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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)

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

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ERRATUM

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Section 3.1.2 Sample homogeneity

For both feeds the 'CV (between samples)' of the homogeneity test was calculated erroneously. The correct CV (between samples) has to be calculated by multiplying with the result of square root of 2. Table 3 must be as follows:

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	1,5	3,9	2,6
Piglet feed	7,5	4,9	3,5	3,1

The correction of CV's (between samples) does not influence the conclusion drawn about the homogeneity.

SUMMARY

This report describes the results of a collaborative study of an HPLC method for the growth promoter olaquindox in three 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).
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 and 7,5 mg/kg and 1 blank piglet feed. 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 19 laboratories. Statistical evaluation was performed according to ISO 5725. The results show that acceptable results are obtained for repeatability (rsd_r < 10 %) and reproducibility (Horrat ratios < 2). However, 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 it is recommended to modify the CANFAS-method in such a way that the ratio between the extraction volume and the sample weight is increased to 5 and to organise a second round of collaborative studies for final validation of the method.
The results of the collaborative study were evaluated in a meeting attended by the participants. It can be concluded that the repeatability and reproducibility of the method is acceptable. The results obtained for the blank feed are also acceptable. The panel agreed that, due to the problems with the practicability of the method, the method cannot be recommended for adoption as an official method. A second collaborative study will be organised with a modified method.

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 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.

The samples that were prepared for the collaborative study were two piglet feeds with declared olaquindox contents of 2 and 7,5 mg/kg respectively and one blank feed. The feed samples were sent to the participants as blind duplicates. Before these samples were shipped, the between- and within-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 collaborative study.

2 PARTICIPANTS

The following laboratories/persons participated in the collaborative study.

- Administration des Services Technique de l'Agriculture Division des Laboratoires, Ettenbruck, Luxemburg; R. Meyers
- 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, E. Azara
- Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy; G. Biancotto, B. Allegretta, D. Berto, V. Capuzzo.
- 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, P. Dapena
- 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; G. Merson, J. Cowles, S.Jayakumar
- LNIV, Lisbon, Portugal; J.M. Nunes da Costa, M.B. Casqueira.
- LUFA – Augustenburg, Karslruhe, Germany; K. Michels, S. Witzemann.
- LUFA-ITL Kiel, Kiel, Germany; F.H. Johannsen, Kollwitz.
- Masterlab, Putten, The Netherlands; K. van Schalm, A. Schaaf.
- National Veterinary Institute, Uppsala, Sweden; E. Nordkvist, A. Stepinska
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Bral, R. van San
- RIKILT, Wageningen, The Netherlands; H. Kleijnen, H. van der Kamp
- State Laboratory Dublin, Ireland, P. Shearan, A. Cunningham, A. Murphy
- Universität Hamburg, Institut für Angewandte Botanik, Hamburg, Germany; H. Putzka, D. Böhm.
- Universität Hohenheim, Landesanstalt für Landwirtschaftliche Chemie, Stuttgart, Germany; B. Eckstein, K. Schwadorf, E. Koenzen.

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	mg/kg	IPC – Dier, Barneveld (NL)	05/09/2000
Piglet feed	7,5	mg/kg	IPC – Dier, Barneveld (NL)	05/09/2000

The feed sample with 2 mg/kg olaquindox also contained 10 mg/kg carbadox, the feed sample with 7,5 mg/kg olaquindox also contained 2,5 mg/kg carbadox. 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 / Ingredient	Piglet feed
Crude protein (%)	18,1
Crude fat (%)	4,3
Starch (%)	39,4
Crude fibre (%)	4,4
Crude ash (%)	4,7
Moisture (%)	12,4

The composition of the feeds was the same as the composition of the products which 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).

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).
Next to the above mentioned samples that contained olaquindox, a blind blank feed was sent to the participants as well as a blank feed labelled "blank feed for olaquindox recovery purposes" (see Appendix 1). The blind blank feed was a bull feed containing 5 mg/kg virginiamycin (see the corresponding CANFAS report). This feed was analysed at LUFA Augustenberg prior to the collaborative studies and was found to contain a small interfering peak at the retention time of olaquindox which corresponds to ca. 0,5 mg/kg. The blank feed for olaquindox recovery purpose was a standard piglet feed produced by IPC-Dier. This feed was also analysed prior to the collaborative study and contained no detectable amounts of olaquindox or interfering substances.

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	1,5	2,8	2,6
Piglet feed	7,5	4,9	2,5	3,1

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 %. All between- and within-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 October 2000 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 Appendix 5), the purity of the reference standard (Lot Nr. 890416) is 99,46 %. The participants were instructed to set the purity of the reference standard to 100 % (see Appendix 1).

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 Appendix 5).

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 column which was recommended in the method is a C18, 250 mm x 4 mm, 5 µm packing or equivalent).
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	As described in the method	As described in the method
15	Hypersil ODS 5 µm 200 x 4,6 mm + guard column	As described in the method
16	Sperisorb ODS 2, 10 µm 250 x 4,6 mm	As described in the method
17	Sperisorb S10 ODS-1 10 µ	As described in the method
18	150 x 4,6 mm; 5 µm; Sperisorb ODS2 C18	As described in the method
20	Alltimo Alltech C18, 250x4,6 mm, 5 µm	As described in the method
21	Supelcosil LC-18 25 cm x 4,6 mm (5 µm) + supelguard LC-18	Acetonitril: ammoniumacetate buffer (0,01M; pH 4,6) Gradient elution
23	Not reported	
24	250 x 4,6 mm C18 5 µm	As described in the method
25	As described in the method	Water/methanol = 800:200 (v/v)
26	Sperisorb ODS 2 250 mm x 4,6 mm 5 µm	As described in the method
27	As described in the method	As described in the method
29	Nova Pack, 4,6 x 250 mm; C18; 4 µ	As described in the method
31	As described in the method	As described in the method
32	Waters symmetry, C18, 5µm, 4,6x250 mm	As described in the method
33	As described in the method	0,01 M ammoniumacetate pH 5: acetonitrile = 95:5 (v/v)
34	As described in the method,	As described in the method
37	Lichrosper RP18-5 endcapped	As described in the method
38	Hypersil ODS C-18, 250 x 4,6 mm, 5 µm	As described in the method
40	C18 sperical 5 µm 3,9 x 15 cm waters	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 6.

5.1 Statistical evaluation

The results reported by the participants are given in Table 6.
Due to problems in obtaining enough solvent after the extraction step two laboratories used a modified method with a higher ratio between extraction volume and sample weight. The results of these laboratories are not included in Table 6 and will be described in par. 5.5.
The results reported by lab 25 clearly show that this lab has interchanged (the results of) the samples (see also par. 5.2): two samples are reported as "nd", two samples at 1,70 mg/kg and two samples at 4,86 - 4,88 mg/kg but only in one case the code corresponds to the right sample. This lab was contacted but was not able to trace back the origin of the interchange. For this reason the results of lab 25 were not taken into account in the statistical evaluation.
Statistical analysis shows that the results of the other 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 method is satisfactory.

Table 5: Horrat ratios of the olaquindox collaborative study

Mean after discarding lab 25 (mg/kg)	Predicted rsd_R	Established rsd_R	Horrat[1]	Conclusion
1,721	14,745	21,12	1,43	Reproducibility OK
5,284	12,454	16,65	1,34	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. The Mandel h plot shows that 3 laboratories (nr. 15, 29 and 37) reported low values for both samples. Laboratories 29 and 37 reported the lowest recoveries, viz. 69 and 49 % while the recovery reported by lab 15 (78 %) is a normal value. Lab 37 is considered as a Grubbs outlier with regard to the recovery (see par. 5.3). Nevertheless it is unjustified to discard the results of lab 37 from statistical evaluation because of the problems encountered by many laboratories (among them lab 37, see par. 5.4) with the extraction step in the method, which is regarded as the main causative factor for the low recovery.

Table 6: Results reported by the participants.

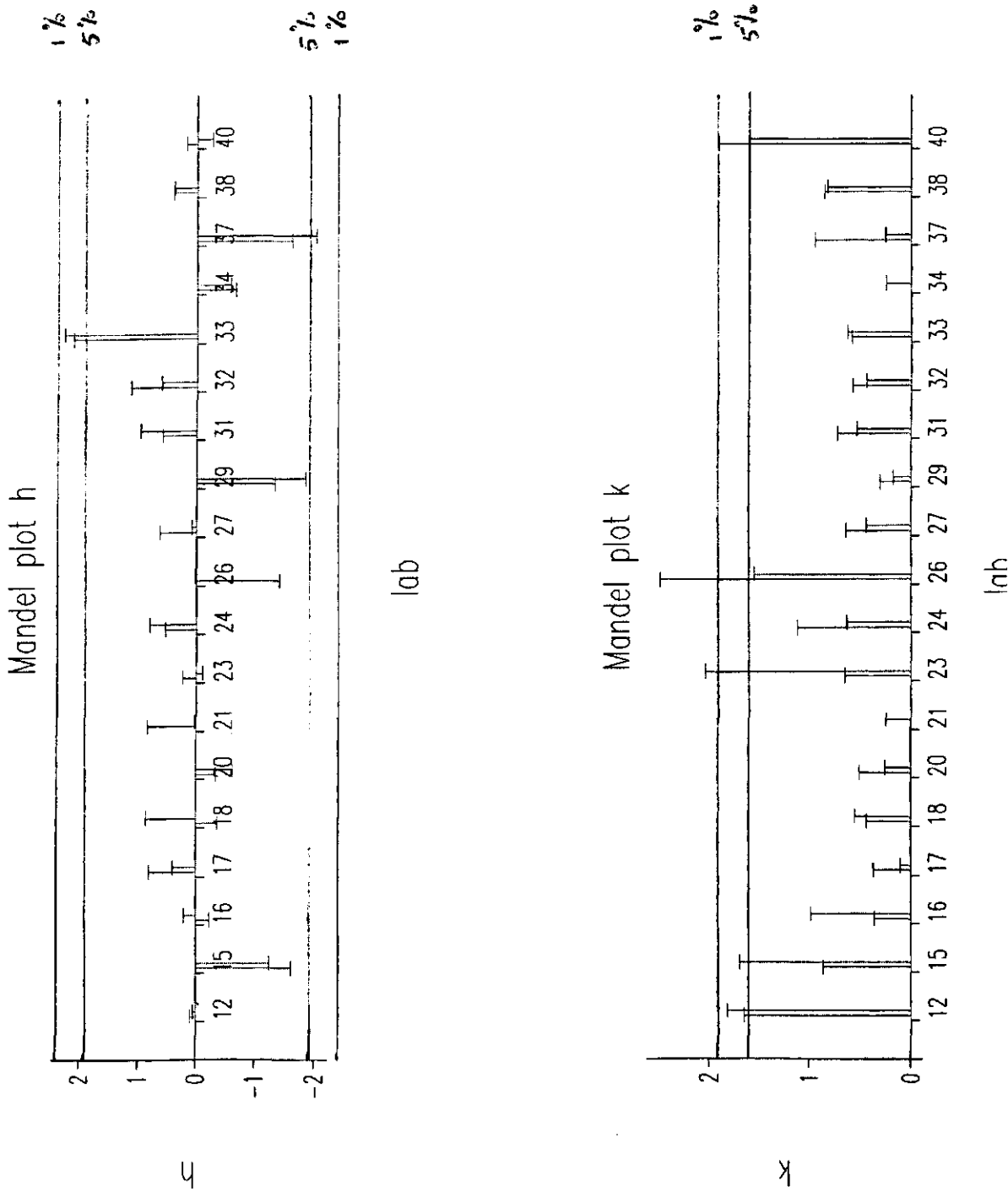
Table 6. Olaquindox in two piglet feeds

	Result (mg/kg)							
Sample	OLA 2 mg/kg	OLA 2 mg/kg	OLA 2 mg/kg	OLA 2 mg/kg	OLA 7,5 mg/kg	OLA 7,5 mg/kg	OLA 7,5 mg/kg	OLA 7,5 mg/kg
Lab								
12	1,97	2,01	1,54	1,51	4,84	4,78	5,84	5,85
15	1,25	1,02	1,12	1,34	4,60	3,52	4,14	4,76
16	1,56	1,70	1,66	1,65	5,66	4,98	5,65	5,54
17	2,07	1,99	1,99	1,92	5,60	5,66	5,58	5,63
18	1,56	1,52	1,67	1,65	5,78	5,92	6,18	6,12
20	1,72	1,60	1,52	1,59	4,85	4,78	4,65	4,78
21	2,00	2,00	2,00	2,00	5,40	5,30	5,30	5,20
23	1,67	1,76	1,91	1,86	4,23	5,77	5,40	5,37
24	2,00	2,10	1,80	1,70	5,70	6,20	6,00	5,90
25	*nd*	*nd*	*nd*	*nd*	*4,88*	*4,88*	*1,70*	*1,70*
26	1,60	1,50	0,70	1,20	5,10	4,80	6,00	5,30
27	2,05	1,98	1,81	1,89	5,22	5,34	5,30	5,56
29	1,20	1,30	1,30	1,30	3,80	3,70	3,80	3,70
31	1,88	1,90	2,08	1,80	6,28	6,13	6,05	5,86
32	2,06	2,24	2,07	2,03	5,91	5,92	5,63	5,71
33	2,30	2,40	2,50	2,50	7,40	6,90	7,20	7,20
34	1,50	1,50	1,50	1,50	4,70	4,80	4,80	4,90
37	1,38	1,22	1,11	1,02	3,70	3,63	3,50	3,58
38	1,72	1,76	2,01	1,94	6,00	5,52	5,53	5,38
40	1,50	1,80	2,21	1,62	4,75	4,56	5,20	5,74

number of labs	19	19
m (mg/kg)	1,721	5,284
rsd_r (%)	9,47	6,22
rsd_R (%)	21,1	16,6

Remark : Italic printed results are not taken into account in the statistical evaluation!

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



5.2 Blank samples

Table 7: Reported results of the blank samples

Partner	Blank sample 1		Blank sample 2	
	Result 1	Result 2	Result 1	Result 2
12	Nsd	nsd	Nsd	Nsd
15	Blank	blank	Blank	Blank
16	Not found	Not found	Not found	Not found
17	-	-	-	-
18	Not detected <0,5	Not detected <0,5	Not detected <0,5	Not detected <0,5
20	Neg	Neg	Neg	Neg
21	0,0 ND	0,0 ND	0,0 ND	0,0 ND
23	<0,11	<0,21	<0,11	<0,21
24	Blank	blank	Blank	Blank
25	1,70	1,70	4,88	4,86
26	0,1	0,1	0,1	0,1
27	Not detect.	Not detect.	Not detect.	Not detect.
29	0	0	0	0
31	0	0	0	0
32	Negative	negative	Negative	Negative
33	<1	<1	<1	<1
34	0	0	0	0
37	ND	ND	ND	ND
38	0	0	0	0
40	-	Not analysed	-	Not analysed

Only laboratories 25 and 26 reported positive results for the blank samples. For lab 25 this was caused by the fact that this lab had interchanged the samples (see par. 5.1).
For lab 26 the reported values (0,1 mg/kg) are at the limit of detection defined for the method. Consequently it can be concluded that no interfering substances are detected in the blank samples.

5.3 Recoveries

Table 8: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	Average recovery in (%)
12	78		78
15	78		78
16	77	76	77
17	85	83	84
18	80	76	78
20	101	101	101
21	83	84	84
23	Not reported		
24	71	86	79
25	84		84
26	75	71	73
27	76		76
29	68	70	69
31	78	86	82
32	82	87	85
33	93	91	92
34	82	86	84
37	48	49	49
38	81		81
40	83		83

Recoveries range from 48 - 101 %. This range is broader than the range (60 - 90 %) which was measured in the between-lab validation of the method (see Second Annual Report CANFAS, J. de Jong, 12-08-2000).
Most probably, the problems encountered by many laboratories with the extraction step in the method are the main causative factor for the low recovery.
The mean recovery value reported by lab 37 (49 %) is a Grubbs outlier.

5.4 Remarks

Table 9: Remarks made by the partners

Partner	Remarks
12	In some cases (we try the method as well with real samples of feedingstuffs), the volume of liquid for the extraction is not enough to get a proper volume of extractant solution, even with centrifugation. We think the weight/volume could be 1/3, not ½. Perhaps, this is one of the reasons because of the revovery percentages are not so good as with carbadox method (where the relation is 1/5).
15	We have encountered some troubles performing the centrifugation step of 50 ml solvent extract in the extraction procedure. However the results were obtained utilising the method without modification. In another occasion, we have tried to increase extraction volume to 100 ml (20 ml methanol and 80 ml water) obtaining an increase of recovery from 78% to 88% at 3,0 mg/kg spiking level and the following results on the samples: Sample code Result 1 (mg/kg) Result 2 (mg/kg) 155711 blank blank 155735 2,07 1,91 155741 1,92 2,09 155761 blank blank 155776 5,90 5,86 155784 5,95 6,02 spike 3,0 mg/kg 2,67 (rec 89%) 2,66 (rec 89%)
16	1. 5.1.2 Recovery test: "..Proceeding with the extraction step (5.2)". Remark: Transfer of 1,5 ml stock standard solution (3.5.1) results in our opinion in the addition of 38,5 ml water and not in 40 ml water (see 5.2). This volume error amounts to about 3 %. 2. 5.2 Extraction: Remark: weight in of 25 g of sample in relation with a volume of 50 ml of liquid strongly recommends centrifugation. Our parameters: 10 minutes with 7200 rpm. The supernatant liquids were subsequently filtered by using membrane filters (Macherey&Nagel, Chromafil Type A-45/25, 0,45 µm).

Partner	Remarks
17	Ad 3.5.1 The olaquindox-standard is soluble within 1 minute by ultrasonic treatment. An ultrasonic treatment of 10 minutes warms up the fluid. Ad. 5.2.1 Attention: the total volume by the recovery test is 51,5 ml!! Ad. 5.2 a) It is hardly possible too moisten 25 g sample with 10 ml methanol. b) It is hardly possible to shake or stir the sample (25g/50 ml liquor) c) It is better to centrifuge the sample than to filter through an folded filter (suck up of the liquid). All samples were analysed by the existing EU-Methode 98/64 mg/kg recovery % 175703 - + 3 mg olaqu/kg: 88% 175718 6,09 175730 2,10 175775 - + 6 mg olaqu/kg: 88% 175817 2,14 175828 6,21
18	HPLC equipment: 15 and 16/11/00: pump; autosampler->HP 1050 (40 µl); DAD-> HP 1100 23/11/00: pump Spectra Physics; autosampler Marathon (50 µl); single wave length Milton Roy; Chromjet Recorder *Differences with CANFAS/ola/02/10/2000 method:* - 3.5.1 Stock standard solution -> 50 mg/1000 ml; weigh to the nearest 1 mg - 3.5.2 Standard solutions -> point at 2,5 µg/ml -> 5 ml of (3.5.1) in a 100 ml graduated flask. - 5.2 Instead of filtration through a folded filter, centrifugation was carried out as mentioned at 7.1. - Receival of sample package on 5/10/00, storage of samples until analysis at < 8 °C, in a refrigerated room - 15 and 16/11/2000: direct analysis of the 6 feeds with DAD; test of recovery; identity confirmation - 23/11/2000: Analysis, with single wavelength detector, of the 2 blank feeds and the lowest content sample to estimate LOD and LOQ Results: - Reported results are the average of height and area results. - Calibration based on height and area (10 points; forced through origin) -> see example
20	No remarks
21	We found difficulties during the extraction because of the large amount of the feed compared to the volume of the solvent. In two samples (n. 770-791) the solvent was almost completely absorbed by the feed and this made the extraction of the samples very difficult. We were forced to centrifuge these extracts in order to obtain supernatant.

Partner	Remarks
23	Not reported
24	The ratio between the sample amount and the extraction solvent volume resulted to be a very critical step of the method which could affect the reproducibility.
25	We had some difficulties in the extraction method (5.2). It was very difficult to have enough filtrate in the filtration step so centrifugation has been applied (4000 rpm for 15 min.) Then we followed the standard procedure: Transfer the sample extract in a 50 ml volumetric flask Filter the solution trough a folded filter Filter an aliquot through a membrane filter (0,45 µm) for analysis by HPLC
26	No remarks
27	As to the samples with the code numbers 275720 and 275773 strong swelling avoided a shakeable suspension to be formed. Along with 275720 the lot of fluid was enlarged to 70 ml,, along with 275773 the volume of extraction fluid was doubled; in this case the injection volume was doubled as well as that of the calibration standards so that the limit of detection (with 0,1 mg/kg) could be ensured.
29	No remarks
31	During HPLC-analyses the area/height ratio changed, the peaks got broader. Quantification was only possible on area. Sample preparation: The samples were centrifuged after extraction, then filtrated on GFA-filter, followed by filtration on acrodisc 0,45 µm
32	No remarks
33	No remarks
34	No remarks
37	We found it impossible to proceed with the method particularly with the use of conical flask + filtration of sample. In this situation no filtrate was collected. The entire study was done using 150 ml glass centrifuge tubes (instead of conical flasks) with a centrifugation step (15 min at 3000) (instead of filtration). It was also necessary to further separate the filtrate and centrifuge this prior to HPLC.
38	Please note that our detection system has been DAD; not a single wavelength UV-detection (as it has been indicated at particularly instruction) because we have not it.
40	No remarks

The remarks clearly indicate that the practicability of the method is unsatisfactory. Ten out of nineteen laboratories reported difficulties in obtaining enough solvent after the extraction step due to the low ratio between the volume of solvent (50 ml) and the weight of feed (25 g). Moreover, two laboratories decided to use a modified method with a higher ratio between extraction volume

and sample weight (see par. 5.5) and one laboratory informed the co-ordinator that they abstained from participation due to the same problem.
The results of laboratories 15 and 17 show that when the ratio between the extraction volume and the sample weight is increased, the recoveries increase and the values for the blind positive samples are higher than with the CANFAS-method.
For these reasons it is proposed to modify the CANFAS-method in such a way that the ratio between the extraction volume and the sample weight is increased to 5 and to organise a second round of collaborative studies for final validation of the method.

5.5 Special requests

The following participants used divergent extraction volumes and/or sample weights, because of difficulties with the prescribed ratio of extraction volume and sample weight (strong swelling, not possible to shake). See also paragraph 5.5.3, remarks.

- Masterlab, Putten, The Netherlands; K. van Schalm, A. Schaaf.
- National Veterinary Institute, Uppsala, Sweden; E. Nordkvist, A. Stepinska

5.5.1 HPLC conditions

Table 10: HPLC conditions

Partner	Column	Mobile phase
Masterlab, Putten, The Netherlands	As described in the method.	As described in the method
National Veterinary Institute, Uppsala, Sweden	Hypersil C18 ODS BDS 250 x 4,6 mm; 5 µm	As described in the method

5.5.2 Recoveries

Table 11: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	Recovery average in %
Masterlab, Putten, The Netherlands	98	82	90
National Veterinary Institute, Uppsala, Sweden	95	96	96

5.5.3 Remarks

Table 12: Remarks made by the partners

Partner	Remarks
Masterlab, Putten, The Netherlands	As prescribed in the method 25 gram sample was mixed with 10 ml methanol and 40 ml water. The whole volume was absorbed by the sample and as a results it was not possible to shake the extract. An extra volume of 10 ml methanol and 40 ml water was added. This extract remained a thick pulp, but it could be shaken. The extract was centrifuged prior to filtration over GF/A.
National Veterinary Institute, Uppsala, Sweden	1. Since the volume extraction solution (50 ml) was found too small for extracting the 25 g sample, a 10 g sample was used for the extraction of olaquindox. 2. The UV-detector wavelength used was 372 nm instead of the recommended 380 nm (the absorbance maximum was detected at this wavelength. 3. The olaquindox content was calculated from the peak area by reference to the calibration graph.

