection volume: **10** µl
- Retention time of Carbadox: **12.9** min

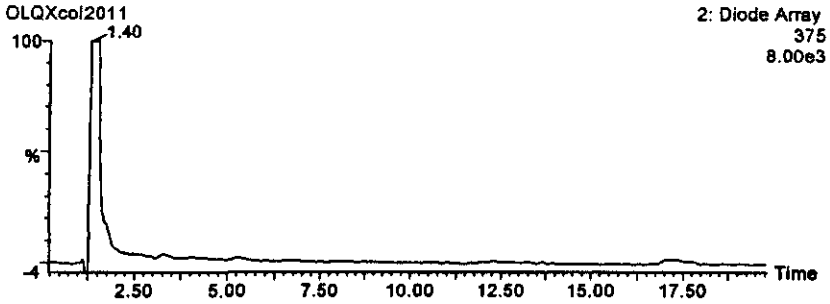
Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank samples

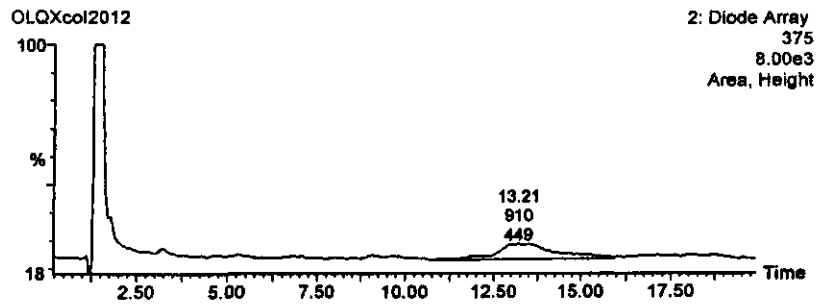
Please indicate the olaquindox peak with an arrow

Recovery results:

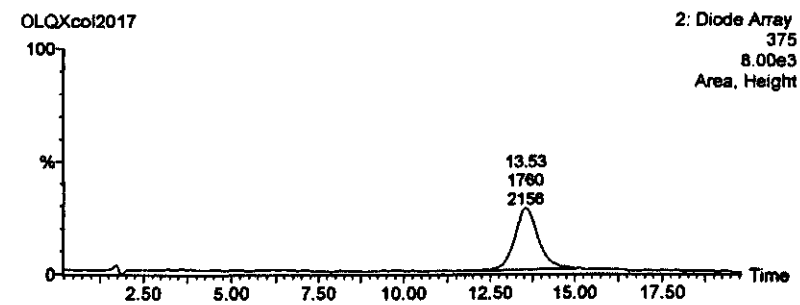
- Percentage recovery: **52.5 %**
- Single / duplicate determinations: ☐ single X duplicate
- If duplicate, please give both percentages: **52%** and **53%**
- Speaking level: **2** mg/kg



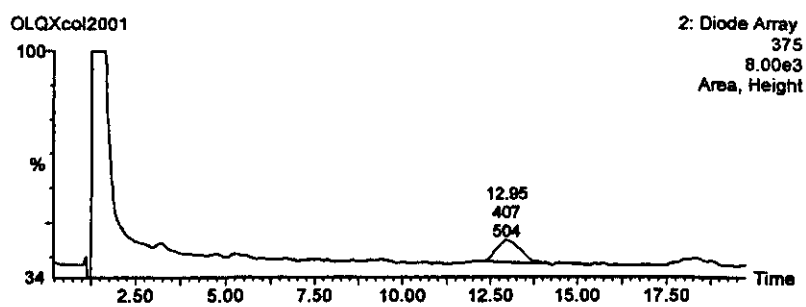
blank



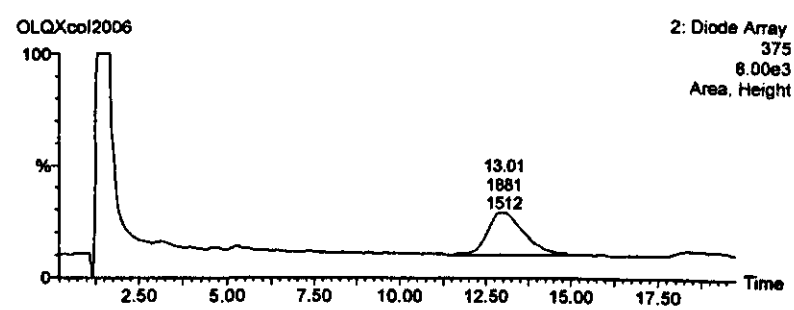
spiked blank (3,83 mg/kg)