5.5.4 Results of the samples

Table 13: Results reported by the partners

Partner	Masterlab, Putten, The Netherlands		National Veterinary Institute, Uppsala, Sweden	
Sample content (mg/kg)	Result 1 (mg/kg)	Result 2 (mg/kg)	Result 1 (mg/kg)	Result 2 (mg/kg)
0	<0,1	<0,1	<1	<1
0	<0,1	<0,1	<1	<1
2	1,5	1,4	2,27	2,2
2	1,6	1,5	2,34	2,21
7,5	4,6	4,5	6,93	7,05
7,5	6,2	6,3	6,81	6,79

The values of Masterlab are similar to the mean values obtained with the CANFAS method. The values of National Veterinary Institute are higher than the mean values obtained with the CANFAS method. Again this shows the applicability of a higher ration between extraction volume and sample weight.

6 EVALUATION AND CONCLUSIONS

The results of the collaborative study were evaluated in a meeting in Tervuren (Belgium) on June 19-20, 2001.
The panel has accepted the results of the statistical evaluation, as described in par. 5.1, Table 6. Consequently it can be concluded that the repeatability and reproducibility of the method is acceptable. The results obtained for the blank feed are also acceptable. Large differences are observed in the recovery (range 49 – 101 %), most probably due to the problems in the extraction step, caused by the unfavourable ratio between extraction volume and sample weight. The panel agreed that, due to the problems with the practicability of the method (see par. 5.4), the method cannot be recommended for adoption as an official method. A second collaborative study will be organised with a modified method. The ratio between the extraction volume and the feed weight will be increased from 2 : 1 to 5 : 1.
The results obtained for the blind blank feed indicate that different columns lead to differences in interfering peaks (large peak eluting prior to olaquindox).
The following columns will be 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 following remarks, related to the method description have been accepted:

- Lab 16, remark 1 (see par. 5.4 of this report).

ACKNOWLEDGEMENTS

Financial support from the European Commission, DG Research, Standards, Measurements and Testing Programme (SMT) is gratefully acknowledged.
Dr. A. Plöger, Danish Plant Directorate, Lyngy, Denmark, 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)

cc Driessen, J. de Jong

DATE
2 October 2000

SUBJECT
collaborative study CANFAS
olaquindox 71.316.24

ENCLOSURE(S)
4

OUR REFERENCE
00/0022094

HANDLED BY
Dr. J. de Jong

DIRECT (TELEPHONE) LINE
+31 317 47 55 81

E-MAIL
j.dejong@RIKILT.WAG-UR.nl

RIKILT
State Institute for Quality Control of Agricultural Products
P.O.Box 230
6700 AB Wageningen
The Netherlands

VISITORS' ADDRESS
Building no. 123
Bornsesteeg 45
6708 PD Wageningen

TELEPHONE
+31 317 47 54 00

FAX
+31 317 41 77 17

CHAMBER OF COMMERCE REGISTRATION NO.
09098104 te Arnhem

THE INTERNET
www.rikilt.wageningen-ur.nl

Dear colleague,

Please find enclosed the samples for the collaborative study for olaquindox:

- 6 feed samples, with the text "additive: OLAQUINDOX" and with a sample code; these samples constitute 2 blind duplicates of feed samples containing olaquindox (contents in the range between 1 and 15 mg/kg) and 1 blind duplicate of a blank feed

The samples must be analysed in *duplicate*.

For recovery purposes we have included a blank sample, with the text "blank feed for olaquindox recovery purposes".

The method which has to be used is included as Annex 1 (please note that this method is a *modified* version of the method which was distributed prior to the kick-off meeting).

Annex 2 contains the reporting form. 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 (please send the results to the following E-mail address: j.j.m.driessen@rikilt.wag-ur.nl). Of course you can also fill in the form and send it by fax or normal mail.
The **deadline** for reporting the results is **8 December 2000**.

Annex 3 contains instructions for handling (milling, storage) of the samples.

Annex 4 is a questionnaire. 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 reference standard of olaquindox which has to be used (980416) was already sent to you with my letter of 31 May 2000. In the calculations this reference standard can be regarded as 100 % pure.

We wish you and your colleagues the best with the collaborative studies. 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

RIKILT
State Institute for Quality C
of Agricultural Products

cc mrs. I. de Froidmont-Görtz, European Commission, DG Research, CII/3, Brussels

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 1 - Description of the method

CANFAS/OLA/02102000/K.MICHELS

SMT4-CT98-2216

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'-(hydrxyethyl)carbamoyl]-3-methylquinoxaline-N^1 ,N^4-dioxide, E 851

3.5.1. *Olaquindox stock standard solution, 50 µg/ml*

Weigh to the nearest 0,1 mg 10 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 0.5, 1.0, 2.5, 5.0 and 10.0 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

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 3 mg/kg, transfer 1.5 ml of the stock standard solution (3.5.1) to a 250 ml conical flask, add 25 g of the blank feed, mix thoroughly and leave for 10 min mixing again several times before proceeding with the extraction step (5.2). 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 25 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 ultrasonic 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)
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: 20 µl -100 µl
Check the stability of the chromatographic system injecting several times the calibration solution (3.5.3) containing 2.5 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 pg/ml 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 a centrifugation step could be carried out.

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

Lab-name:

Contact person: e-mail:

fax:

telephone:

Date of analysis:

Analyte: OLAQUINDOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
315707		
315709		
315710		
315794		
315801		
315811		

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - 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 OCTOBER 2000 - 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

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 OCTOBER 2000 - 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 cooperation !

APPENDIX 2

Composition of the feed samples

BESTMIX - Afdruk mengopdracht - 05/09/00 - IPC DIER BARNEVELD

2 250.00 Biggen opfok korrel Rikilt Piglet
biggenvoer van 12 tot 25/30 kg
10 ppm carbadox + 2 ppm olaquindox

Grondstof	Silo	%	Gewicht kg	Tol. +/-Afw.	Cumul Gew. kg	Charge 6	Charge
Weegschaal DW 1							
113 Zonbl.schr.290re	(2)	2.00	10.00	0.30	10.00	✓	.....
460 Tapioca65%zetmeel	(4)	7.50	37.50	1.13	47.50	✓	.....
77 Soja 45/46(arg/braz)	(9)	13.00	65.00	1.95	112.50	✓	.....
Weegschaal DW 2							
145 Tarwe (voer)	(9)	10.00	50.00	1.50	50.00	✓	.....
14 Gerst	(11)	37.10	185.50	5.57	235.50	✓	.....
40 Mais	(12)	12.00	60.00	1.80	295.50	✓	.....
Bijstort SP4							
34 Lynzaad	(0)	5.00	25.00	0.75	25.00	✓	.....
105 Vismeel 65.9% re	(0)	4.40	22.00	0.66	47.00	✓	.....
Bijstort SP6							
476 Powerfood Twil melkv	(0)	4.00	20.00	0.60	20.00	✓	.....
Bijstort SP7							
21 Fumaarzuur	(0)	0.25	1.25	0.01	1.25	✓	.....
78 L-lysine HCl	(0)	0.17	0.85	0.01	2.10	✓	.....
79 DL-Methio-nine	(0)	0.03	0.15	0.00	2.25	✓	.....
117 Krijt/kalksteen	(0)	0.45	2.25	0.02	4.50	✓	.....
228 Monocal Belgie	(0)	0.50	2.50	0.03	7.00	✓	.....
485 Zout	(0)	0.10	0.50	0.01	7.50	✓	.....
508 Prem biggen Rikilt	(0)	1.00	5.00	0.05	12.50	✓	.....
1,0 g/kg Carb + Olaquindox 0,2 g/kg							
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. : 2,5..... mm
H.M. : hoog/laag toeren
kringloop : ja/nee
L.M. : voormengen ..0.. sec
namengen 300 sec
M.D. : ..73.. l/h

Meel temp : ..35.. °C korreldemp 78 °C
Matrijs diam. : 2,5 x 35. mm
K.P. : 28.. Amp
Laagdikte Ko : 35. cm
Zeef Ko : fijn mm
Kruimelen : ja/neen
Holmen : 96,8 %
Vocht : %

BESTMIX - Afdruk mengopdracht - 05/09/00 - IPC DIER BARNEVELD

2 250.00 Biggen opfok korrel Rikill Piglet
biggenvoer van 12 tot 25/30 kg
2,5 mg/kg carbadox + 7,5 mg/kg olaquindox

Grondstof	Silo	%	Gewicht kg	Tol. +/-Afw.	Cumul Gew. kg	Charge 5	Charge
Weegschaal DW 1							
113 Zonbl.schr.290re	(2)	2.00	10.00	0.30	10.00	V	
460 Tapioca65%zetmeel	(4)	7.50	37.50	1.13	47.50	V	
77 Soja 45/46(arg/braz)	(8)	13.00	65.00	1.95	112.50	V	
Weegschaal DW 2							
145 Tarwe (voer)	(9)	10.00	50.00	1.50	50.00	V	
14 Gerst	(11)	37.10	185.50	5.57	235.50	V	
40 Mais	(12)	12.00	60.00	1.80	295.50	V	
Bijstort SP4							
34 Lynzaad	(0)	5.00	25.00	0.75	25.00	V	
105 Vismeel 65.9% re	(0)	4.40	22.00	0.66	47.00	V	
Bijstort SP6							
476 Powerfood Twil melkv	(0)	4.00	20.00	0.60	20.00	V	
Bijstort SP7							
21 Fumaarzuur	(0)	0.25	1.25	0.01	1.25	V	
78 L-lysine HCl	(0)	0.17	0.85	0.01	2.10	V	
79 DL-Methio-nine	(0)	0.03	0.15	0.00	2.25	V	
117 Krijt/kalksteen	(0)	0.45	2.25	0.02	4.50	V	
228 Monocal Belgie	(0)	0.50	2.50	0.03	7.00	V	
485 Zout	(0)	0.10	0.50	0.01	7.50	V	
508 Prem biggen Rikill	(0)	1.00	5.00	0.05	12.50	V	
0,25 g/kg CARB; 7,5 g/kg OLA							
Vloeistoffen							
474 Melasse riet >450s	(3)	2.50	12.50	0.38	12.50	V	
			Totaal :		500.00		

RETOURPRODUKT ....

INSTELLINGEN

T.R. : aut. 50%
V.Z. : grof (fijn) .80... %
Z.F. : .2,5.... mm
H.M. (hoog)/laag toeren
kringloop : ja/(nee)
L.M. : voormengen .0.. sec
namengen 300 sec
M.D. : ..73. l/h

Meel temp : 55.. °C korrels 77 °C
Matrijs diam. : 2,5 x 35 mm
K.P. : 28.. Amp
Laagdikte Ko : 35 cm
Zeef Ko : fijn mm
Kruimelen : ja/(nee)
Holmen : 96,8 %
Vocht : %

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 collaborative study

Additive : Olaquindox
Product : Feed sample: 2 ppm

Date of determination : September 22th, 2000

Sample	Content mg/kg	Duplicate average mg/kg
341024 A	1,6	1,6
341024 B	1,5	
341030 A	1,5	1,5
341030 B	1,5	
341028 A	1,5	1,5
341028 B	1,5	
341029 A	1,5	1,5
341029 B	1,5	
341021 A	1,5	1,5
341021 B	1,5	
341023 A	1,4	1,5
341023 B	1,5	
341027 A	1,5	1,5
341027 B	1,5	
341026 A	1,5	1,5
341026 B	1,4	
341022 A	1,4	1,4
341022 B	1,4	
341025 A	1,5	1,5
341025 B	1,5	

Homogeneity	**OK**		
Criterion : $CV_{between}$ = < 15%			
Average		1,5	
SD (between samples)		0,04	
CV (between samples)		2,8	Result Grubb's test
Grubb's test, single lower		2,065	no outlier
Grubb's test, single upper		1,579	no outlier
Grubb's test, double lower		0,3279	no outliers
Grubb's test, double upper		0,6557	no outliers

Repeatability		
SD (within samples)	(sd_r)	0,04
CV (within samples)	(CV (%))	2,6

CANFAS

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

Homogeneity test collaborative study

Additive : **Olaquindox**
Product : **Feed sample: 7.5 ppm**

Date of determination : September 22th, 2000

Sample	Content mg/kg	Duplicate average mg/kg
345016 A	4,8	5,1
345016 B	5,3	
345014 A	5,0	5,0
345014 B	4,9	
345013 A	5,0	5,1
345013 B	5,2	
345012 A	5,0	4,9
345012 B	4,8	
345020 A	5,0	5,0
345020 B	4,9	
345015 A	4,8	4,8
345015 B	4,7	
345017 A	4,9	4,8
345017 B	4,7	
345019 A	5,0	5,0
345019 B	4,9	
345011 A	4,7	4,8
345011 B	4,8	
345018 A	4,9	4,8
345018 B	4,7	

Homogeneity OK Criterion : $CV_{between}$ = < 15%		
Average	**4,9**	
SD (between samples)	**0,12**	
CV (between samples)	**2,5**	Result Grubb's test
Grubb's test, single lower	**1,225**	**no outlier**
Grubb's test, single upper	**1,633**	**no outlier**
Grubb's test, double lower	**0,5833**	**no outliers**
Grubb's test, double upper	**0,4236**	**no outliers**

Repeatability		
SD (within samples)	(sd_r)	**0,15**
CV (within samples)	(CV (%))	**3,1**

APPENDIX 4

Sample codes

Sample codes supplied to the participants in the olaquindox collaborative study

OLAQUINDOX number of participants 23	OLA piglet 7,5ppm OLA 1a	OLA piglet 7,5ppm OLA 1b	OLA piglet 2ppm OLA 2a	OLA piglet 2ppm OLA 2b	VIRG bull 5ppm OLA blank 1a	VIRG bull 5ppm OLA blank 2b
Participant code						
12	125820	125795	125785	125760	125818	125748
15	155776	155784	155741	155735	155761	155711
16	165708	165816	165774	165719	165802	165751
17	175828	175718	175730	175817	175775	175703
18	185693	185695	185731	185733	185758	185823
20	205796	205822	205729	205739	205809	205713
21	215797	215827	215715	215813	215791	215770
22	225727	225736	225806	225790	225725	225810
23	235694	235699	235787	235712	235793	235767
24	245746	245829	245825	245723	245781	245780
25	255782	255696	255728	255819	255814	255742
26	265749	265768	265743	265763	265764	265755
27	275805	275700	275830	275745	275773	275720
29	295704	295721	295762	295786	295732	295756
31	315794	315709	315811	315710	315707	315801
32	325747	325705	325807	325716	325744	325798
33	335772	335759	335804	335706	335702	335753
34	345778	345750	345717	345752	345698	345826
35	355714	355738	355808	355812	355777	355824
37	375792	375740	375788	375765	375754	375722
38	385815	385726	385783	385789	385737	385803
40	405800	405779	405799	405734	405701	405697
41	415724	415771	415769	415821	415757	415766

APPENDIX 5

Olaquindox reference standard profile, identity and purity

Fat 500g portion modtaget 26-1-99.
Er i fryseren i kælder.

11 FEB. 1999

APL

CERTIFICATO DI ANALISI N. 01/99
CERTIFICATE OF ANALYSIS No.01/99

Nome prodotto:			
Product name:	OLAQUINDOX PURE		
Codice Prodotto/Product code:	311363	**Analisi N. / Analysis no.**	LC 02
Lotto N/Batch no:	980416		
Data Produzione/Mfg date :	Aprile 98/Apr 98	**Data scadenza/Exp date :**	Aprile 01/Apr 01

RISULTATI/RESULTS:

Metodo/Method	Descrizione/Description	Specifica/Specification	Risultato/Result
01ASP	Aspetto/Appearance	polvere cristallina/ crystalline powder	Corrisponde/Corresponds
01COL	Colore/Colour	giallo/yellow	Corrisponde/Corresponds
01ODO	Odore/Odour	inodore/odourless	Corrisponde/Corresponds
UV016	Titolo/Assay	min 98 max 101.5 %	99.46%
01PPE	Perdita di peso per essicamento/Loss on drying	max 0.5 %	0.07%
	Mono-N1-Ossido/Mono-N1-Oxide	max 0.5 %	max 0.5 %
	Mono-N4-Ossido/Mono-N4-Oxide	max 0.25 %	max 0.25 %
	Metilestere	max 0.2 %	max 0.2 %

Il soprannominato Prodotto è stato analizzato secondo i metodi analitici DOX-AL ITALIA SpA ed è stato approvato per la vendita dal CONTROLLO QUALITA'/The above mentioned product has been analyzed according to Dox-al Italia Spa analytical methods and has been approved for sale by QUALITY CONTROL

Analista/Analyst
Lucia Corbetta

DOX-AL ITALIA SPA
Direttore Tecnico/
Technical Director
pp Dr. G. Astegiano

Data analisi/Date of analysis : 14/01/99

DOX-AL ITALIA S.p.A.: 20050 CORREZZANA (MI) - I - Tel. 039-6860701 - Telefax 039-6065816 - Cap. Soc. L. 10.000.000.000
MI 103057 - C.C.I.A.A. Milano 827985 - Tribunale Monza Reg. Soc. 11271 - Reg. Ord. 2216M
P.I./VAT IT-00729770966 - Codice Fiscale 02117690152
New Address e-mail: doxalitgalenica.it

Sede Operativa: DOX-AL ITALIA S.p.A. - Via BINI, 20 - 20050 SULBIATE SUPERIORE (MI)
Tel. 039-6020252
Fax 039-623844

CANFAS
71.316.24

Verification of identity and purity of Olaquindox and Carbadox standard substance

J.A. van Rhijn, A. Lommen and H.C.H. Kleijnen
RIKILT, Wageningen, The Netherlands
May 2001

Introduction

In order to ensure that the standard substances purchased in the framework of the CANFAS collaborative studies were fit-for-purpose, UV spectroscopy, ^{1}H-NMR and mass spectrometry were used to verify their identity. Purity was determined by ^{1}H-NMR.

Materials

Carbadox

Supplier	Pfizer
Lotnr	3E121-84QCS
Drying loss (%)	0.02
Purity (%)	99.3

Olaquindox

Supplier	DOX-AL Italia
Product ID code	311363
Lotnr	980416
Drying loss (%)	<0.5
Purity (%)	99.5

Experimental

UV spectroscopy

UV sample preparation: Canfas substances of olaquindox and carbadox each were dissolved in a mixture of acetonitril and methanol (50/50, v/v) and diluted with the same solvent to obtain for each substance a solution containing a concentration of exactly 4 μg/ml.
UV experiments: UV spectra in the wavelength range 220 to 500 nm were recorded using a Beckman DU60 UV-VIS spectrometer. The spectra were matched with the spectra of reference substances of Olaquindox (Bayer, purity 99.4%) and Carbadox (Sigma, lot 030H0349, purity >99%) regarding both the absorbance maxima observed, indicative of the analytes identity, and the absorbance, indicative of their quantitative equivalence.

Table 1 UV-VIS Spectral information for the reference standards carbadox and olaquindox and the deviations obtained for the corresponding Canfas standard substances.

Compound	Absorbance maxima (nm)	Δ (nm)	Absorbance (AU)	Δ (%)
Carbadox	243.5	-1.0	0.2216	+6.0
	308.0	0	0.5924	+2.3
	382.5	-1.0	0.2036	+3.5
Olaquindox	230.5	+1.0	0.3251	-2.8
	266.5	0	0.3649	-4.5
	383.5	+0.5	0.1856	-4.9

Results: Table 1 presents the spectral data of both the known standards and the deviation of those parameters observed for the Canfas-standard substances. The spectra of the Canfas-substances were found to be identical to the reference standard substances within the tolerances set for standard comparability for absorbance maxima and absorbance[1] .

^{1}H-NMR

^{1}H-NMR sample preparation: Typically, an exact amount of TMSP (trimethylsilylpropionic-2,2',3,3'-d4 acid, sodium salt; certificate present) is dissolved in DMSO-d6 (99.8%) corresponding to a concentration of ca. 5 mM. Part of this solution is stored for a control measurement and part is used to dissolve an exact amount of carbadox/olaquindox (ca. 5 mM).

^{1}H-NMR experiments: ^{1}H-NMR experiments were performed on a Bruker AMX 400 WB spectrometer. A 90 degree pulse was used; the total relaxation delay was set to 62.7 seconds; spectral width was 12195 Hz; number of scans was 64. The data were acquired in 64K data points. Before Fourier transformation a zero-filling to 256 K was applied. Calibration of spectra was achieved by setting the methyl resonance of TMSP to 0.00 ppm. A number of checks on the equipment were performed on a weekly basis, such as temperature calibration and stability checks as well as line width checks as described elsewhere.[2,3]

^{1}H-NMR structural conformation: The resonances of the samples, which were to be examined, were compared to those of known commercial origin. Multiplet structures, integrals and resonance positions were fully compatible. Assignments of resonances were done on the basis of expert knowledge. Thus sample identity could be confirmed.

^{1}H-NMR quantification: Integrals of non-overlapping resonances of non-exchangeable protons were determined and calibrated with regard to the internal standard (TMSP). Knowing the exact amount of the sample of interest and the internal standard (100% pure) the concentration of the sample of interest can be calculate relative to the internal standard from the integrals.

Results (see also Figure 1 to 3).

1. Both carbadox and olaquindox were confirmed with respect to identity.

2. The carbadox content was determined in duplo giving a purity of resp. 95.5% and 94.5% on a w/w basis
3. The olaquindox content was determined in duplo giving a purity of resp. 93.3% and 96.3% on a w/w basis
4. In both samples traces of impurities in the procent range could be detected in the ^{1}H-NMR spectrum.

Mass spectrometry

MS sample preparation: The Canfas-substances of olaquindox and carbadox each were dissolved in a mixture of acetonitril and methanol (50/50, v/v). The stock solution was diluted to obtain for each substance a solution containing 10 µg/ml of the analyte in a mixture of acetonitril / methanol / 1 mM ammonium acetate (25/25/50, v/v). The same solutions were made from reference standards of olaquindox and carbadox.
MS experiments: The mass spectrometer was calibrated according to the manufacturers instructions prior to use.
Using a syringe pump at a flow rate of 5 µl/min, the 10 µg/ml solutions were subsequently infused continuously, into an LCQ ion-trap mass spectrometer equipped with an ESI interface. The ESI interface was operated in positive ion mode at standard settings with regard to capillary temperature, sheath gas and auxiliary gas flows. Positive ion mass spectra were recorded in MS^1.mode as well as in MS^n mode (n ranging from 2 to 4) using the protonated molecule and adduct ions and fragment ions present in the MS^1 spectrum as the primary precursor ions in the MS^n experiments. Several MS^n product ions were used in further MS^n experiments (n>2) as precursors for further fragmentation.
Results: Figure 4 gives a schematic representation of the ions formed by carbadox in the MS^n experiment. The molecular mass of carbadox was confirmed and the same fragmentations were observed, using identical experimental conditions, in the Canfas-substance and the reference standard.
Figure 5 gives a schematic representation of the ions formed by olaquindox in the MS^n experiment. The molecular mass of olaquindox was confirmed and the same fragmentations were observed using identical experimental conditions, in the Canfas-substance and the reference standard.

Conclusions

Carbadox

The identity of the Canfas standard substance Carbadox could be confirmed by UV, ^{1}H-NMR as well as mass spectrometry.
Its purity was determined in duplicate by ^{1}H-NMR to be on average 95.0 %. This is slightly lower than the purity declared by the manufacturer (99.3%). Trace level (percentage range) amounts of unknown impurities were present in the NMR spectra. By UV spectroscopy the purity of the Canfas standard substance was shown to be of similar purity as the reference standard to within 5% which is in agreement with the results from ^{1}H-NMR.

Olaquindox

The identity of the Canfas standard substance Olaquindox could be confirmed by UV, ^{1}H-NMR as well as mass spectrometry.
Its purity was determined in duplicate by ^{1}H-NMR to be on average 94.8%. This is slightly lower than the purity declared by the manufacturer (99.5%). Trace level (percentage range)

amounts of unknown impurities were present in the NMR spectra. By UV spectroscopy the purity of the Canfas standard substance was shown to be of similar purity as the reference standard to within 5% which is in agreement with the results from ^{1}H-NMR.

References

1) RIKILT standard operating procedure A0628, Veterinary drugs - preparation and quality control of standard substances.
2) Lommen, J.M. Weseman, G.O.Smith and H.P.J.M. Noteborn (1998), Special issue "NMR in Environmental Sciences". *Biodegradation*, **9**, 513-525.
3) H.P.J.M. Noteborn, J.M. Weseman, R. van de Jagt and A. Lommen (2000), Special issue "NMR in Biotechnology", *Journal of Biotechnology*, **77**, 103-114.

FIGURE 1

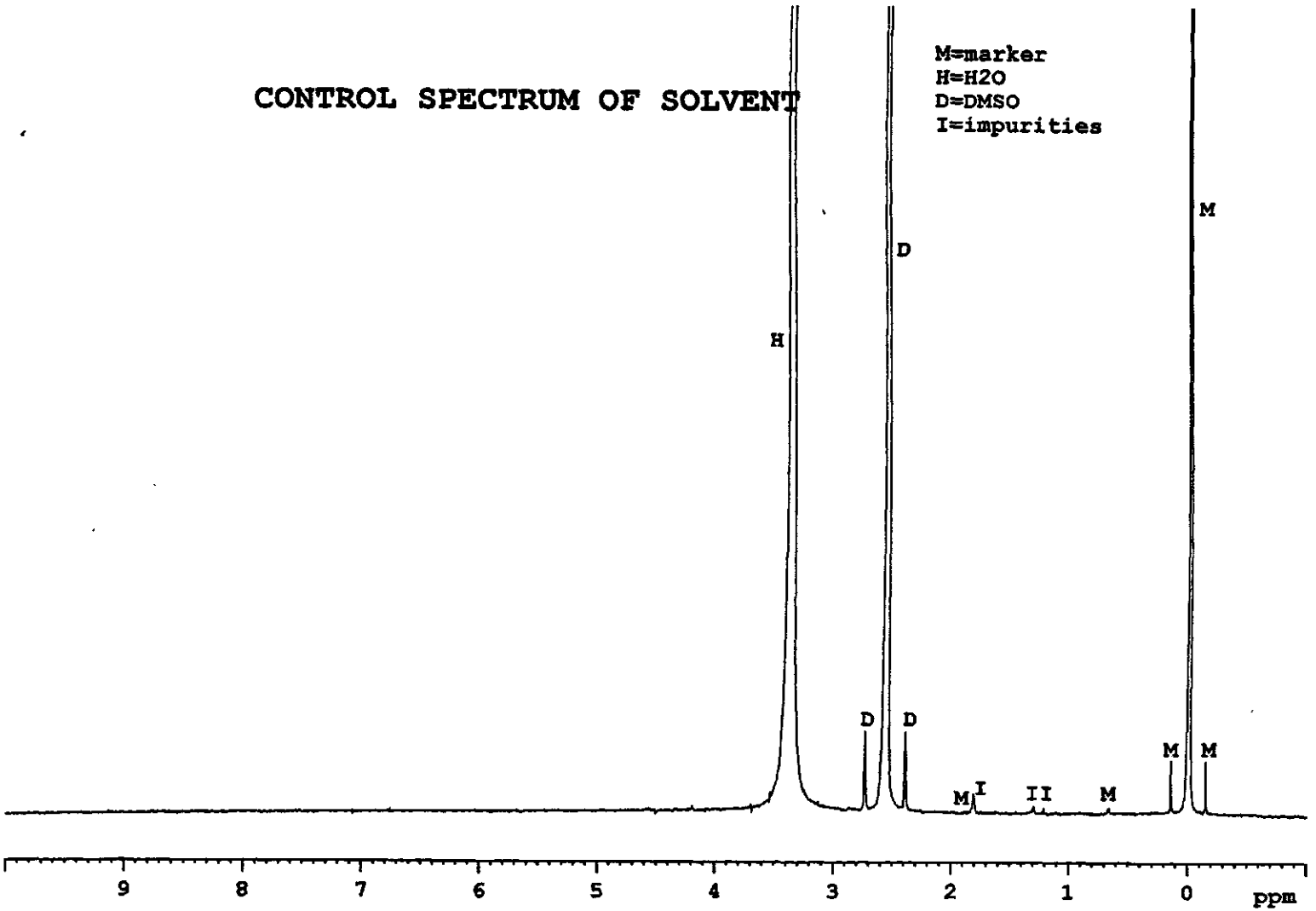

CONTROL SPECTRUM OF SOLVENT
M=marker
H=H2O
D=DMSO
I=impurities
H
D
D
D
M
M
M
M
I
II
M
9
8
7
6
5
4
3
2
1
0
ppm

FIGURE 2

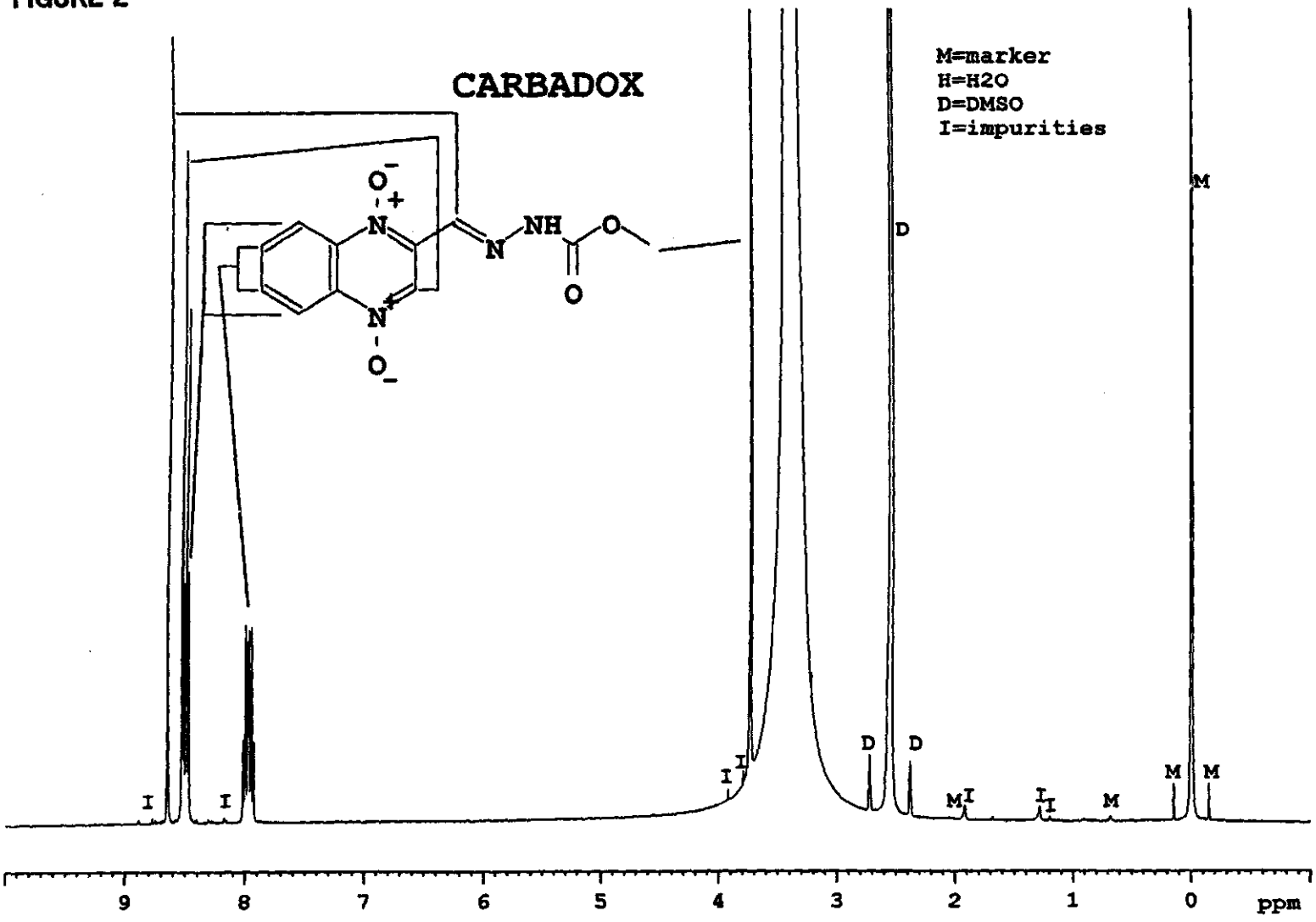
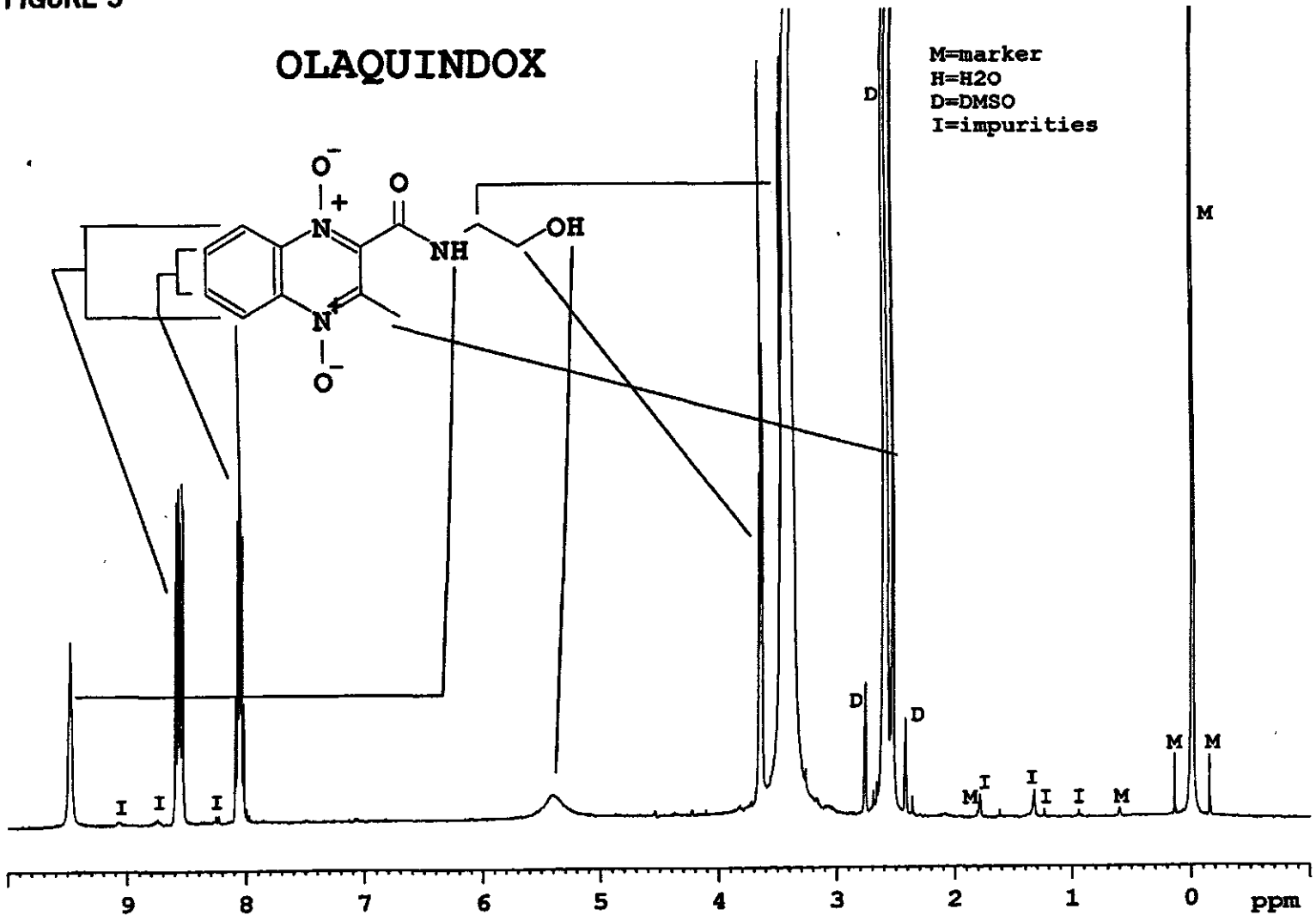

CARBADOX
M=marker
H=H2O
D=DMSO
I=impurities
O
N
NH
O
O
N
O
I
I
I
I
D
D
D
M
I
I
I
M
M
M
9
8
7
6
5
4
3
2
1
0
ppm

FIGURE 3


OLAQUINDOX
M=marker
H=H2O
D=DMSO
I=impurities
O⁻
N⁺
O
NH
OH
N⁺
O⁻
D
D
D
M
M
M
I
I
I
I
I
I
M
M
9
8
7
6
5
4
3
2
1
0
ppm

Carbadox

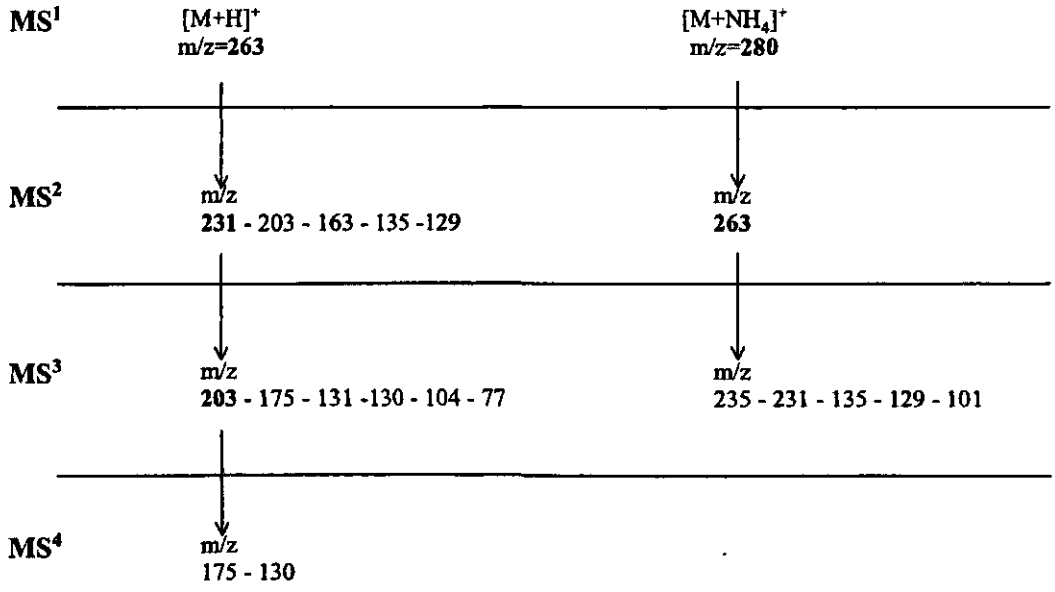


Figure 4 Schematic representation of the fragmentations observed for Carbadox in an MS^n experiment.

Olaquindox

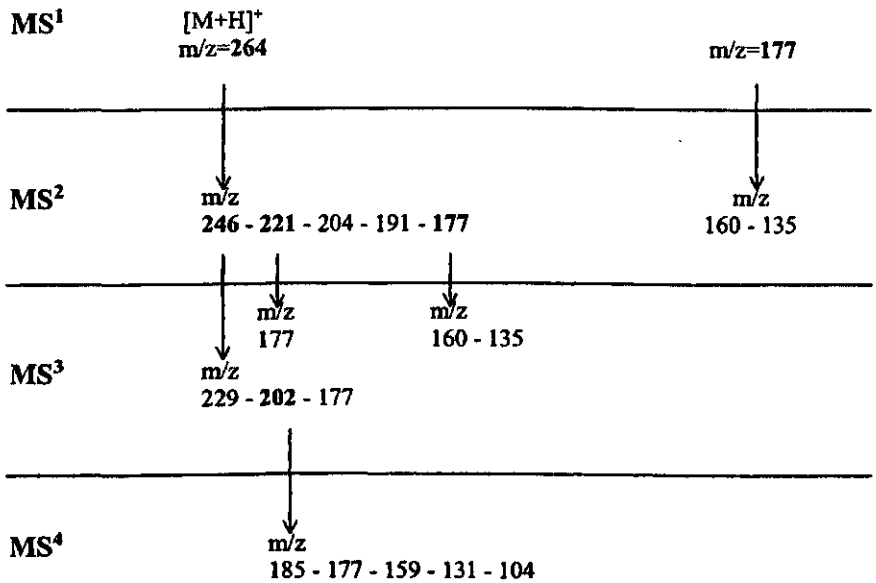


Figure 5 Schematic representation of the fragmentations observed for Olaquindox in an MS^n experiment.

APPENDIX 6

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**
Lab-name:
Contact person: e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
125748	N.S.D.	N.S.D.
125760	1,97	2,01
125785	1,54	1,51
125795	4,84	4,78
125818	N.S.D.	N.S.D.
125820	5,84	5,85

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: **22 Noviembre 2000**

Chromatographic conditions:

- Column:
  - ☒ As described in the method.
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method.
  - ☐ Other: .....
- Flow-rate: **1,2 ml/min**
- Injection volume: **50 µl**
- Retencion time of olaquindox: **≈ 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: **78%**
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: .....% and .....%
- Spiking level: **3 mg/kg**

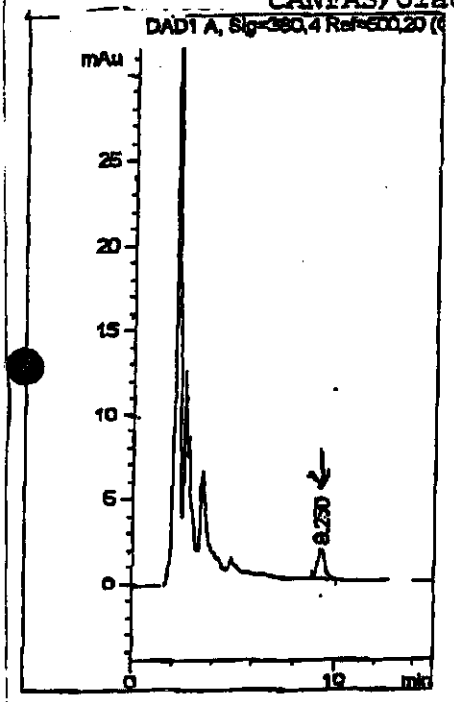
Injection Date : 22/11/00 13:10:36
Sample Name :
Acq. Operator :

Vial : 1

Inj Volume : 50 µl

Acq. Method : C:\HPCHEM\1\ \OLAQCOL.M
Last changed : 22/11/00 13:08:59 by
Analysis Method : C:\HPCHEM\1\ \OLAQCOL.M
Last changed : 22/11/00 19:51:44 by
(modified after loading)

CANFAS/Olaquindox



OLAQ 125785

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,4 Ref=500,20

Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	9.250	BB	0.3105	43.85185	1.75619	100.0000
Totals :				43.85185	1.75619	

*** End of Report ***

(12)

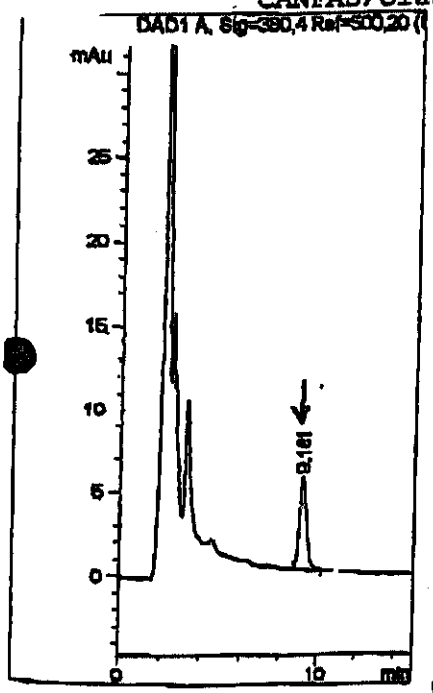
Injection Date : 22/11/00 14:01:28
Sample Name :
Acq. Operator

Vial : 1

Inj Volume : 50 µl

Acq. Method : C:\HPCHEM\1\ _\OLAQCOL.M
Last changed : 22/11/00 13:08:59 by
Analysis Method : C:\HPCHEM\1\ \OLAQCOL.M
Last changed : 22/11/00 19:51:44 by
(modified after loading)

CANFAS/Olaquindox



OLAQ 125745

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=380,4 Ref=500,20
Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	9.181	BV	0.3120	139.76018	5.39218	100.0000
Totals :				139.76018	5.39218	

*** End of Report ***

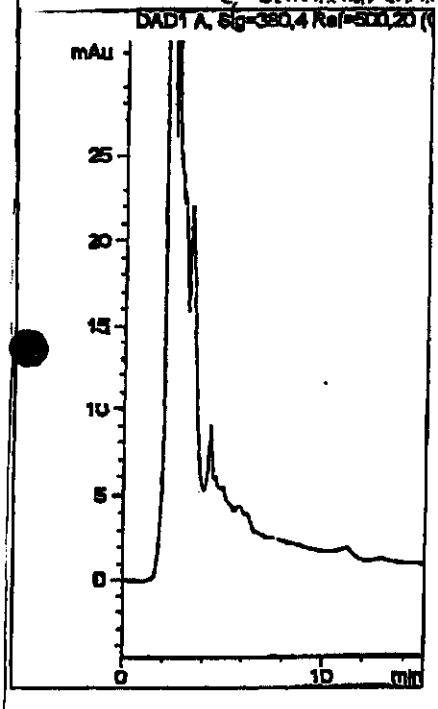
Injection Date : 22/11/00 14:27:06
Sample Name :
Acq. Operator :

Vial : 1

Inj Volume : 50 µl

Acq. Method : C:\HPCHEM\1\ \OLAQCOL.M
Last changed : 22/11/00 13:08:59 by
Analysis Method : C:\HPCHEM\1\ \OLAQCOL.M
Last changed : 22/11/00 19:51:44 by
(modified after loading)

CANFAS/Olaquindox



OLAQ. 125748

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

No peaks found

*** End of Report ***

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 15

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
155711	blank	blank
155735	1,25	1,02
155741	1,12	1,34
155761	blank	blank
155776	4,60	3,52
155784	4,14	4,76

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 03 November 2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: HYPERSIL ODS 5 µm 200 x 4.6 mm + GUARD COLUMN
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.5 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: 5.1 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: 78 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 3.0 mg/kg

```
Injection Date   : 11/3/00 4:22:22 PM          Seq. Line :   6
Sample Name      : 155711                         Vial :   6
Acq. Operator    : adl                             Inj :   1
                                            Inj Volume : 20 µl
Sequence File    : C:\HPCHEM\1\SEQUENCE\MOLAQVAL.S
Acq. Method      : C:\HPCHEM\1\METHODS\MOLAQVAL.M
Last changed     : 11/3/00 2:34:40 PM by adl
Analysis Method  : C:\HPCHEM\1\METHODS\MOLAQVAL.M
Last changed     : 11/6/00 2:27:24 PM by
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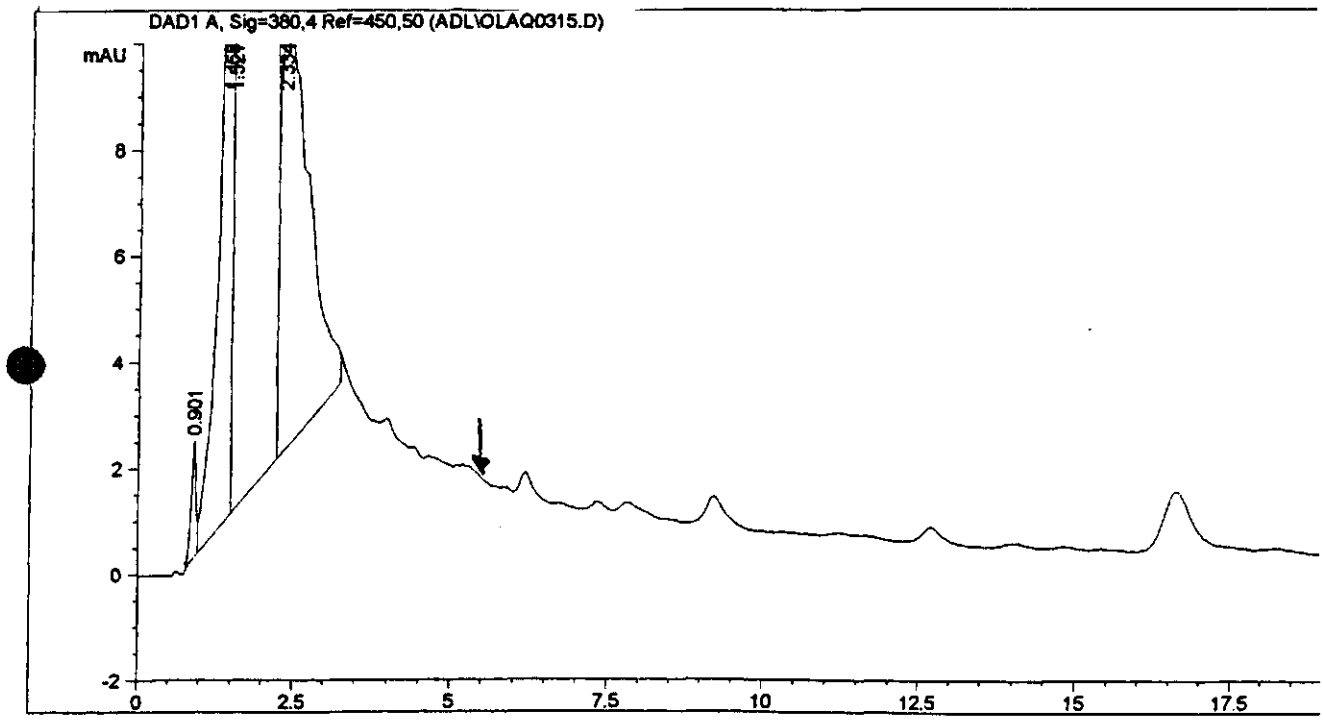


External Standard Report