standard olaquindox
1,92 ppm



385369/1



385336/6

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 40

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - *Report form*

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: **Task 4 COLLABORATIVE STUDY - 2nd round**

Lab-name:

Contact person: e-mail:
fax:
telephone:

Date of analysis: 26.11.-6.12.01

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
405315	2,67	2,82
405381	8,84	8,87
405385	8,93	8,96
405391	2,87	2,84

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Laboratory: ..

Contact person: .....

Date(s) of analysis: .....26november – 6december 2001.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: .....C18 spherical 5µm 3,9x150mm; WATERS.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....1,3..... ml/min
- Injection volume: ..50....µl
- Retention time of olaquindox: ..3,7... min

Chromatograms: Please include representative chromatograms of:

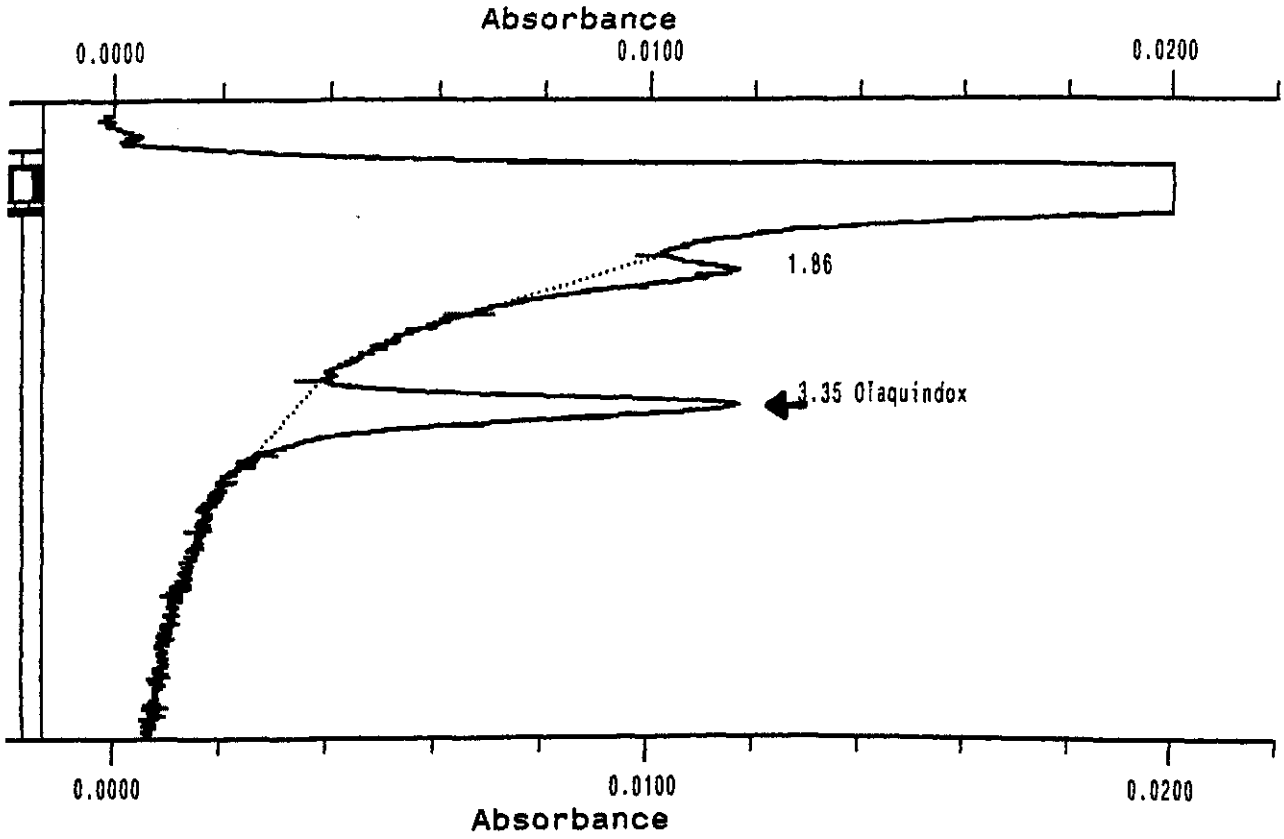
- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow

Recovery results:

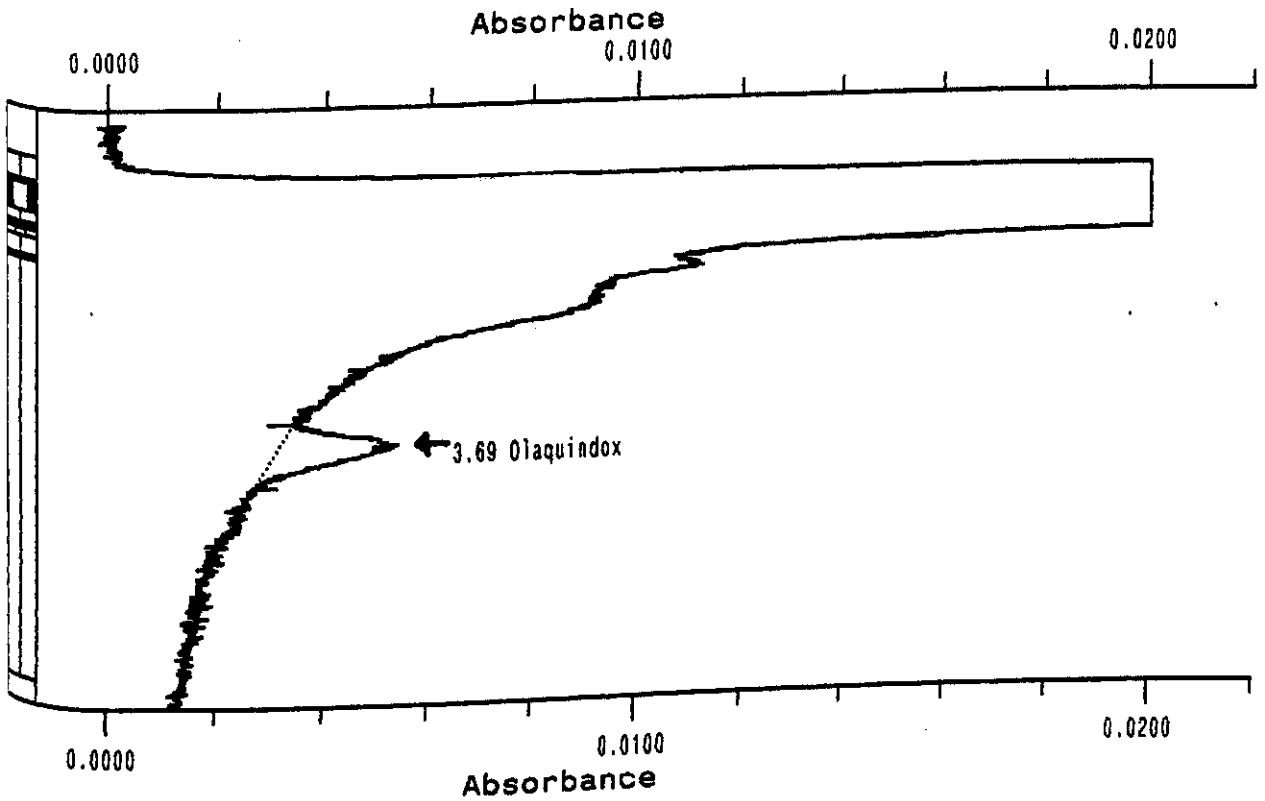
- Percentage recovery: ..97.. %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 96... % and 98... %
- Spiking level: ..2,5... mg/kg