```
Sorted By             :      Signal
Calib. Data Modified  :      Monday, November 06, 2000 2:14:45 PM
Multiplier            :      1.0000
Dilution              :      1.0000

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

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

```
Totals :                                  0.00000

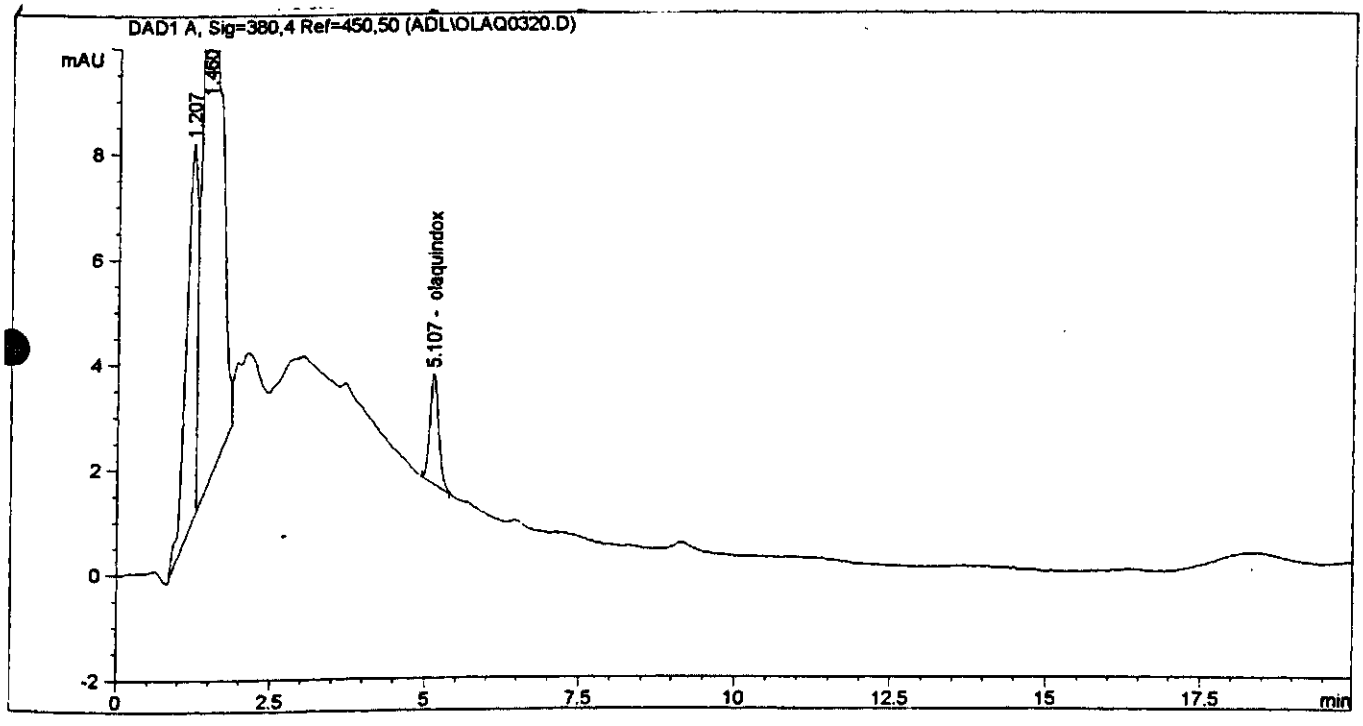
 Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found
```

(15)

```
==========================================================================
Injection Date  : 11/3/00 6:08:37 PM          Seq. Line :  11
Sample Name     : 155741                          Vial :  11
Acq. Operator   : adl                              Inj :   1
                                            Inj Volume : 20 µl

Sequence File   : C:\HPCHEM\1\SEQUENCE\MOLAQVAL.S
Acq. Method     : C:\HPCHEM\1\METHODS\MOLAQVAL.M
Last changed    : 11/3/00 2:34:40 PM by adl
Analysis Method : C:\HPCHEM\1\METHODS\MOLAQVAL.M
Last changed    : 11/6/00 2:27:24 PM by
```




```
==========================================================================
                      External Standard Report
==========================================================================

Sorted By             :      Signal
Calib. Data Modified  :      Monday, November 06, 2000 2:14:45 PM
Multiplier            :      1.0000
Dilution              :      1.0000

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

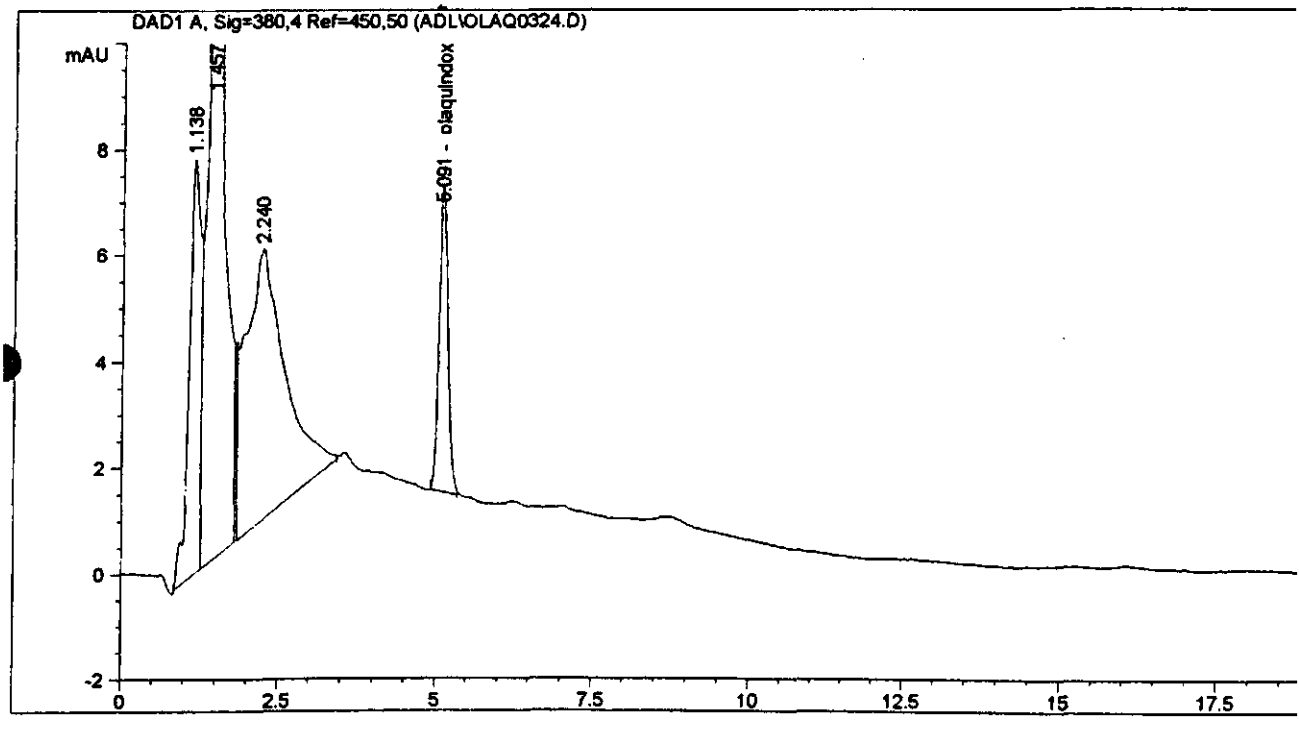
 RetTime  Type      Area      Amt/Area    Amount    Grp   Name
  [min]           [mAU*s]                [ng/ul]
-------|------|----------|----------|----------|--|-----------------
   5.107 PB       20.12256 3.34201e-2 6.72497e-1     olaquindox

Totals :                                6.72497e-1 * 2 = 1.34 mg/kg

 Results obtained with enhanced integrator!
==========================================================================
                        *** End of Report ***
```

```
Injection Date   : 11/3/00 7:33:32 PM          Seq. Line :  15
Sample Name      : 155776                        Vial :  15
Acq. Operator    : adl                           Inj :   1
                                              Inj Volume : 20 µl

Sequence File    : C:\HPCHEM\1\SEQUENCE\MOLAQVAL.S
Acq. Method      : C:\HPCHEM\1\METHODS\MOLAQVAL.M
Last changed     : 11/3/00 2:34:40 PM by adl
Analysis Method  : C:\HPCHEM\1\METHODS\MOLAQVAL.M
Last changed     : 11/6/00 2:27:24 PM by
```



External Standard Report

```
Sorted By             :      Signal
Calib. Data Modified  :      Monday, November 06, 2000 2:14:45 PM
Multiplier            :      1.0000
Dilution              :      1.0000

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

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
5.091	BB	55.72574	3.16900e-2	1.76595		olaquindox

Totals : 1.76595 × 2 = 3.52 ug/kg

Results obtained with enhanced integrator!

*** End of Report ***

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 16

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
Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis:

Analyte: OLAQUINDOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
165708	5,66	4,98
165719	1,56	1,7
165751	not found	not found
165774	1,66	1,65
165802	not found	not found
165816	5,65	5,54

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 2000-10-23, 2000-11-14, 2000-11-15 (each assay one day)

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - x Other: Spherisorb ODS 2, 10 µm, 250 x 4.6 mm
- Mobile phase:
  - x As described in the method
  - ☐ Other:

  .....
- Flow-rate: 1.7 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: 9.5 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

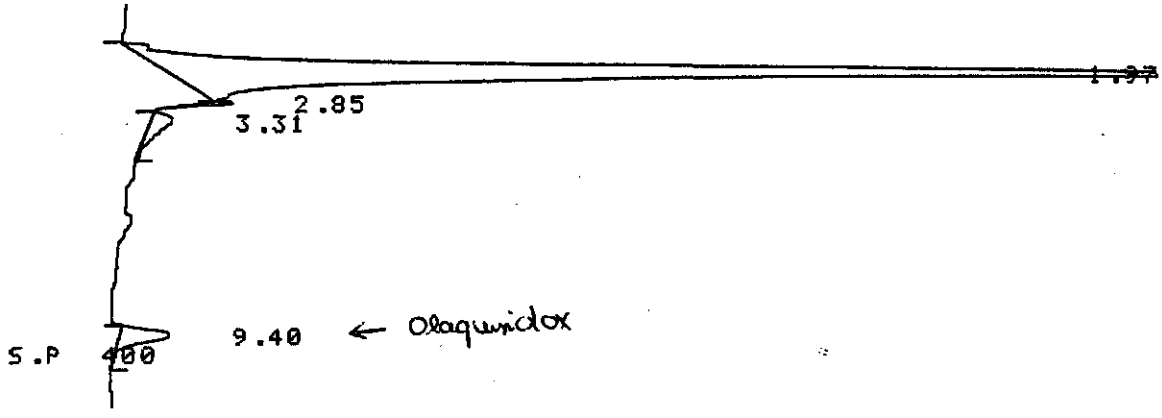
Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 76.5 %
- Single / duplicate determinations: ☐ single x duplicate
- If duplicate, please give both percentages: 76.9 and 76.0 %
- Spiking level: 3.0 mg/kg

Olaquindox

CH. 1 C.S 5.00 ATT 2 OFFS 10 11/15/00 14:06 Std 1



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

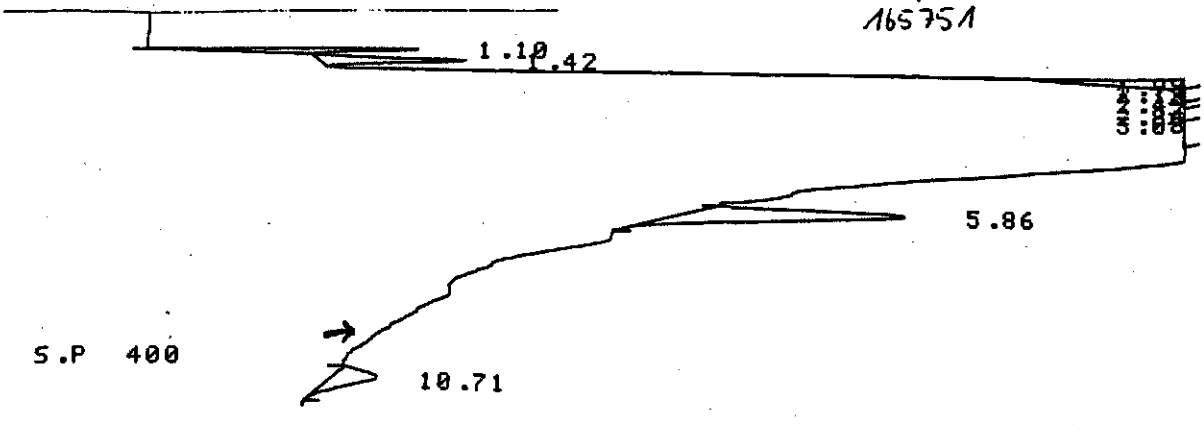
D-2500 11/15/00 14:06

METHOD: TAG: 495 CH: 1

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME
4	9.40	4351	172		OLA

CH. 1 C.S 5.00 ATT 2 OFFS 10 11/15/00 14:19 sample code 165751



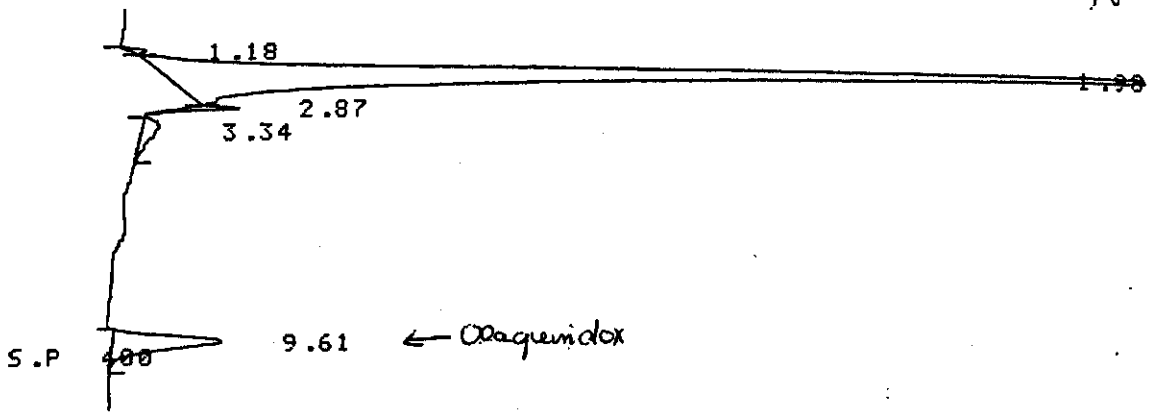
D-2500 11/15/00 14:19

METHOD: TAG: 496 CH: 1

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

Olaquindox (16)

CH. 1 C.S 5.00 ATT 2 OFFS 10 11/15/00 12:14 Standard 1.0116 µg/ml



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

0-2500 11/15/00 12:14

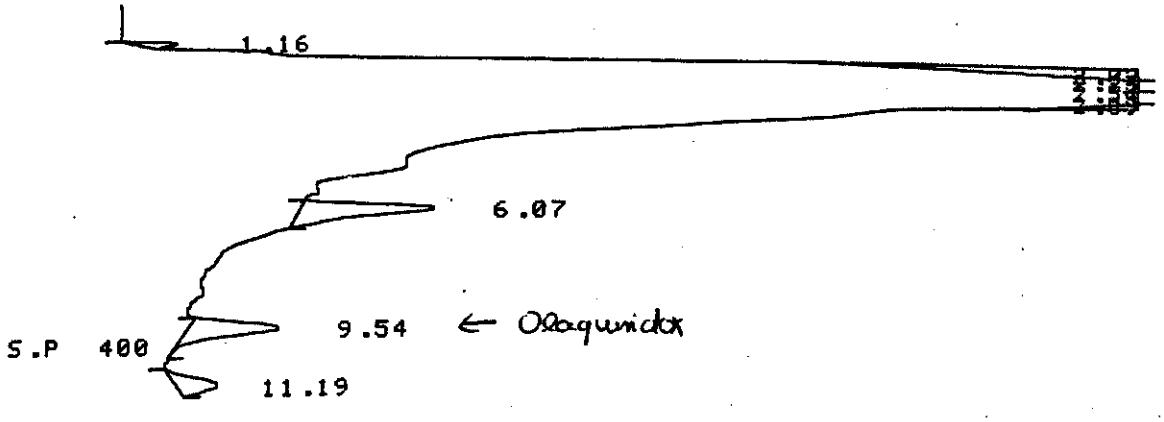
METHOD: TAG: 489 CH: 1

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME
5	9.61	10173	386		OLA

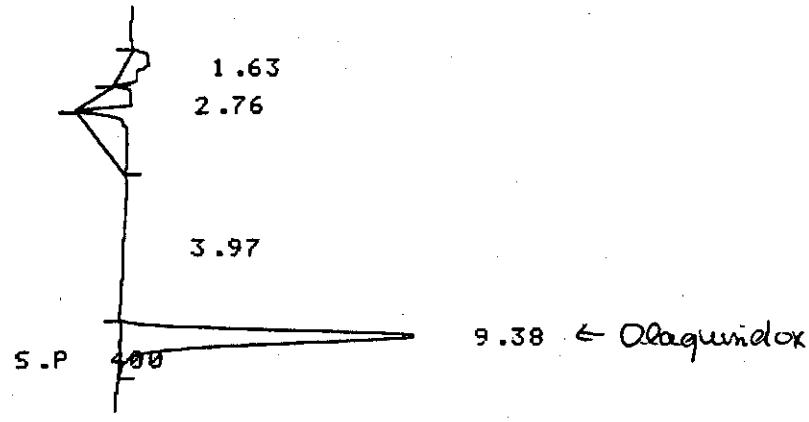
sample code 165719

CH. 1 C.S 5.00 ATT 2 OFFS 10 11/15/00 12:27



Olaquindox

CH. 1 C.S 5.00 ATT 2 OFFS 10 11/15/00 10:23 standard 2,540 µg/ml



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

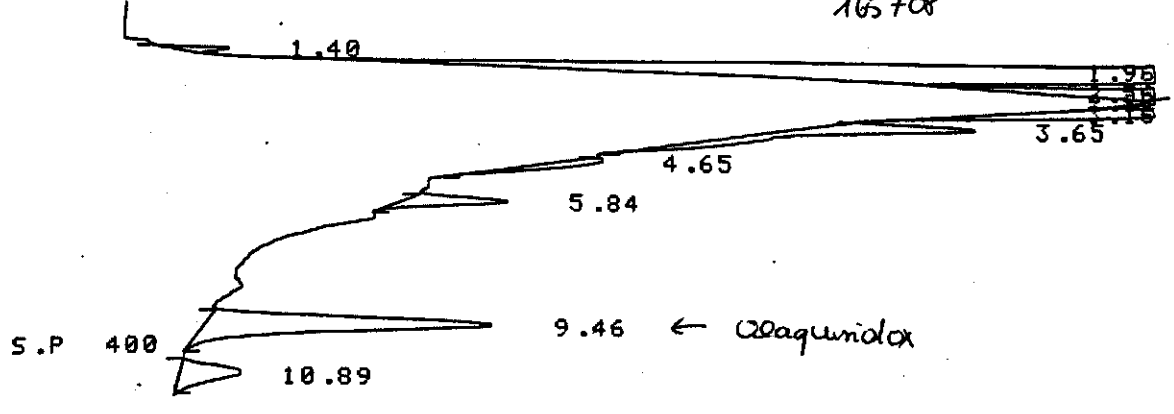
D-2500 11/15/00 10:23

METHOD: TAG: 483 CH: 1

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME
4	9.38	27974	1025	2.540	OLA

CH. 1 C.S 5.00 ATT 2 OFFS 10 11/15/00 10:36 sample code 16570B



D-2500 11/15/00 10:36

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 17

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
175703	0	0
175718	5,60	5,66
175730	2,07	1,99
175775	0	0
175817	1,99	1,92
175828	5,58	5,63

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 11.10.2000

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: 7.12 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: 83.9 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 84.5 % and 83.3 %
- Spiking level: 3.0 mg/kg

The recovery was not respected in calculation the results.

D-7000 HSM: Olaquindox **Series: 0199**

Sample Name: Standard 2,5µg/ml

Analyzed: 11.10.00 13:39

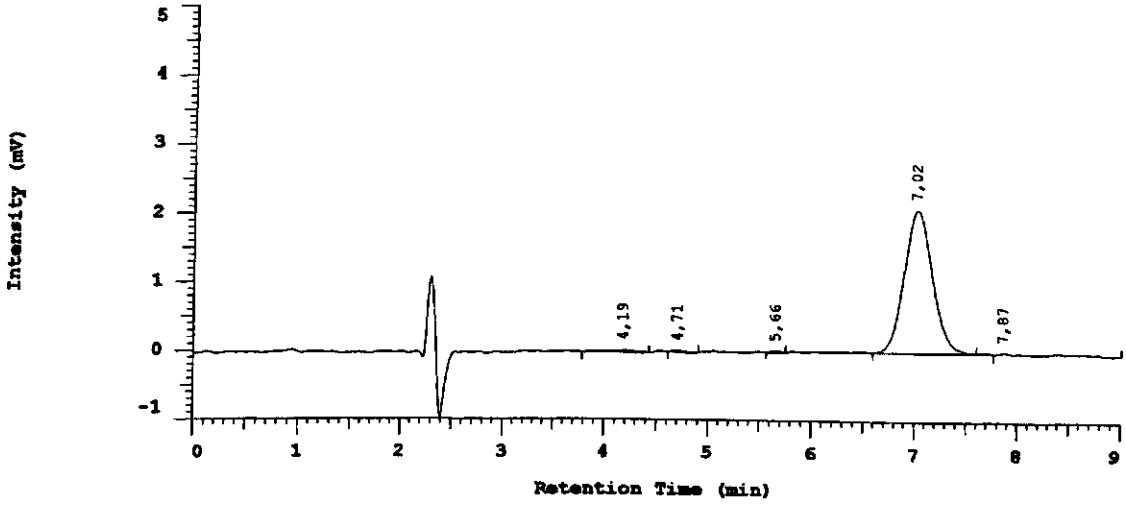
Reported: 13.11.00 10:46
Processed: 13.11.00 10:46

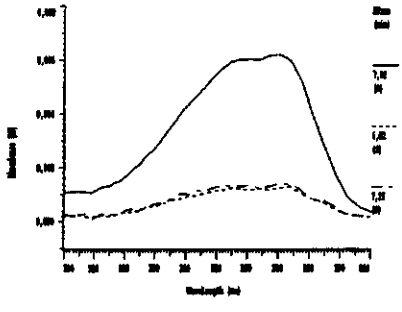
Data Path: C:\Win32App\HSM\OLAQU\DATA\0199\
Application: Olaquindox
Injection from this vial: 1 of 1

Series:0199
Vial Number: 1
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 C: MeOH/H2O
Peak Quantitation: AREA
Calculation Method: EXT-STD

Developed by:

Solvent B: MeOH/H2O
Solvent D: MeOH/H2O
Sample Amount: 1,000
Scale Factor 1: 1,000

Name	RT	Area	Conc 1	BC
	4,19	558	0,000	BB
	4,71	141	0,000	BB
	5,66	124	0,000	BB
Olaqu	7,02	39485	2,607	MC
	7,87	0	0,000	
		40308	2,607	

D-7000 HSM: Olaquindox **Series: 0200**

Sample Name: 175775

Analyzed: 11.10.00 17:40

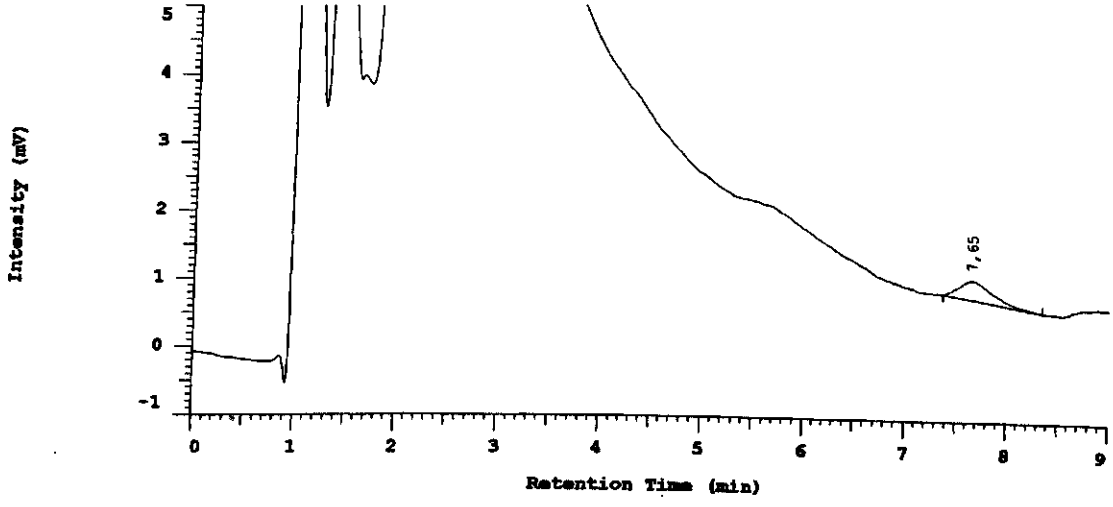
Reported: 13.11.00 10:35
Processed: 13.11.00 10:34

Data Path: C:\Win32App\HSM\OLAQU\DATA\0200\
Application: Olaquindox
Injection from this vial: 1 of 1

Series:0200
Vial Number: 9
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 C: MeOH/H2O
Peak Quantitation: AREA
Calculation Method: EXT-STD

Developed by:

Solvent B: MeOH/H2O
Solvent D: MeOH/H2O
Sample Amount: 0,500
Scale Factor 1: 1,000

Name	RT	Area	Conc 1	BC
	7,65	6506	0,000	BB
		6506	0,000	

Peak rejection level: 0

D-7000 HSM: Olaquindox **Series: 0199**

Sample Name: 175828

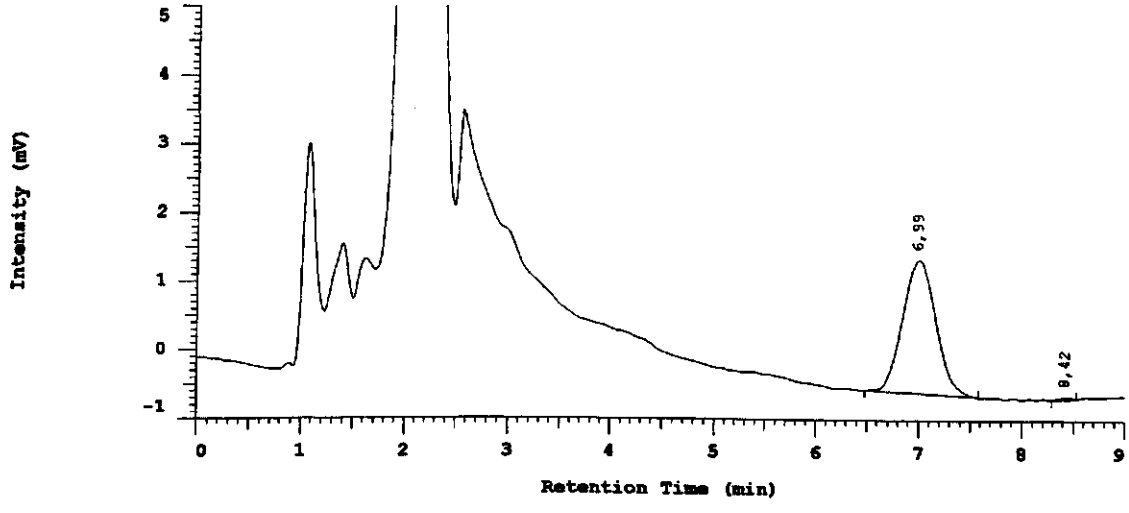
Analyzed: 11.10.00 15:39
Reported: 13.11.00 10:25
Processed: 13.11.00 10:25

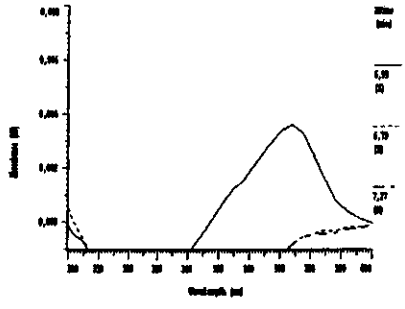
Data Path: C:\Win32App\HSM\OLAQU\DATA\0199\
Application: Olaquindox
Injection from this vial: 1 of 1

Series:0199
Vial Number: 13
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 C: MeOH/H2O
Peak Quantitation: AREA
Calculation Method: EXT-STD

Developed by:

Solvent B: MeOH/H2O
Solvent D: MeOH/H2O
Sample Amount: 0,500
Scale Factor 1: 1,000

Name	RT	Area	Conc 1	BC
Olaqu	6,99	42790	5,634	MC
	8,42	253	0,000	BB
		43043	5,634	

Peak rejection level: 0

D-7000 HSM: Olaquindox **Series: 0200**

Sample Name: 175817

Analyzed: 11.10.00 17:50

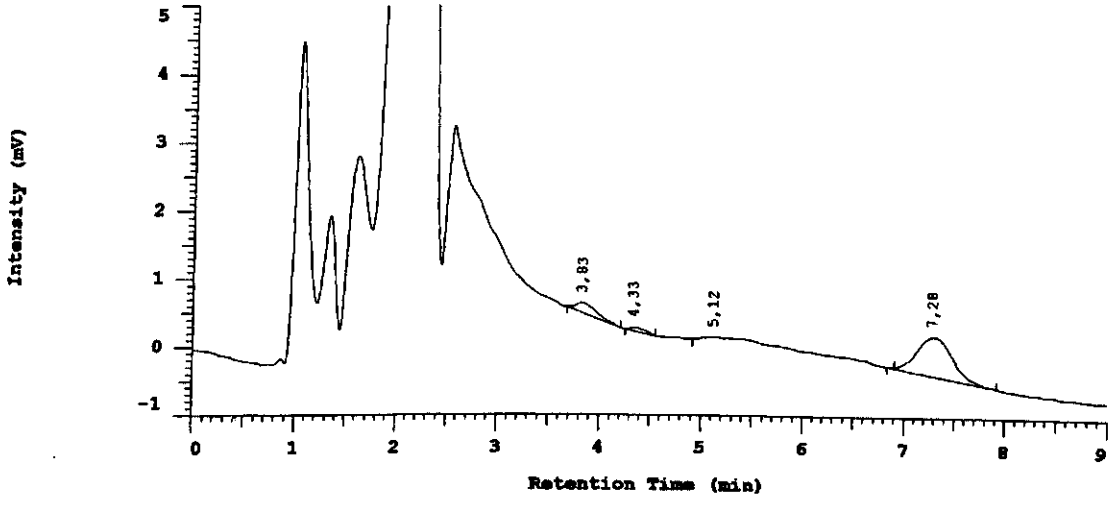
Data Path: C:\Win32App\HSM\OLAQU\DATA\0200\
Application: Olaquindox
Injection from this vial: 1 of 1

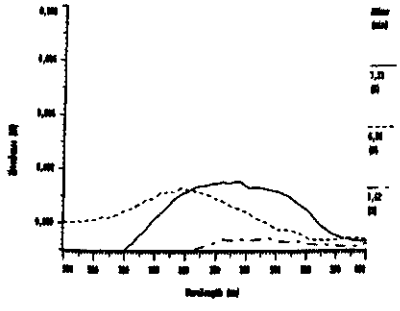
Sample Description:

Reported: 13.11.00 10:37
Processed: 13.11.00 10:37

Series:0200
Vial Number: 10
Volume: 20,0 ul

Chrom Type: Fixed WL Chromatogram, 380 nm





Acquisition Method: Olaquindox
Column Type: RP 18
Developed by:
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: 0,500
Calculation Method: EXT-STD
Scale Factor 1: 1,000

Name	RT	Area	Conc 1	BC
	3,83	2176	0,000	BB
	4,33	663	0,000	BB
	5,12	0	0,000	
Olaqu	7,28	14085	1,990	MC
		16924	1,990	

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 18

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**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
185693	5,78	5,92
185695	6,18	6,12
185731	1,56	1,52
185733	1,67	1,65
185758	Not Detected ; LOD<0,5	Not Detected ; LOD<0,5
185823	Not Detected ; LOD<0,5	Not Detected ; LOD<0,5

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: ..15, 16/11/00 and 23/11/00.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: .....150 x 4,6 mm ; 5 µm ; Spherisorb ODS 2 C18.....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....1,0.... ml/min
- Injection volume: .40-50 µl
- Retention time of olaquindox: .8,4. min

Chromatograms: Please include representative chromatograms of:

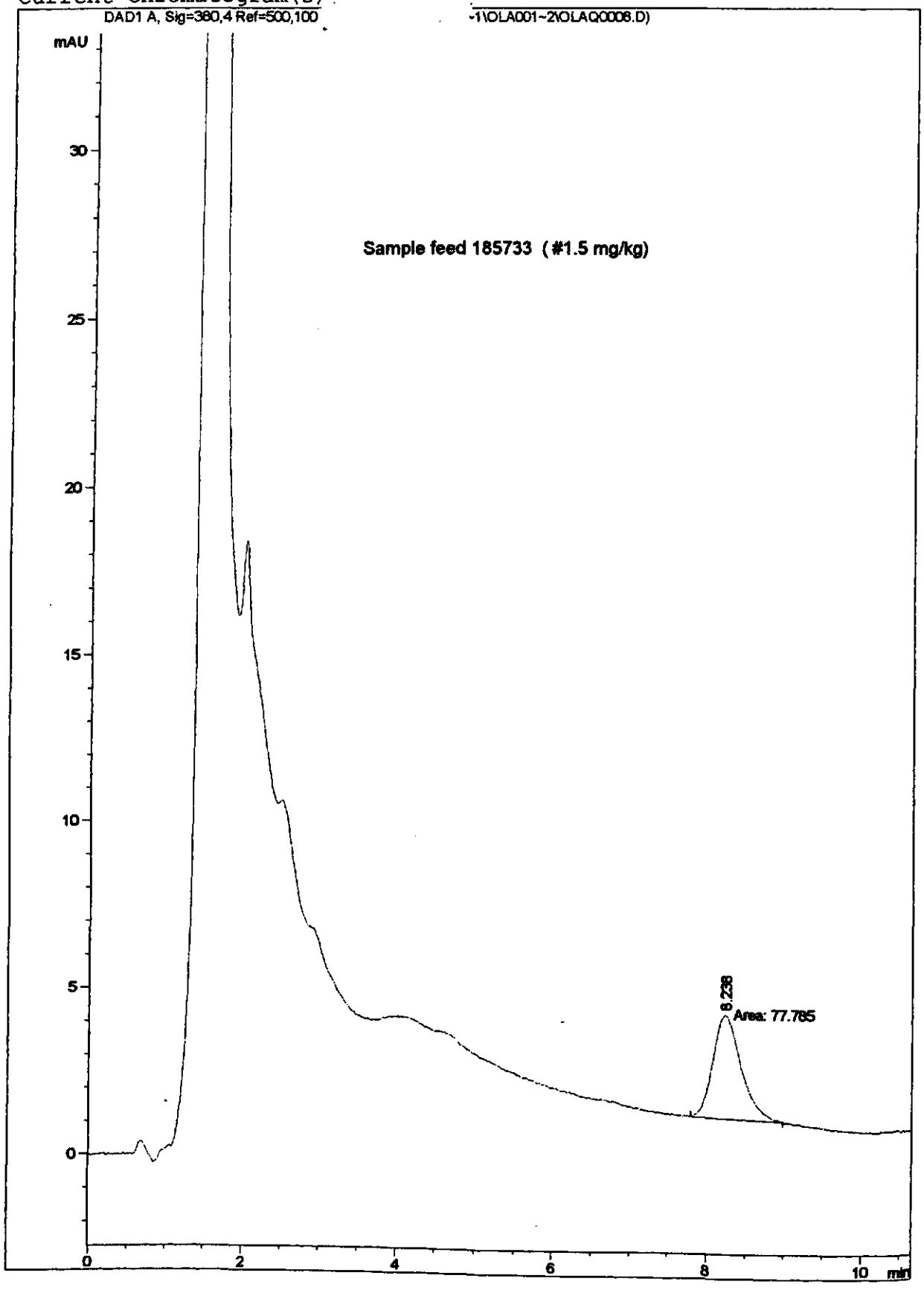
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

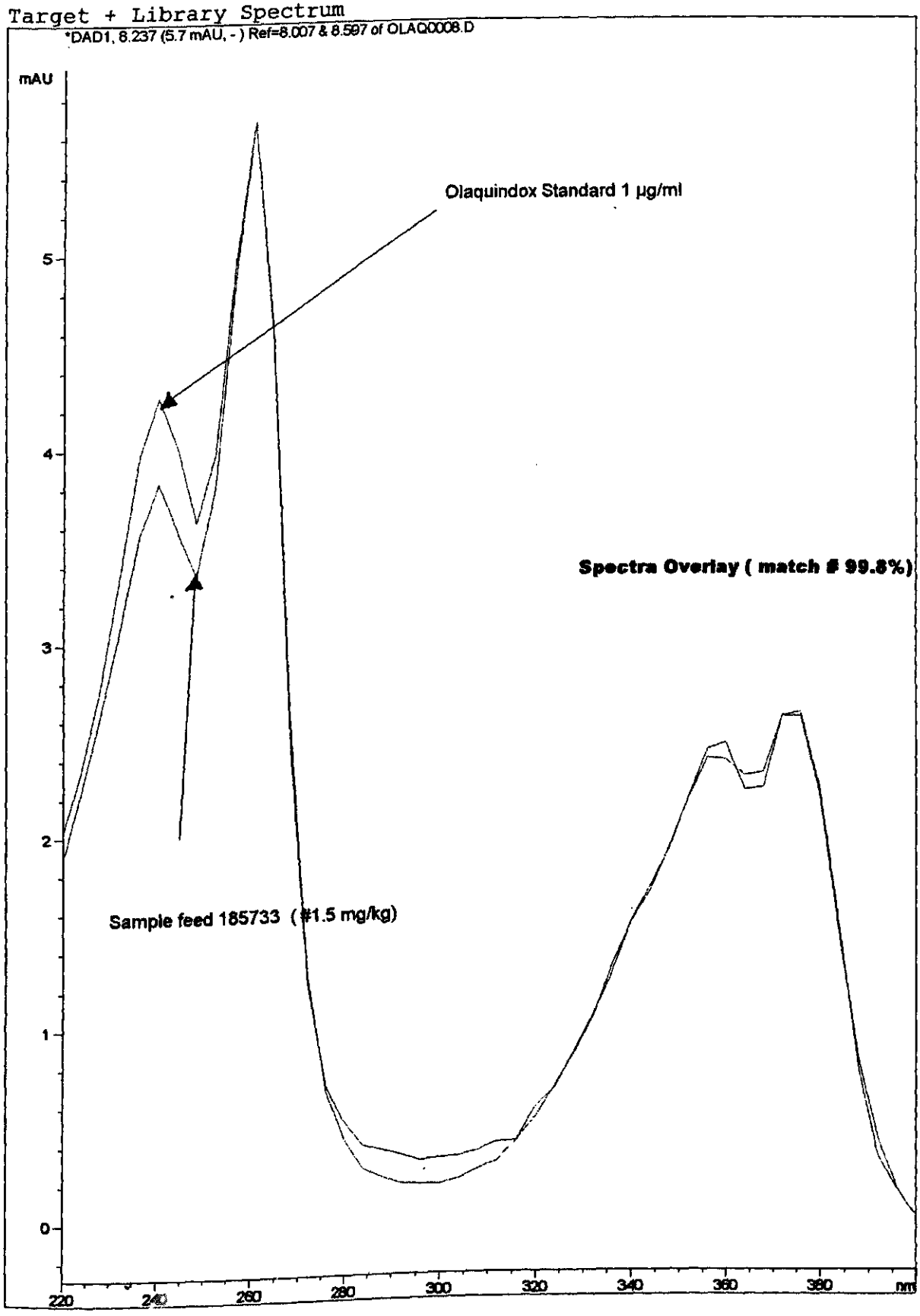
Recovery results:

- Percentage recovery: 77,1 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 78,6 % and 76,1 %
- Spiking level: ...3..... mg/kg

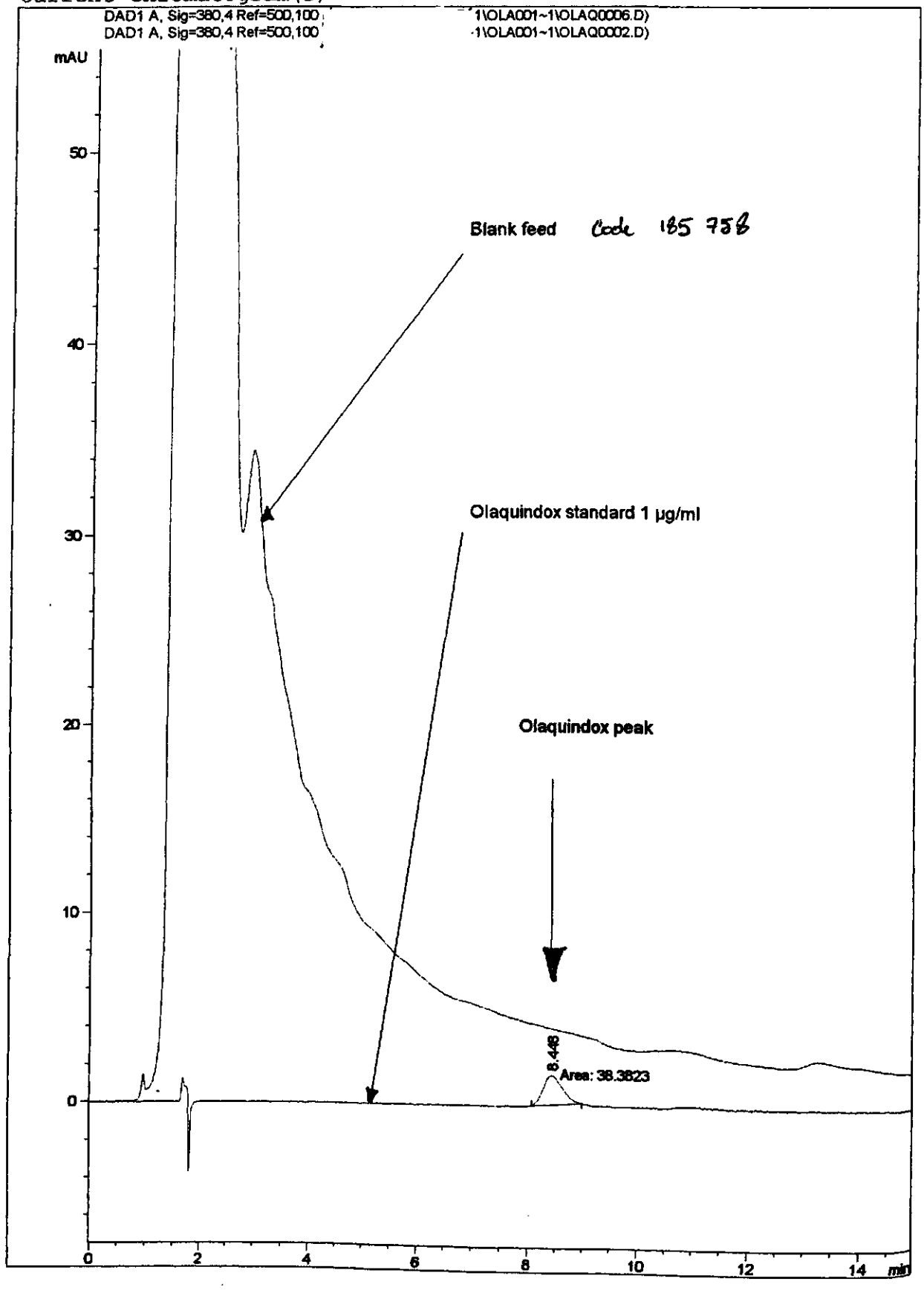
Current Chromatogram(s)



Target + Library Spectrum



Current Chromatogram(s)



APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 20

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**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
205713	neg	neg
205729	1,72	1,6
205739	1,52	1,59
205796	4,85	4,78
205809	neg	neg
205822	4,65	4,78

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 28/11/2000

Chromatographic conditions:

- Column:
  - ☒ As described in the method AlltimA AlltCch C18. 250 x 4,6 mm 5 µm
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ...1.5..... ml/min
- Injection volume: ...50.....µl
- Retention time of olaquindox: 5,4.. 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: 100,7 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 100,8 % and 100,5 %
- Spiking level: 3,05 mg/kg

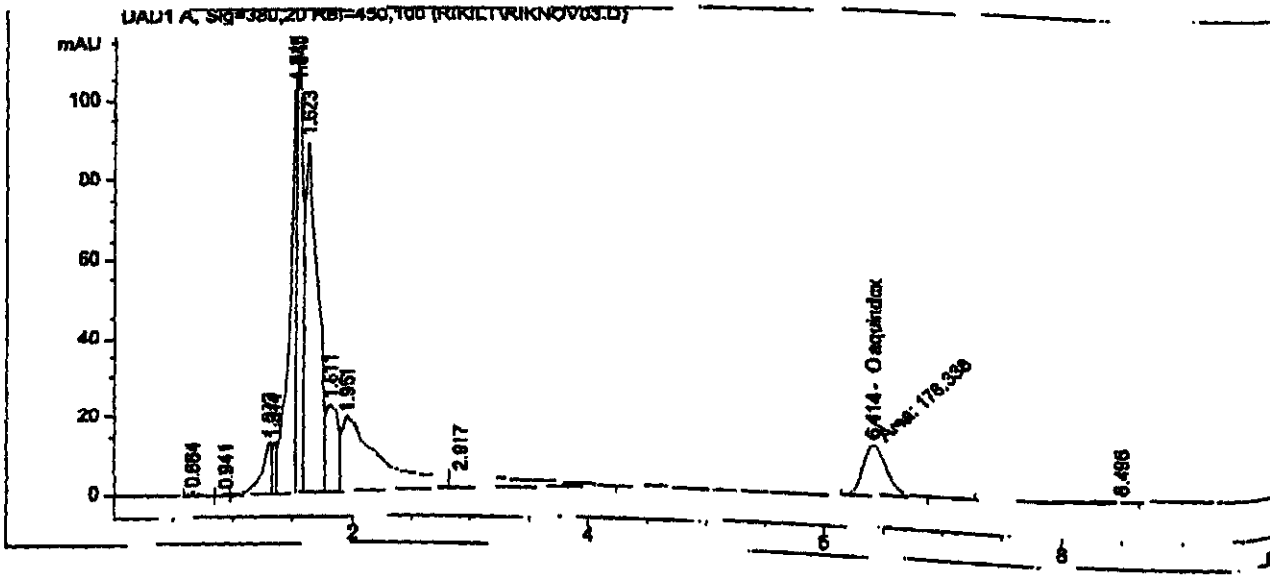
Data File D:\ANAL_7\RIKILT\RIKNOV03.D Sample Name: 205796

Injection Date : 11/28/2000 1:26:31 PM
Sample Name : 205796 a
Acq. Operator : ea

Seq. Line : 3
Vial : 22
Inj : 1
Inj Volume : 50 µl

Acq. Method : C:\HPCHEM\1\METHODS\OLAQ-RK.M
Last changed : 11/28/2000 1:25:55 PM by ea
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\OLAQ-RK.M
Last changed : 11/28/2000 3:11:17 PM by ea
(modified after loading)

Col Alltima 250 mm 3-4-98



External Standard Report

Sorted By : Signal
Calib. Data Modified : Tuesday, November 28, 2000 3:10:06 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=300,20 Ref=450,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ug/ml]	Grp	Name
6.414	MM	178.33818	1.47681e-2	2.63371		Olaquindox

Totals : 2.63371

Results obtained with enhanced integrator!

*** End of Report ***

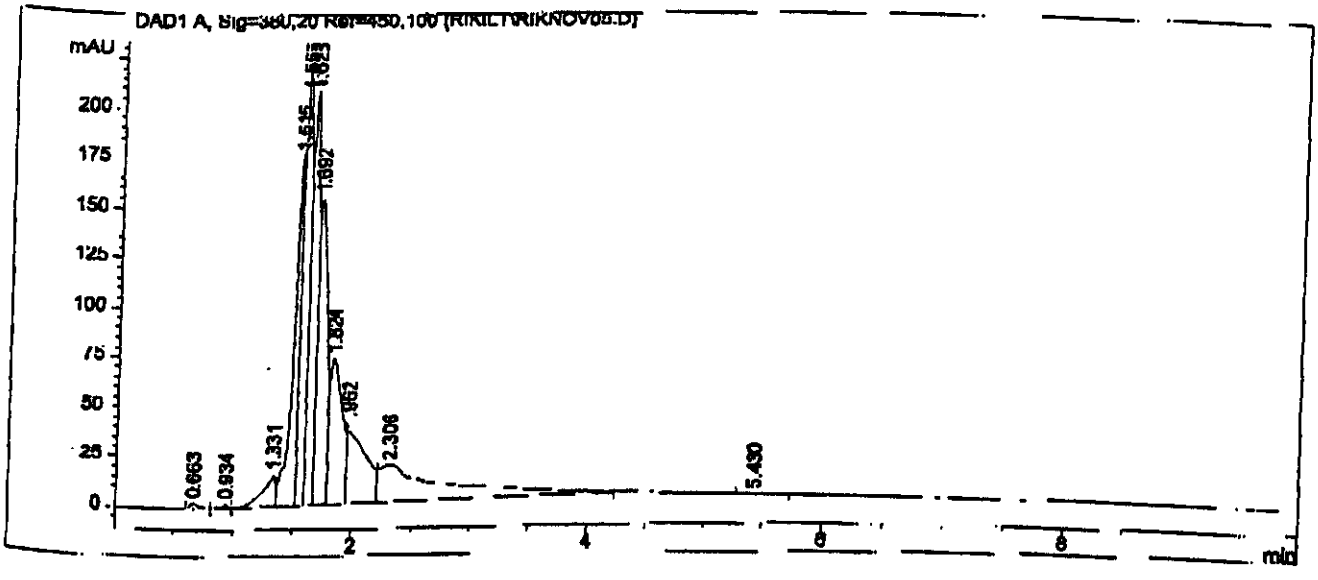
4.81 µg/g

HP 1090 11/28/2000 3:15:03 PM ea

Data File D:\ANAL...\RIKILT\RIKNOV05.D Sample Name: 205713 a

Injection Date	: 11/28/2000 1:51:46 PM	Seq. Line	: 5
Sample Name	: 205713 a	Vial	: 24
Acq. Operator	: ea	Inj	: 1
		Inj Volume	: 50 µl
Acq. Method	: C:\HPCHEM\1\METHODS\OLAQ-RK.M		
Last changed	: 11/28/2000 1:51:09 PM by ea (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\OLAQ-RK.M		
Last changed	: 11/28/2000 3:11:17 PM by ea (modified after loading)		

Col Alltima 250 mm 3-4-98



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Tuesday, November 28, 2000 3:10:06 PM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=380,20 Ref=450,100

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

Totals : 0.00000 Neg

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 21

CANFAS

Development and Validation of HPLC-methods for the official control Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2

Subtitle: **Task 4 COLLABORATIVE STUDY**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
215715	2,0	2,0
215770	0,0 N.D.	0,0 N.D.
215791	0,0 N.D.	0,0 N.D.
215797	5,4	5,3
215813	2,0	2,0
215827	5,3	5,2

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 23/10/2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: SUPELCOSIL LC-18 25cm x 4,6 mm (5 µm) + SUPELGUARD LC-18
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: GRADIENT ELUTION (See TIMETABLE enclosed)
- Flow-rate: 1,2 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: 7,6 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

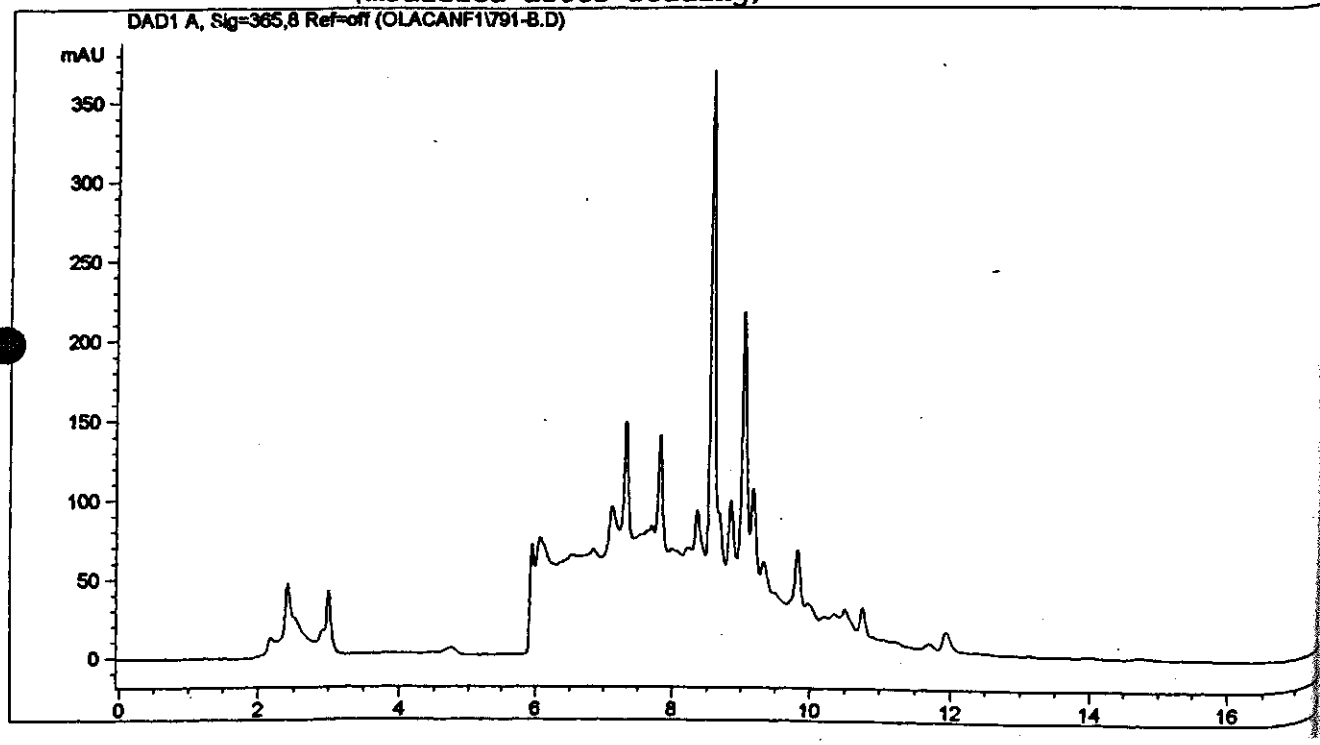
Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 83,5 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 83 % and 84 %
- Spiking level: 3,0 mg/kg

(21)