	TIME	DATE
INJECTION	15:46:37	4 DEC 2001
ANALYSIS	15:14:32	5 DEC 2001
REPORT	16:13:03	6 DEC 2001



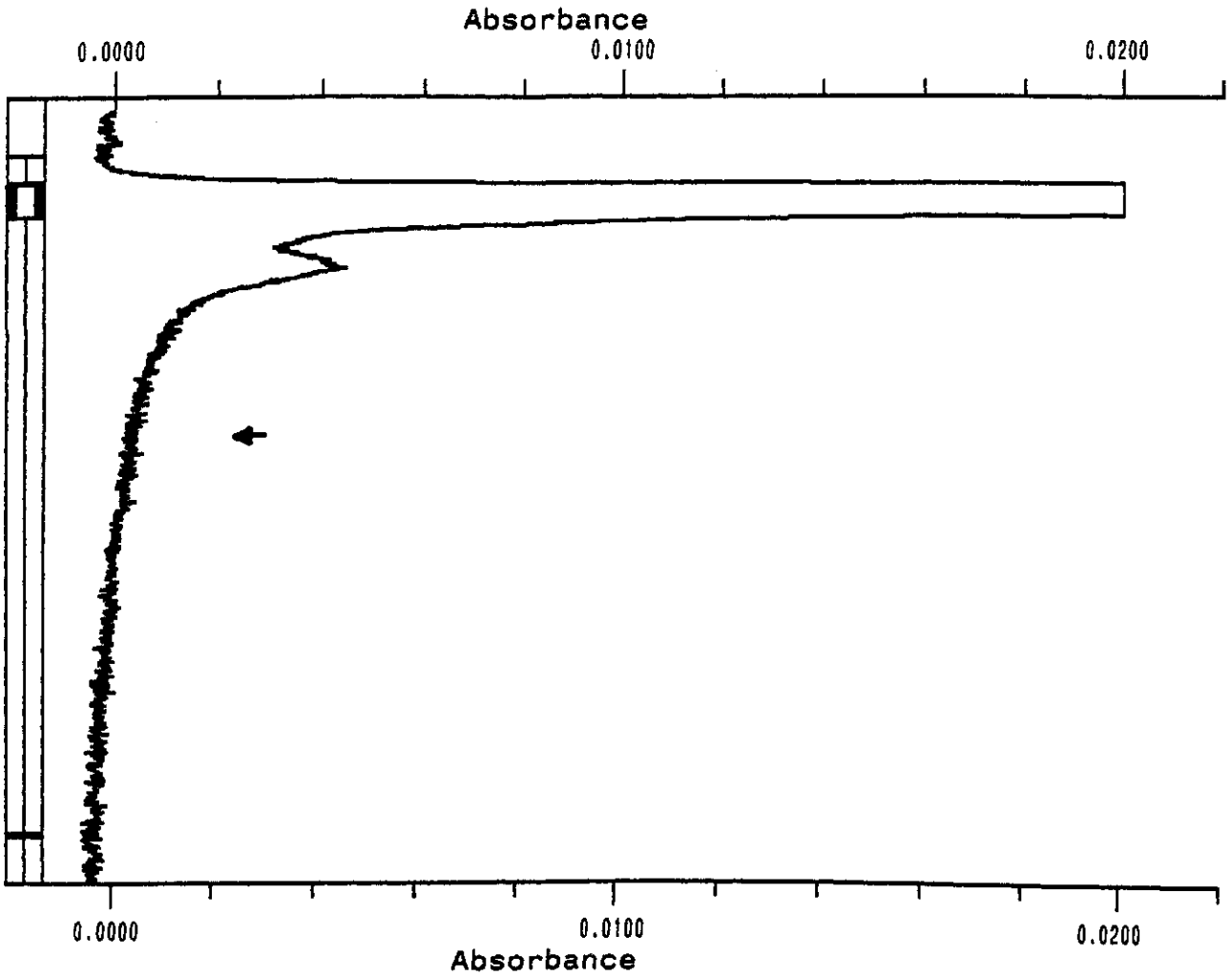
CANFAS sample 405381

	TIME	DATE
INJECTION	15:37:01	4 DEC 2001
ANALYSIS	12:28:34	5 DEC 2001
REPORT	16:13:49	6 DEC 2001



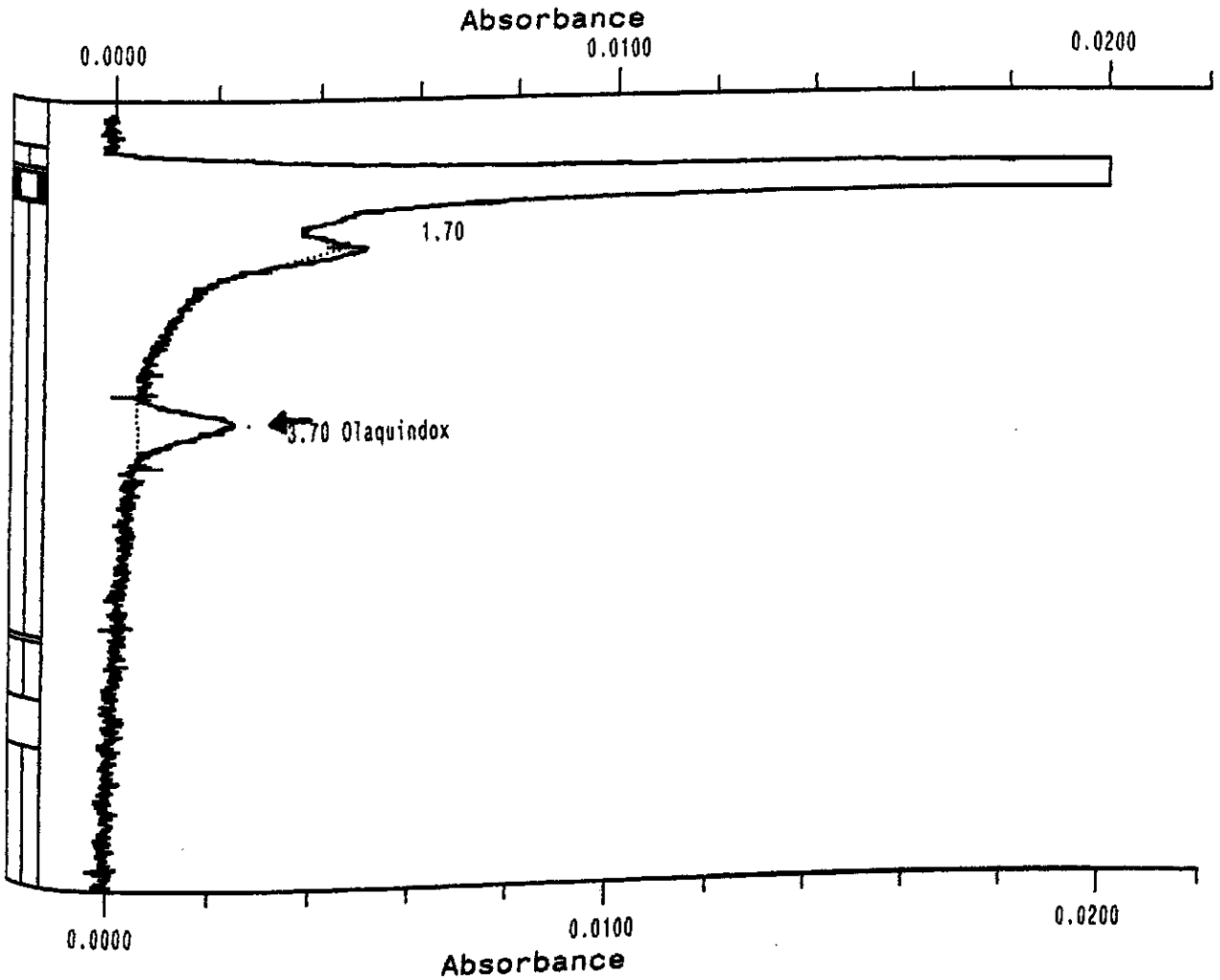
CANFAS sample 405315

	TIME	DATE
INJECTION	16:04:02	6 DEC 20
ANALYSIS	16:13:21	6 DEC 20
REPORT	16:16:36	6 DEC 20



Blank piglet feed

	TIME	DATE
INJECTION	16:26:34	4 DEC 2001
ANALYSIS	12:23:25	5 DEC 2001
REPORT	16:15:03	6 DEC 2001



Spiked blank for recovery test 1

APPENDIX 5

Table with results, questionnaire (page 1) and chromatograms

of partner 41

CANFAS COLLABORATIVE STUDIES - 2ND ROUND - NOVEMBER 2001

ANNEX 2 - Report form

CANFAS

Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY - 2nd round

Lab-name:

Contact person:

e-mail:

fax:

telephone:

Date of analysis: 19-11-2001

Analyte: OLAQUINDOX

Sample code / Unit	Result 1 (mg/kg)	Result 2 (mg/kg)
415320	2,67	2,82
415330	2,48	2,60
415396	9,85	9,93
415430	9,77	9,98

CANFAS COLLABORATIVE STUDIES – 2nd round - NOVEMBER 2001

ADDITIVE: OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: ....19.11.2001.....

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....1.5..... ml/min
- Injection volume: ..100..µl
- Retention time of olaquindox: 10.5 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample (from your own collection and to be used for recovery purposes)

Please indicate the olaquindox peak with an arrow Sample 4117 used for recovery purposes

Recovery results:

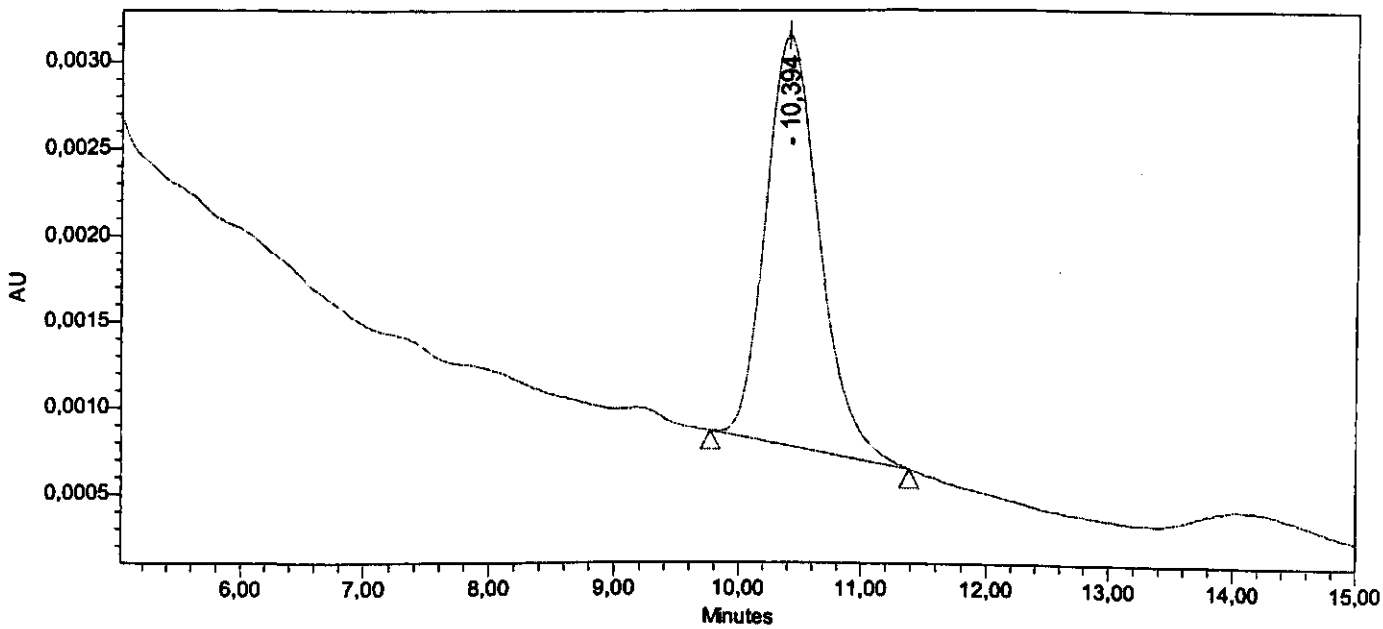
- Percentage recovery: 100.0 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 101.2 % and 98.8 %
- Spiking level: 2.5 mg/kg

Current Date 10.12.2001

Sample Information

SampleName	4118	Sample Type	
Vial	9	Date Acquired	
Injection	1	Acq Method Set	
Injection Volume	100,00 ul	Processing Method	
Channel	486	Date Processed	
Run Time	15,0 Minutes		

Auto-Scaled Chromatogram



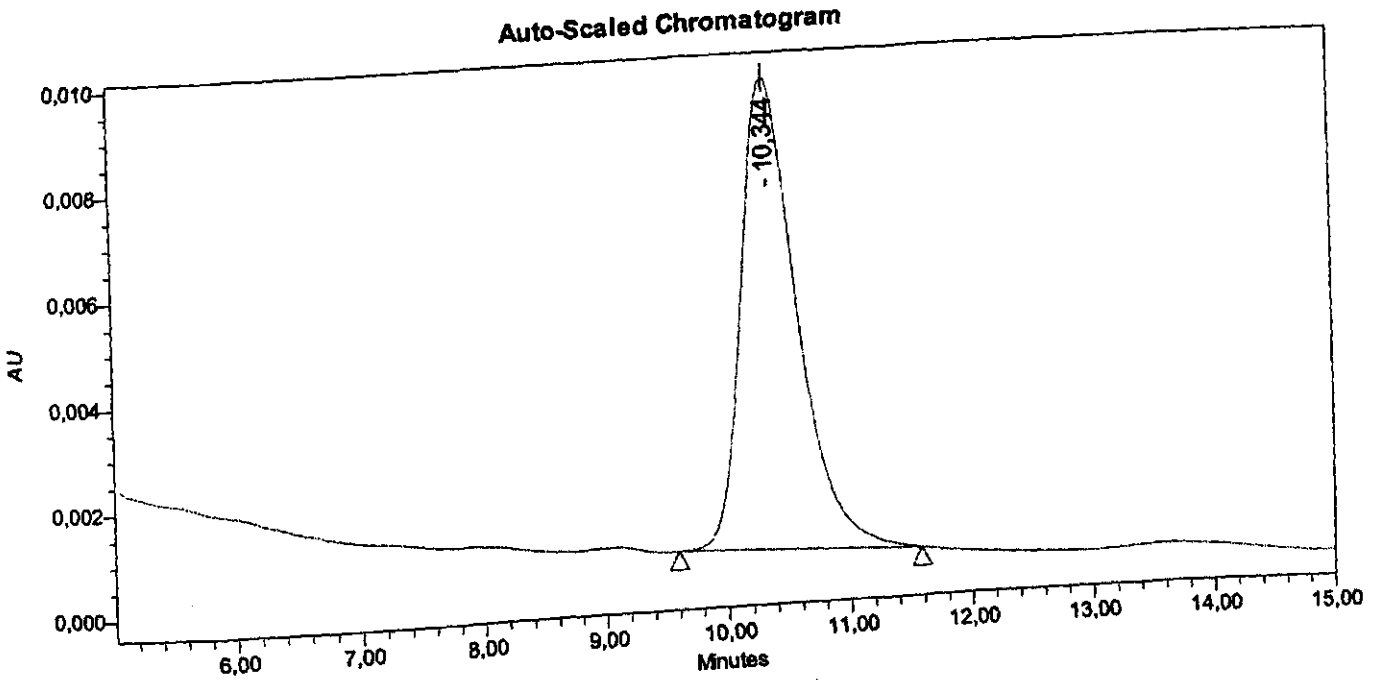
Peak Results

	Name	RT	Area	Height	Amount	Units
1		10,394	72042	2369	4773,406	ug

Current Date 10.12.2001

Sample Information

SampleName	4119	Sample Type	
Vial	10	Date Acquired	
Injection	2	Acq Method Set	
Injection Volume	100,00 ul	Processing Methox	
Channel	486	Date Processed	
Run Time	15,0 Minutes		



Peak Results

	Name	RT	Area	Height	Amount	Units
1		10,344	281552	9010	18669,052	ug