```
Injection Date    : 23/10/2000 21.30.34          Seq. Line :  16
Sample Name       : 791-B                          Vial :  13
Acq. Operator     :                                 Inj :   1
                                                Inj Volume : 10 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 20 µl
Acq. Method       : C:\HPCHEM\1\
Last changed      : 28/08/2000 16.09.10  by
Analysis Method   : C:\HPCHEM\1\
Last changed      : 04/12/2000 15.32.39
                    (modified after loading)
```



External Standard Report

```
Sorted By             :      Signal
Calib. Data Modified  :      04/12/2000 15.32.39
Multiplier            :      1.0000
Dilution              :      1.0000

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

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

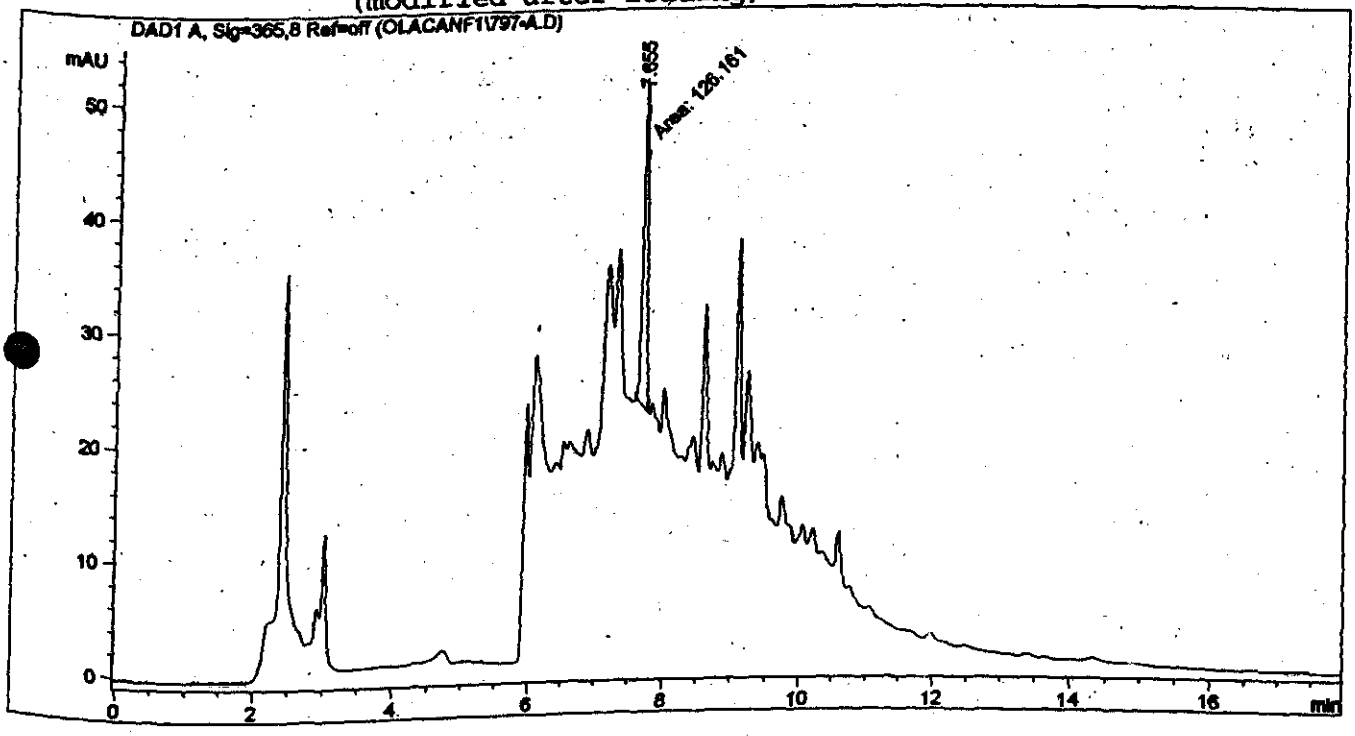
Totals :                                  0.00000

 Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found
```

(21)

Injection Date : 23/10/2000 21.53.52 Seq. Line : 17
Sample Name : 797-A Vial : 14
Acq. Operator : Inj : 1
Inj Volume : 10 µl
Different Inj Volume from Sequence ! Actual Inj Volume : 20 µl
Acq. Method : C:\HPCHEM\1\
Last changed : 28/08/2000 16.09.10 by
Analysis Method : C:\HPCHEM\1\
Last changed : 24/10/2000 14.40.09
(modified after loading)



External Standard Report

Reported By : Signal
Calib. Data Modified : 24/10/2000 13.49.03
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
7.655	MM	126.16067	4.26466e-1	53.80323		OLAQ-olaquindox

Totals : 53.80323

Results obtained with enhanced integrator!

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 23

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**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
235694	4,23	5,77
235699	5,40	5,37
235712	1,67	1,76
235767	< 0,11	< 0,21
235787	1,91	1,86
235793	< 0,11	< 0,21

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 24

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
245723	2,0	2,1
245746	5,7	6,2
245780	blank	blank
245781	blank	blank
245825	1,8	1,7
245829	6,0	5,9

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 26 October 2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: 250 mm × 4.6 mm C18 5 µm
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1,5 ml/min
- Injection volume: 50 µl
- Retention time of olaquindox: 8,2 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:

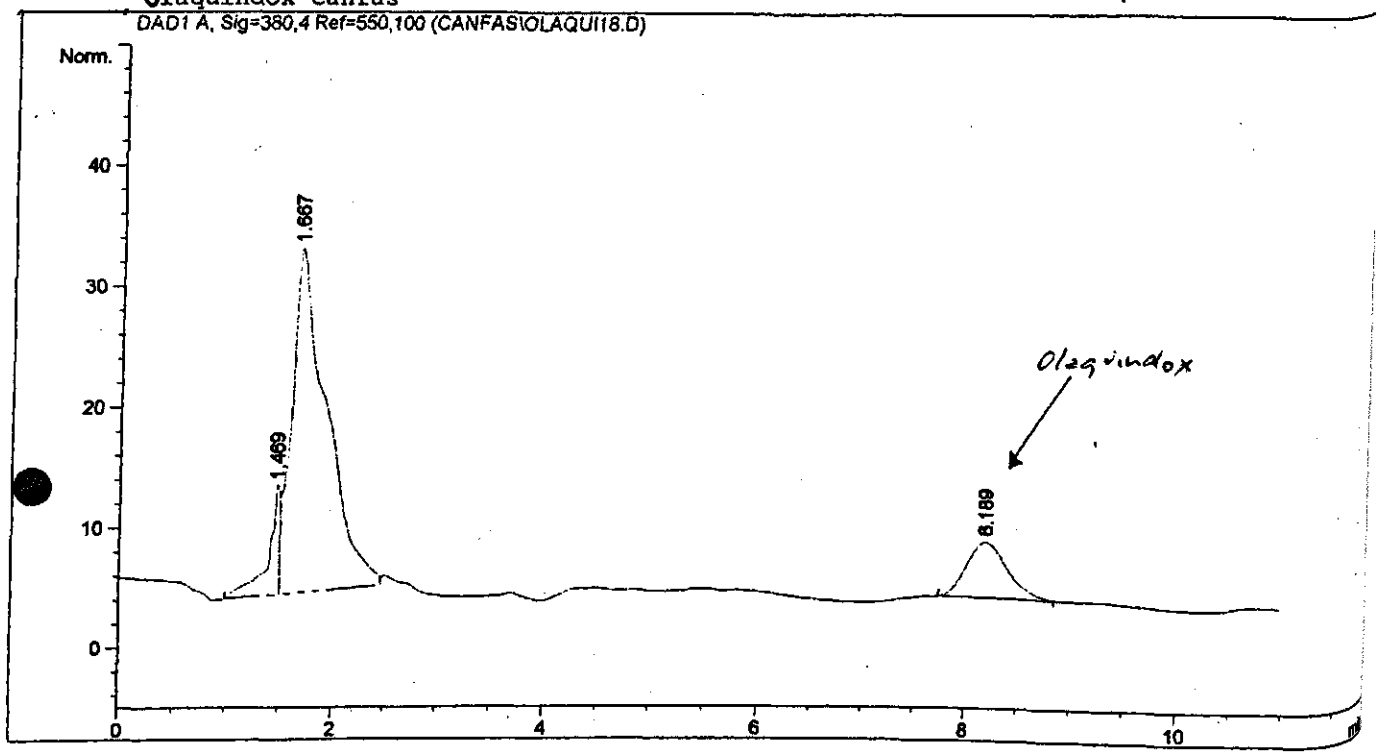
- Percentage recovery: 79 % (average)
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 71 % and 86 %
- Spiking level: 3 mg/kg

Data File C:\HPCHEM\1\DATA\...

29 Sample n° 245+46 (Olaquindox)

Injection Date : 26/10/00 14.12.05
Sample Name : Location : Vial 1
Acq. Operator :
Method : C:\HPCHEM\1\METHODS\IZS_ME~1\OLAQUID.M
Last changed : 26/10/00 13.00.32 by
(modified after loading)

Olaquindox canfas



External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 26/10/00 12.09.03
Multiplier : 1.0000
Dilution : 1.0000

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

RetTime [min]	Sig	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
8.189	1	BB	124.84376	2.27448e-2	2.83955		olaquidox

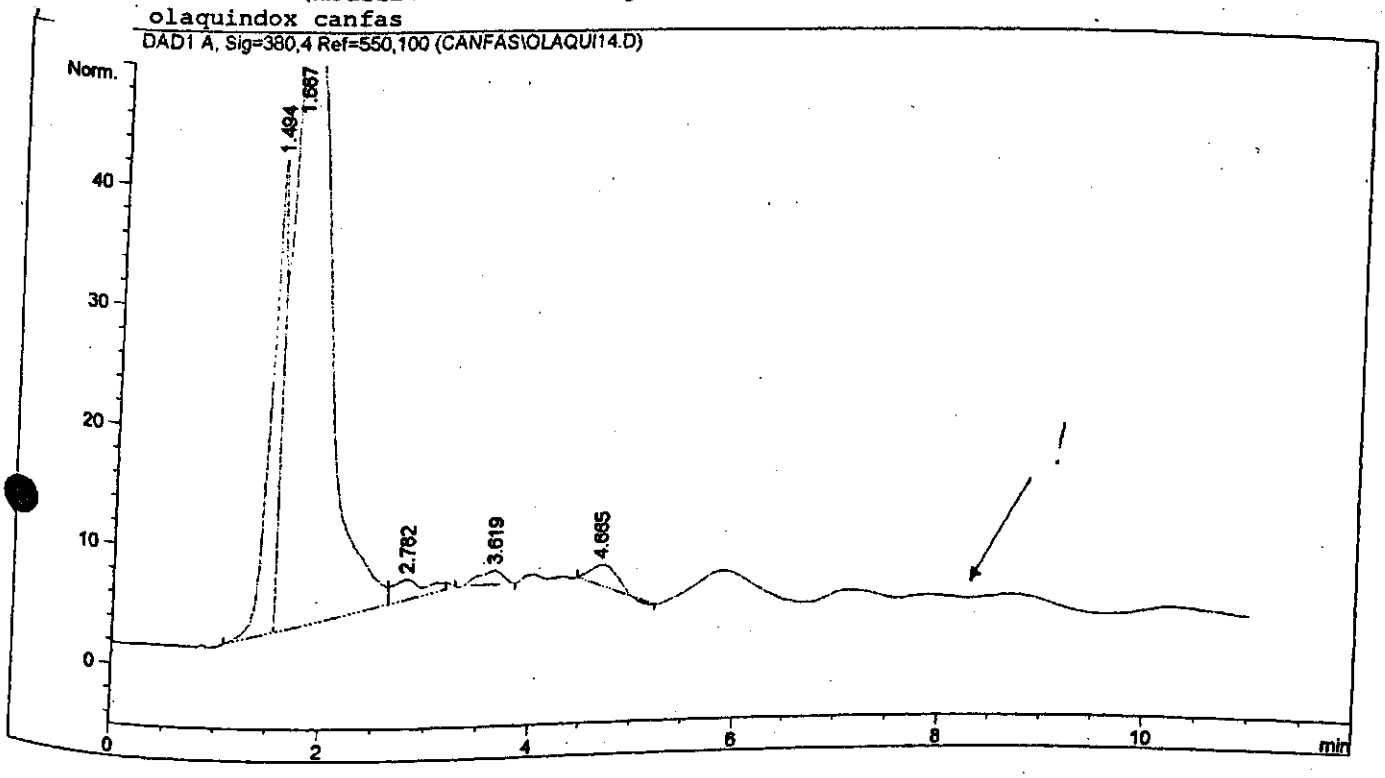
Totals : 2.83955

Results obtained with enhanced integrator!

*** End of Report ***

40 Sample n° 245781 (Olaquindox) 24

Injection Date : 26/10/00 13.17.33
Sample Name :
Location : Vial 1
Acq. Operator : Miss
Method : C:\HPCHEM\1\METHODS\IZS_ME~1\OLAQUID.M
Last changed : 26/10/00 13.00.32 by Miss
(modified after loading)



External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 26/10/00 12.09.03
Multiplier : 1.0000
Dilution : 1.0000

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

RetTime [min]	Sig	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
8.338	1		-	-	-		olaquidox

Totals : 0.00000

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 25

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
255696	4,88	4,88
255728	nd	nd
255742	1,70	1,70
255782	1,70	1,70
255814	4,88	4,86
255819	nd	nd

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 2-12-2000

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: WATER - METHANOL 800+200 (V+V)
- Flow-rate: 1 ml/min
- Injection volume: 40 µl
- Retention time of olaquindox: 5,33 min

Chromatograms: Please include representative chromatograms of:

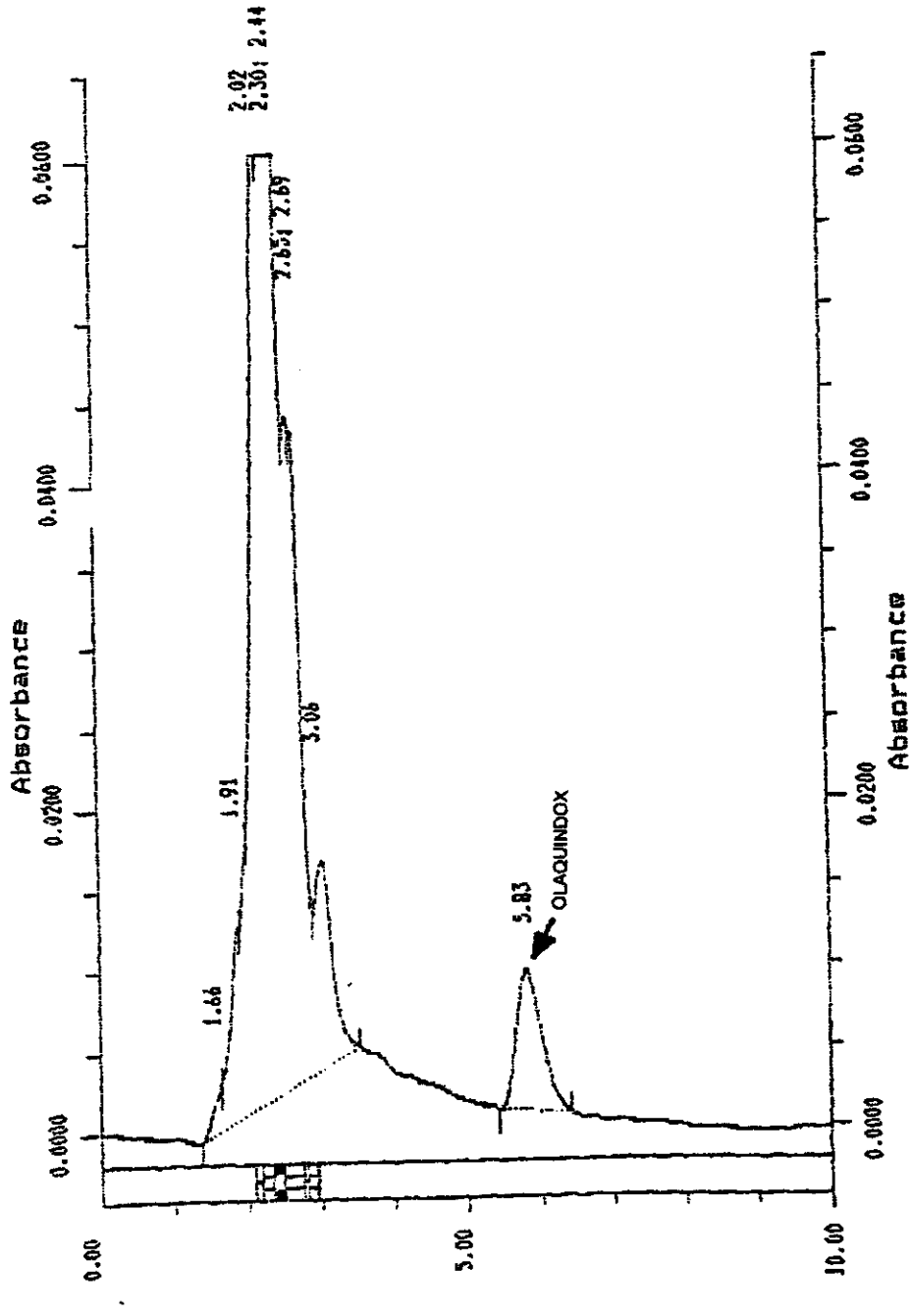
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

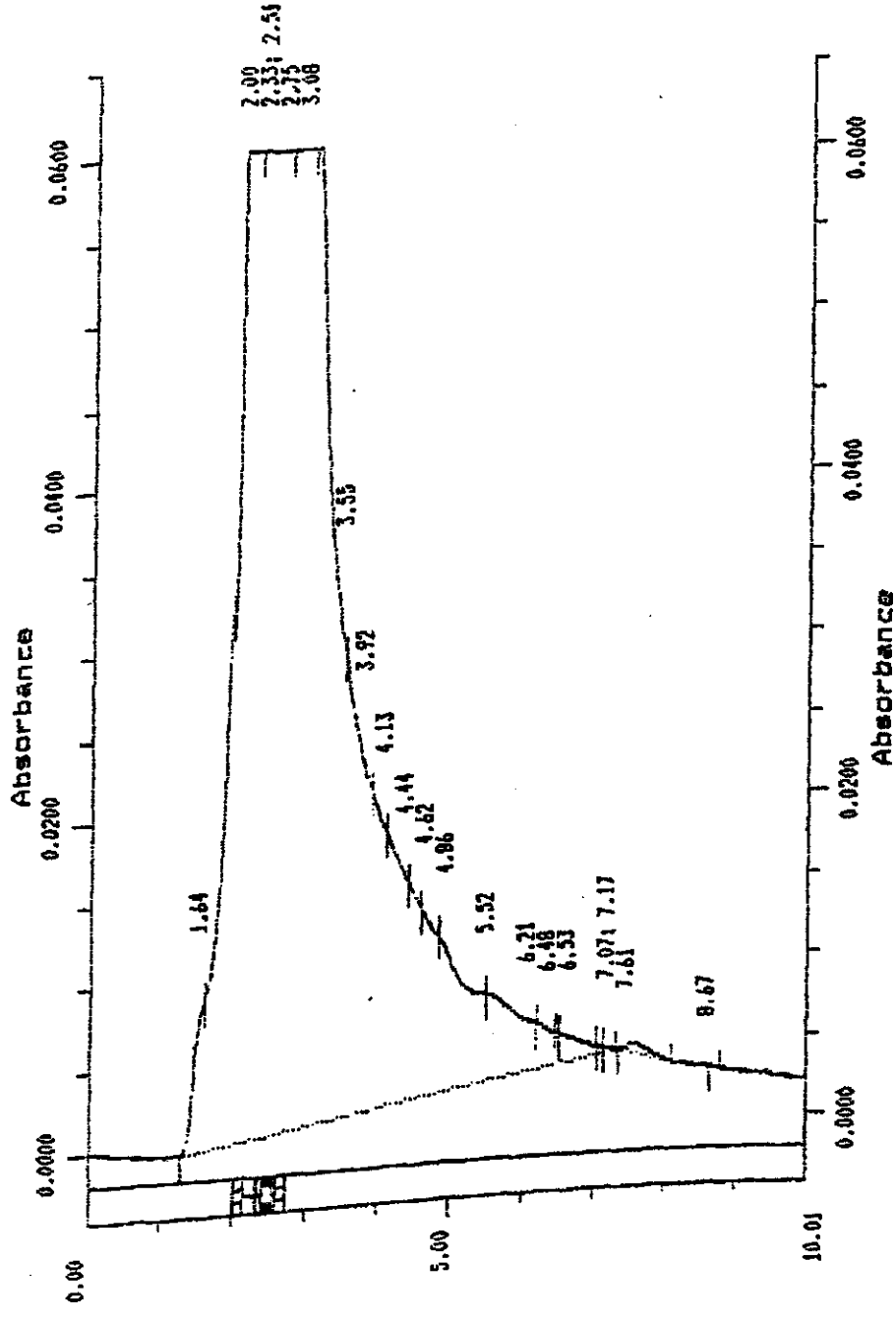
- Percentage recovery: 84 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 5 mg/kg

Chromatogram for olaquindox study



Feed n° 255696

Chromatogram for olaquindox study



Feed n° 255819 (Blank)

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 26

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis: 7/12/00

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
265743	1,6	1,5
265749	5,1	4,8
265755	0,1	0,1
265763	0,7	1,2
265764	0,1	0,1
265768	6,0	5,3

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 7/12/00

Chromatographic conditions:

- Column:
  - As described in the method
  - ✓ Other: SPHERISORB ODS 2 5µm 250mm × 4.6mm.
- Mobile phase:
  - ✓ As described in the method
  - Other:
- Flow-rate: 1.5 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: 9.8 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

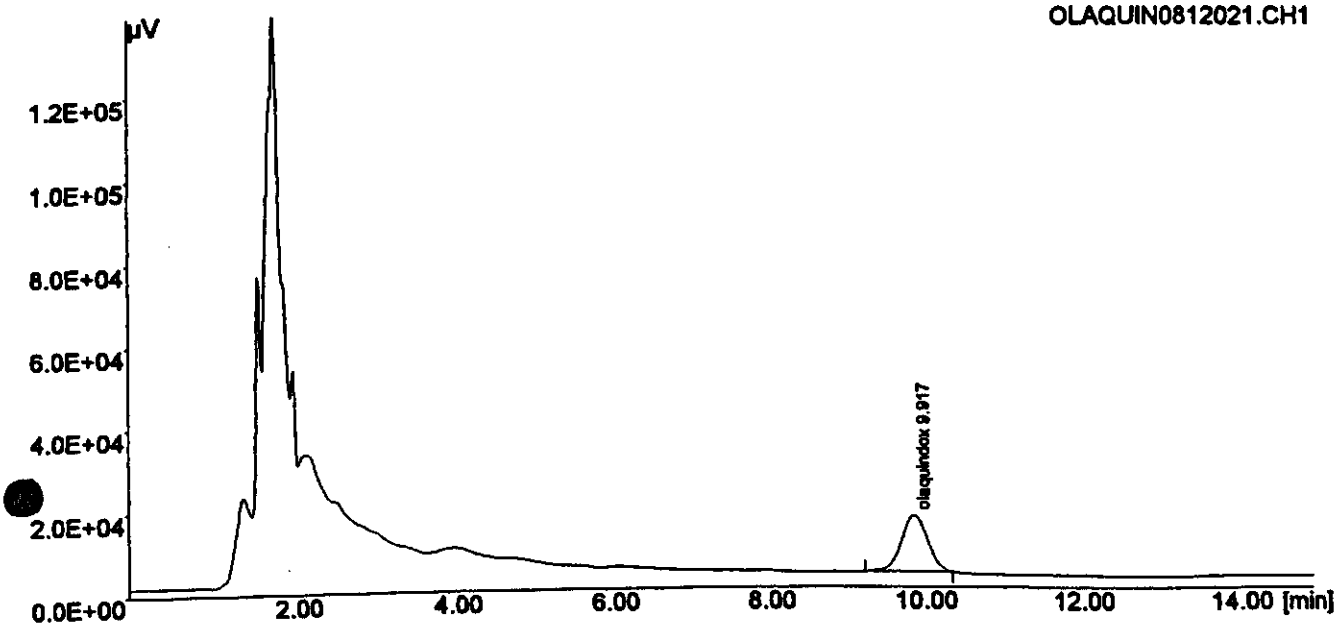
Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: 73 %
- Single / duplicate determinations: single duplicate
- If duplicate, please give both percentages: 75 % and 71 %
- Spiking level: 3 mg/kg

POSITIVE FEED

50A1



File name : OLAQUIN0812021.CH1

Info :
50A1

Vial # = 21 Rack # = 1
Injection Date : 8-Dec-2000 20:15:20
Curr. Date : 18-Dec-2000 16:49:54
User :
Group : OLAQUIN
Control Method :

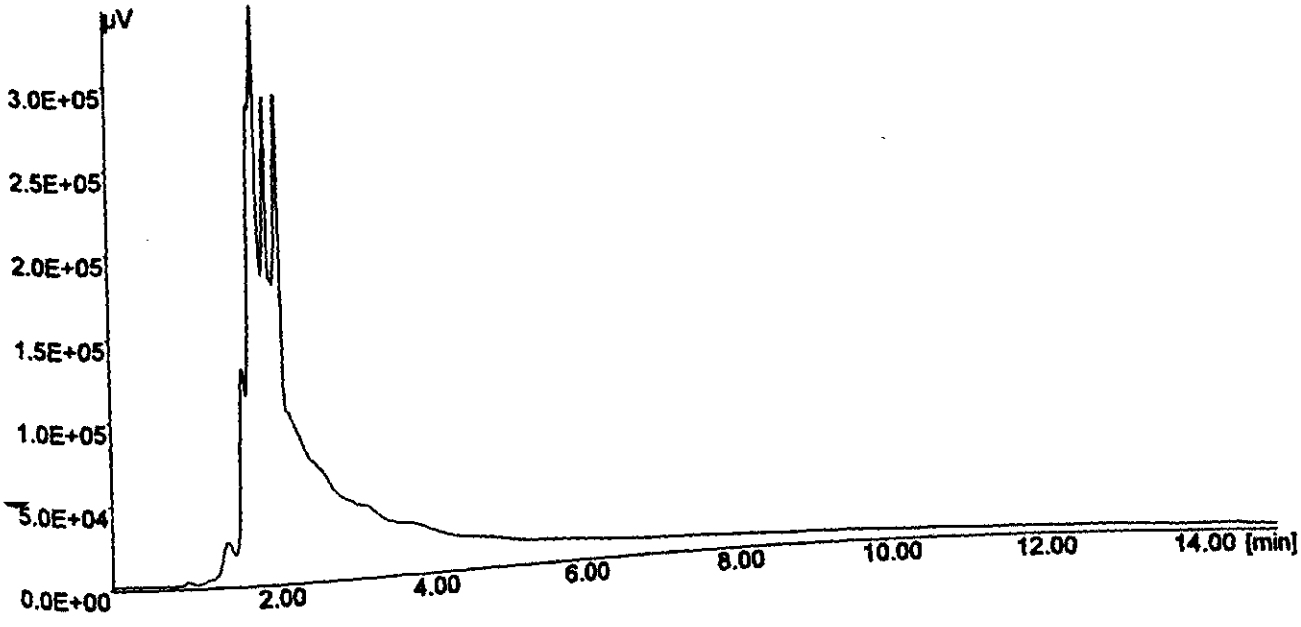
#	Name	RT	Area[µV.Sec]	Quantity
1	olaquindox	9.917	303752.400	0.000

Total Area of Peak = 303752.400 [µV.Sec]

BLANK FEED

51A1

OLAQUIN0812025.CH1



File name : OLAQUIN0812025.CH1

Info :
51A1

Vial # = 25 Rack # = 1
Injection Date : 8-Dec-2000 21:25:02
Curr. Date : 18-Dec-2000 16:49:58
User :
Group : OLAQUIN
Control Method :

Peak Detection Not Available

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 27

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
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: OLAQUINDOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
275700	5,22	5,34
275720	not detectable	not detectable
275745	2,05	1,98
275773	not detectable	not detectable
275805	5,30	5,56
275830	1,81	1,89

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 41st week 2000

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 0,8-1,1 ml/min , depending on the back pressure of the column
- Injection volume: 50 µl , for code number 275773: 100 µl
- Retention time of olaquindox: 10-14 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:

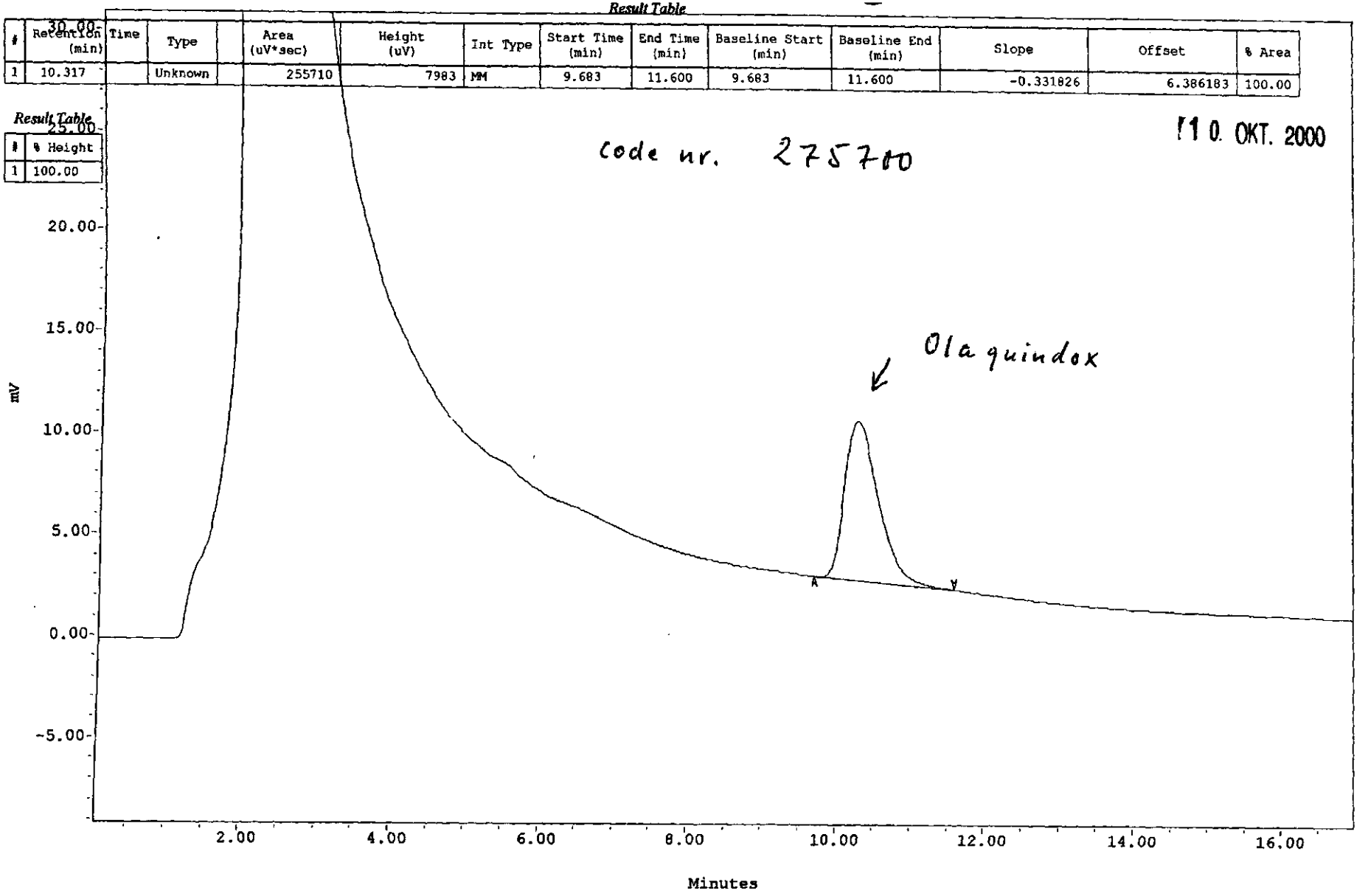
- Percentage recovery: 75,7 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 3 mg/kg

Result Table

#	Retention Time (min)	Type	Area (uV*sec)	Height (uV)	Int Type	Start Time (min)	End Time (min)	Baseline Start (min)	Baseline End (min)	Slope	Offset	% Area
1	10.317	Unknown	255710	7983	MM	9.683	11.600	9.683	11.600	-0.331826	6.386183	100.00

Result Table

#	% Height
1	100.00

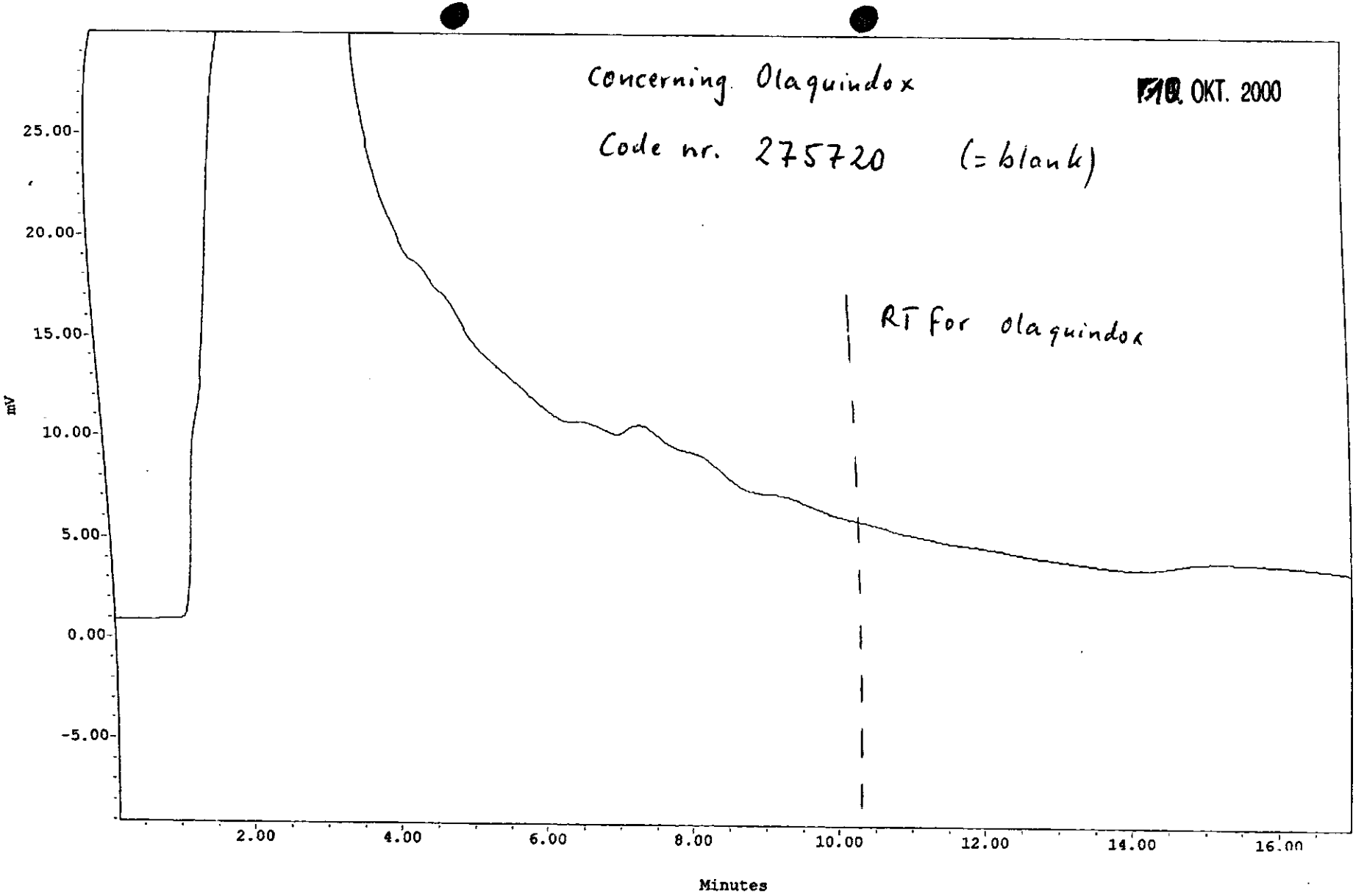


Code nr. 275700

Olaquindox

10. OKT. 2000

SampleName: OLA 1a Vial: 6 Inj: 1 Ch: SATIN Type: Unknown Page: 1 of 1

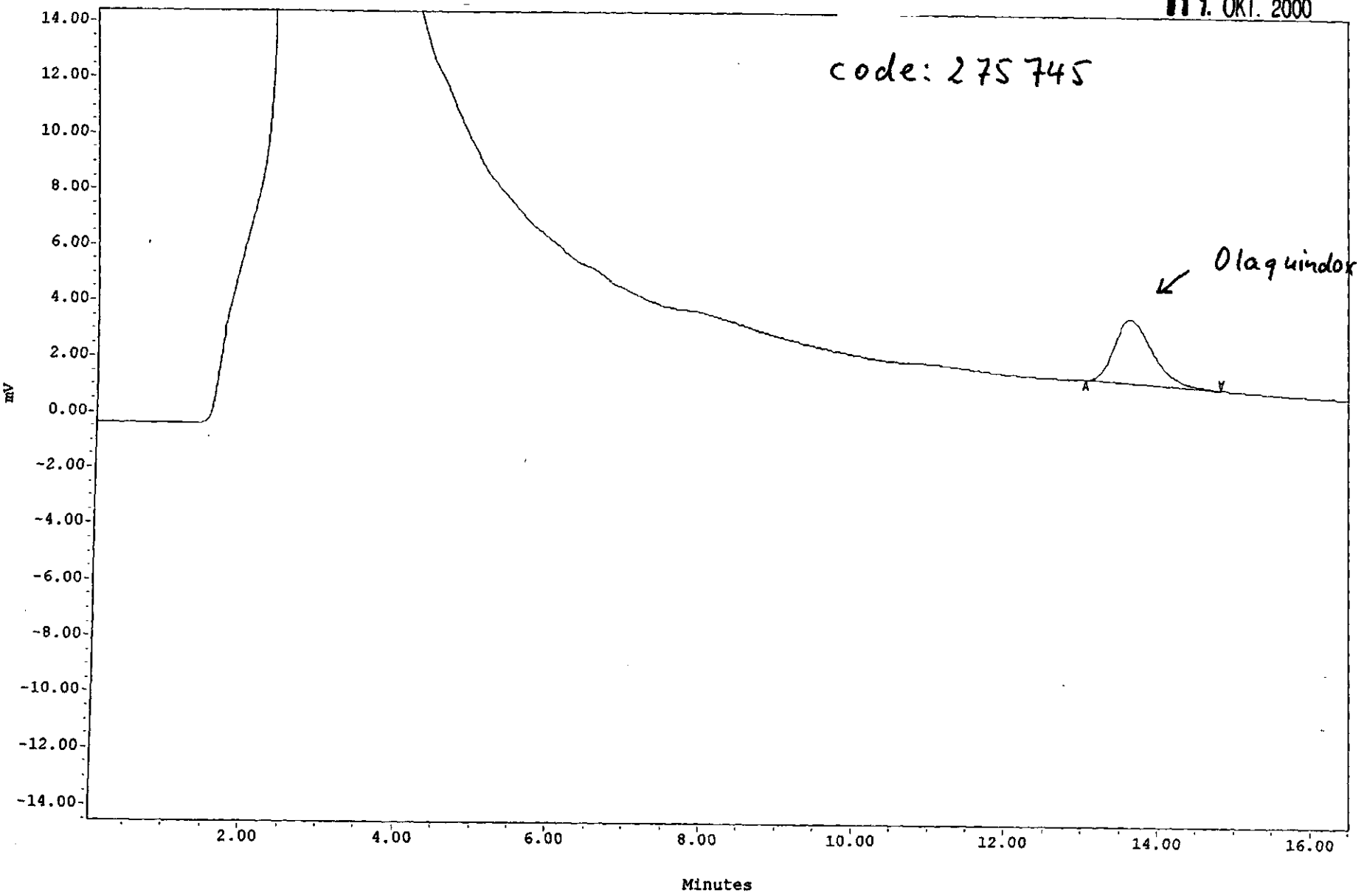


SampleName: OLA 2a Vial: 8 Inj: 1 Ch: SATIN Type: Unknown

27

17. OKT. 2000

code: 275745



SampleName: OLA 3a Vial: 6 Inj: 1 Ch: SATIN Type: Unknown

27

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 29

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**
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
295704	3,8	3,7
295721	3,8	3,7
295732	0	0
295756	0	0
295762	1,2	1,3
295786	1,3	1,3

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: ....22.11.2000.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: Nova-Pac C8, 4.6x250mm, C18, 4µ
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: .....1.5.... ml/min
- Injection volume: .100..µl
- Retention time of olaquindox: .6.1. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

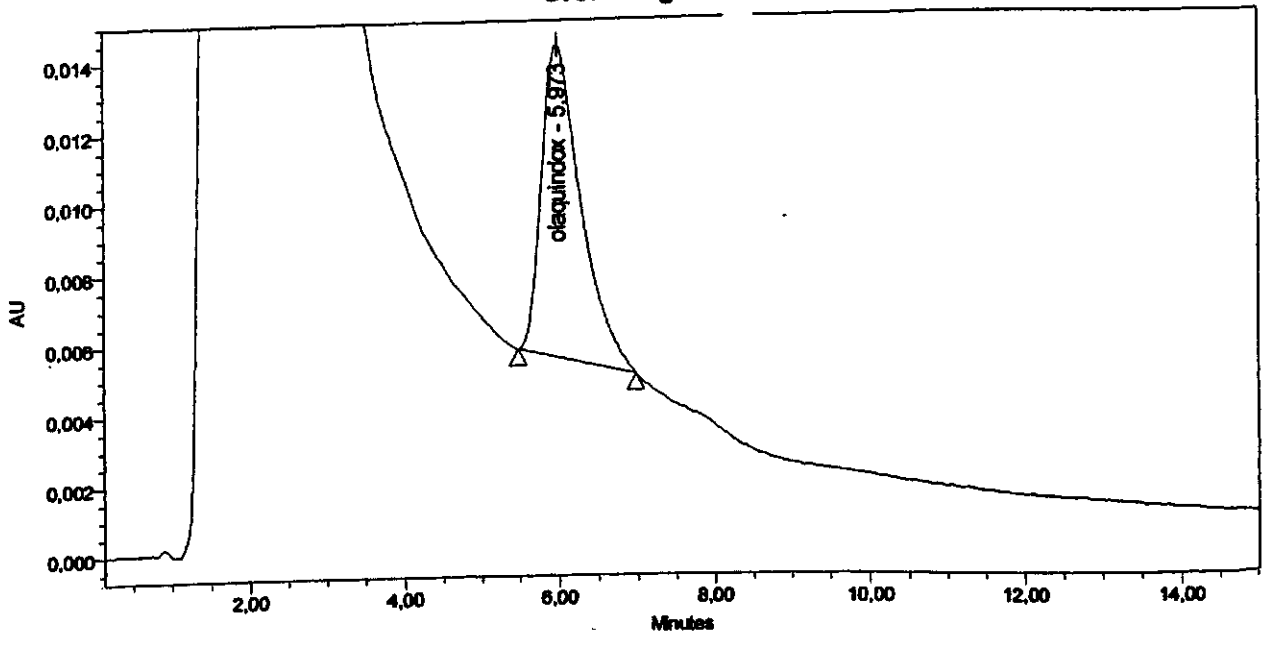
Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: .68. % and .70. %
- Spiking level: ....3..... mg/kg

Sample Name:	**olaquindox 721I**	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	16-11-2000 15:34:13
Vial:	32	Acq. Method Set:	Olaquindox
Injection #:	1	Date Processed:	18-11-2000 18:03:28
Injection Volume:	100,00 ul	Processing Method:	**'olaquidox 18 11 00**
Run Time:	15,0 Minutes		
Sample Set Name:	**OLAQUINDOX**	Proc. Chnl. Descr.:	PDA 380,0 nm

Cromatogram

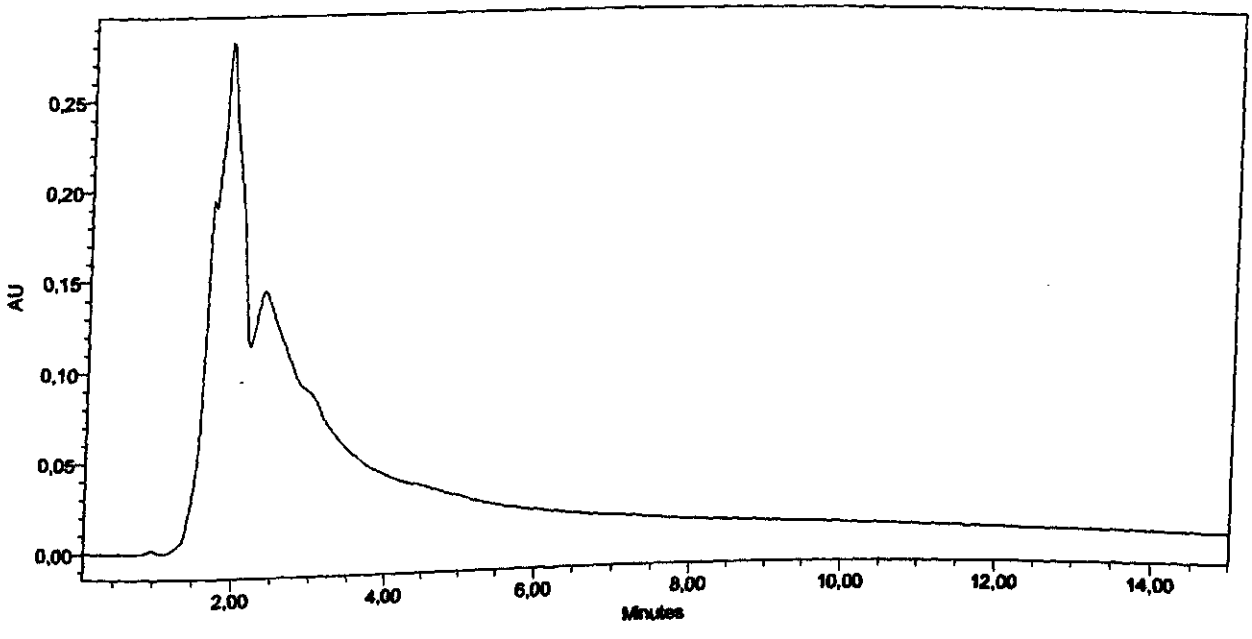


	Name	RT	Area	Height	Amount	Units
1	olaquindox	5,973	308140	8855	1,947	ug/ml

(3,8 mg 1kg)

Sample Name:	**olaquindox 732 I / II**	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	16-11-2000 18:01:25
Vial:	34	Acq. Method Set:	Olaquindox
Injection #:	1	Date Processed:	18-11-2000 18:03:28
Injection Volume:	100,00 ul	Processing Method:	**. olaquidox 18 11 00**
Run Time:	15,0 Minutes		
Sample Set Name:	**olaquindox 2**	Proc. Chnl. Descr.:	PDA 380,0 nm

Cromatogram



	Name	RT	Area	Height	Amount	Units
1	olaquindox	6,137				

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 31

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**
Lab-name:
Contact person: e-mail:
fax:
telephone:

Date of analysis:

Analyte: OLAQUINDOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
315707	0	0
315709	6,28	6,13
315710	1,88	1,90
315794	6,05	5,86
315801	0	0
315811	2,08	1,80

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: ...6-11-2000.....

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: .7,3. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

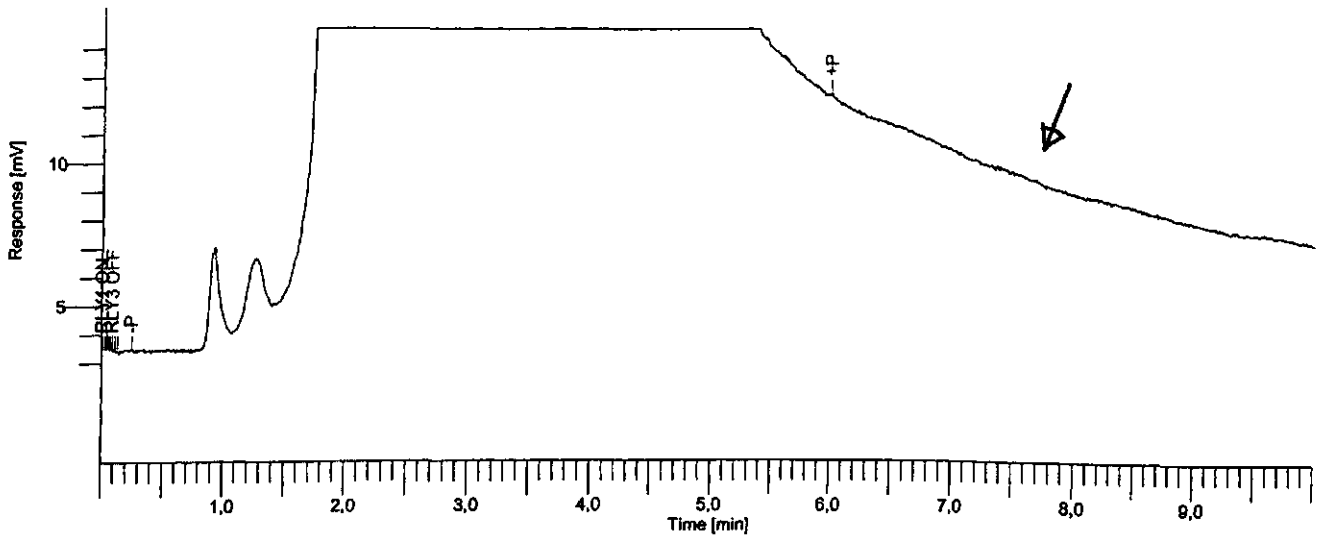
Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: .78. % and .86. %
- Spiking level: ....3..... mg/kg

Software Version	: 6.1.1.0.0:K20	Date	: 7-11-00 9:03:51
Sample Name	: 0022012	Data Acquisition Time	: 6-11-00 20:19:35
Instrument Name	: HPLC-1	Channel	: A
Rack/Vial	: 0/0	Operator	:
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 19		

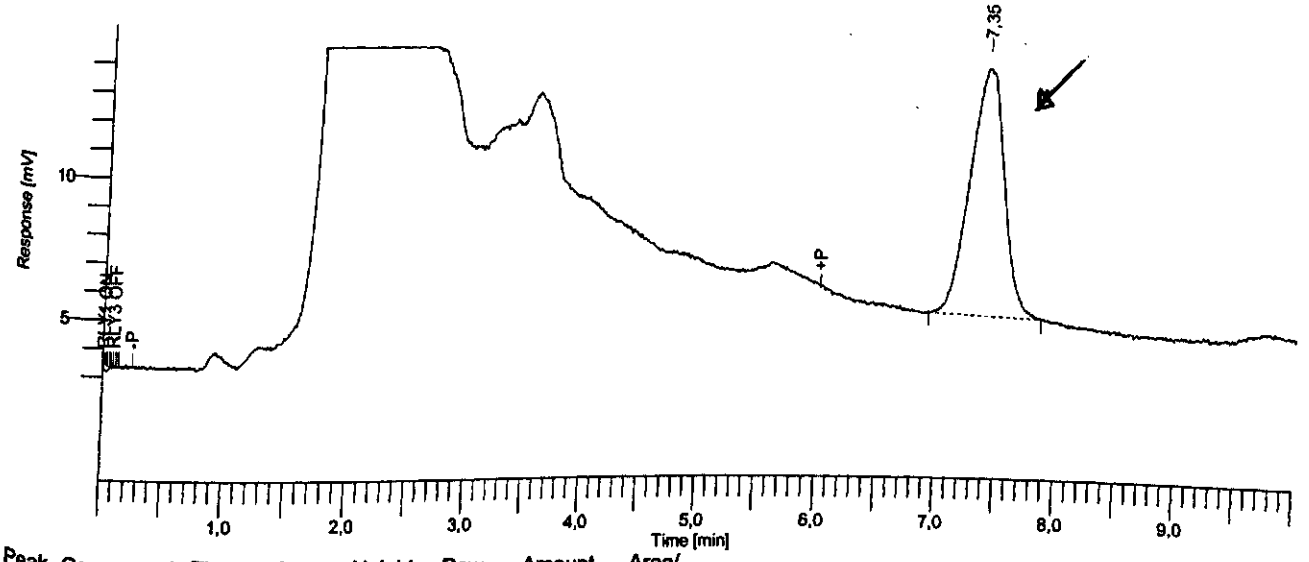
Result File : \\ 04s\TCDATA\ Residue\HPLC-1\olaquindox\061100-019.rst
Sequence File : \\ 04s\TCDATA\ Residue\HPLC-1\olaquindox\testolx.seq



No peaks available to report

Software Version	: 6.1.1.0.0:K20	Date	: 7-11-00 9:02:51
Sa[illegible]le Name	: 0022010-	Data Acquisition Time	: 6-11-00 15:34:47
I[illegible]ument Name	: HPLC-1	Channel	: A
Rack/Vial	: 0/0	Operator	:
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 10		

Result File : \\[illegible]004s\TCDATA\[illegible] Residue\HPLC-1\olaquindox\061100-010.rst
Sequence File : \\[illegible]04s\TCDATA\[illegible] Residue\HPLC-1\olaquindox\testolx.seq



Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Raw Amount	Amount [Norm. %]	Area/ Amount
1		7,353	176368	8792	176368	100,00	1,0000
			176368	8792	176368	100,00	1,0000

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 32

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**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
325705	5,91	5,92
325716	2,06	2,24
325744	Negative	Negative
325747	5,63	5,71
325798	Negative	Negative
325807	2,07	2,03

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Waters Symmetry, C18, 5 um, 4.6mmX250mm (Part N° WAT 054215)
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.4 ml/min
- Injection volume: 20 (µL)
- Retention time of olaquindox: 7.05 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:

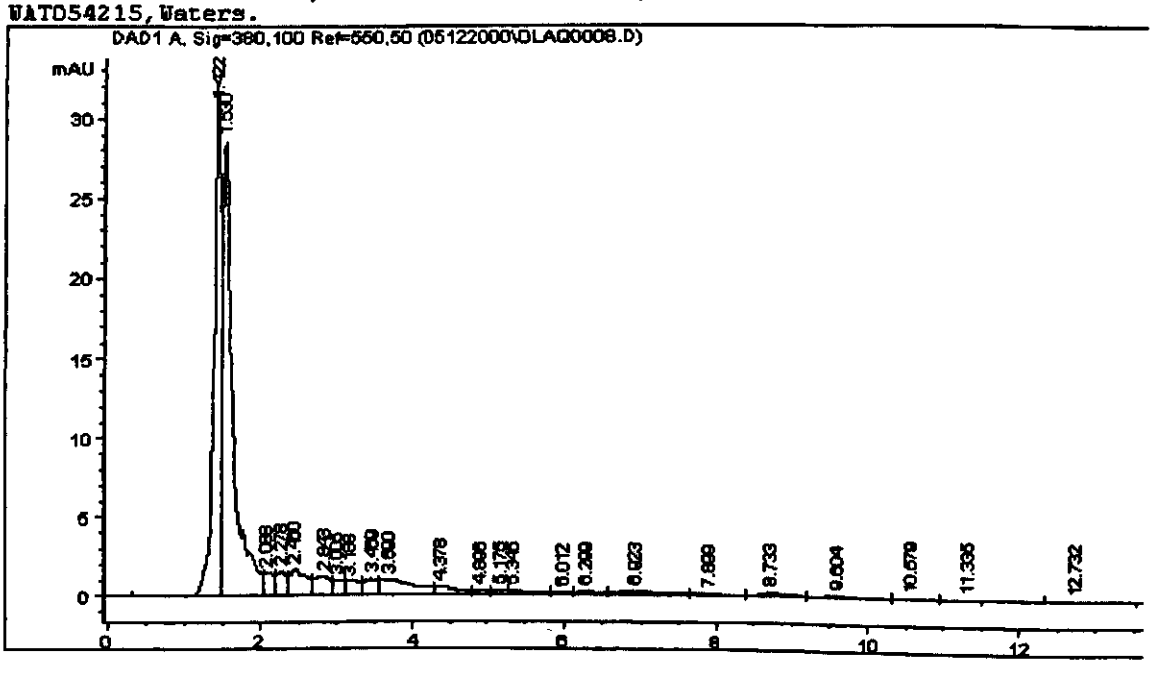
- Percentage recovery: 84.5 %
- Single/duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 81.99% and 86.97%
- Spiking level: 3 mg/kg

Data File C:\HPCHEM\1\DATA\05122000\OLAQ0008.D Sample Name:

BLANK SAMPLE

Injection Date	: 12/5/00 9:23:37 PM	Seq. Line	: 8
Sample Name	:	Vial	: 8
Acq. Operator	:	Inj	: 1
		Inj Volume	: 20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\OLAQUIN.M		
Last changed	: 12/5/00 9:21:37 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\OLAQUIN.M		
Last changed	: 12/6/00 12:23:11 PM by (modified after loading)		

PE-032-HP/QT, Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	12/6/00 12:23:08 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	Amount [ug/ml]	Grp	Name
6.923	VV	10.86601	5.52238e-2	6.00063e-1		Olaquindox
Totals :				6.00063e-1		

Instrument 1 12/6/00 12:27:36 PM

(32)

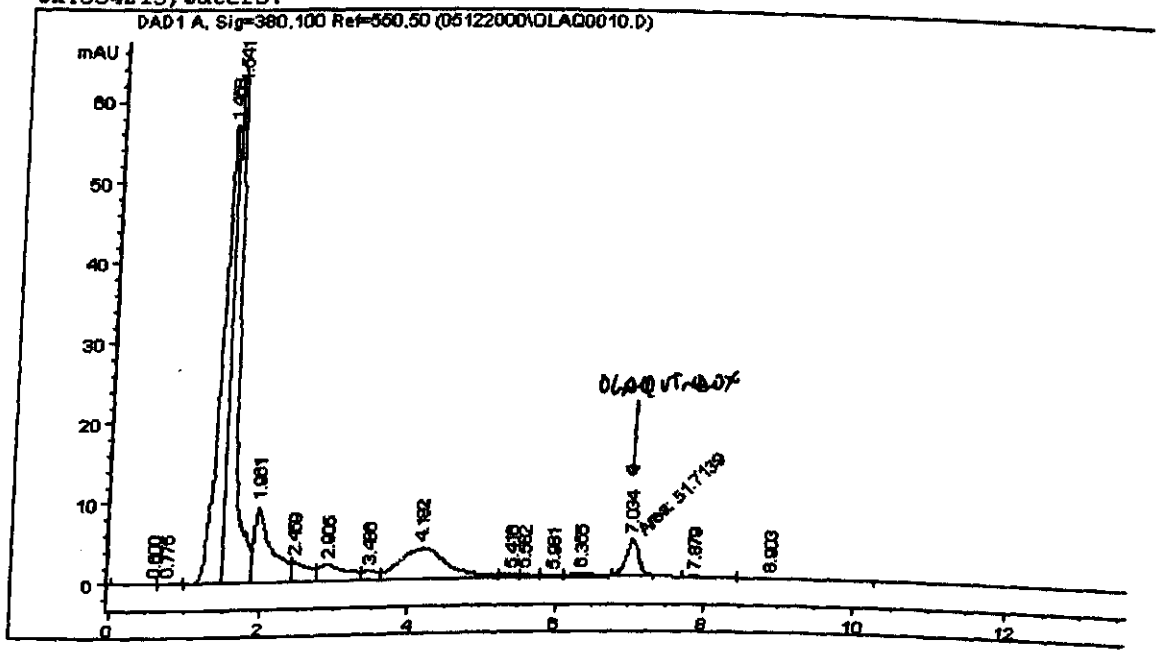
Data File C:\HPCHEM\1\DATA\05122000\OLAQ0010.D

Sample Name: 325705/

Code 325705, massa - 25.0049g

Injection Date	: 12/5/00 9:57:44 PM	Seq. Line	: 10
Sample Name	: 325705/	Vial	: 10
Acq. Operator	:	Inj	: 1
		Inj Volume	: 20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\OLAQUIN.M		
Last changed	: 12/5/00 9:55:42 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\OLAQUIN.M		
Last changed	: 12/6/00 12:23:11 PM by (modified after loading)		

.C, PE-032-HP/QT, . Symmetry, 250 x 4.6 mm, ref. WAT054215, Waters.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	12/6/00 12:23:08 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	Amount [ug/ml]	Grp	Name
7.034	MM	51.71388	5.60974e-2	2.90101		Olaquindox
Totals :				2.90101		

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 33

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**
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
335702	< 1	< 1
335706	2,3	2,4
335753	< 1	< 1
335759	7,4	6,9
335772	7,2	7,2
335804	2,5	2,5

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

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

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: 95/5 0,01M Sodiumacetaat pH 6 / acetonitrile
- Flow-rate: 0,5 ml/min
- Injection volume: 20 µl
- Retention time of olaquindox: 9,1 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: 93 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 93 % and 91 %
- Spiking level: 10 mg/kg

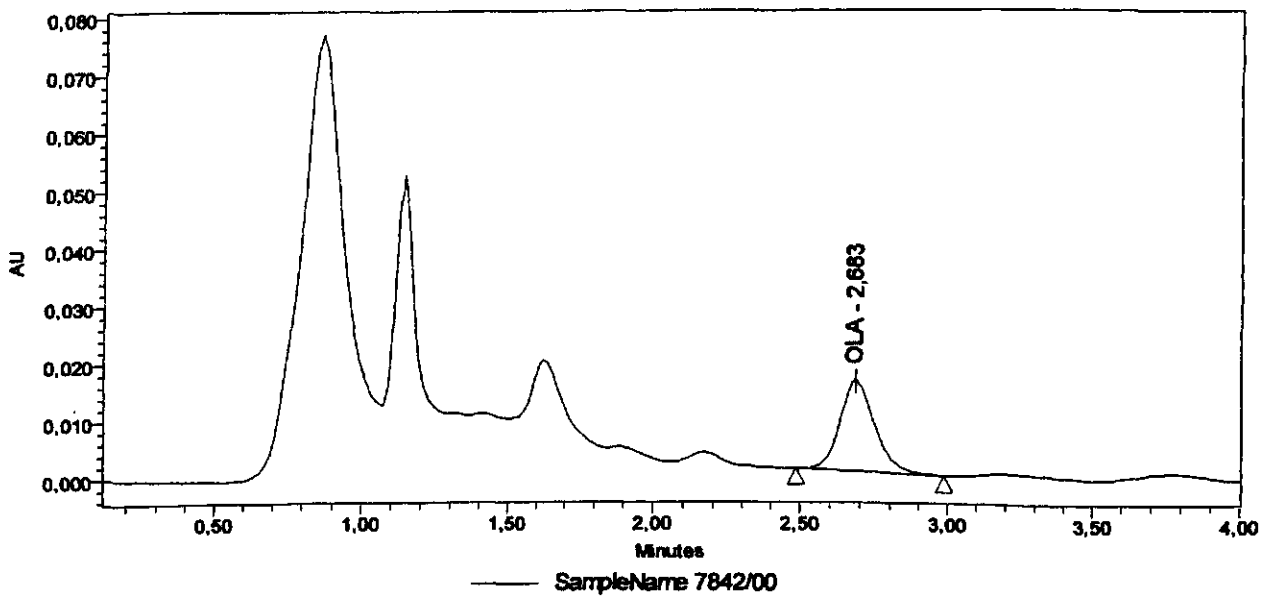
Sample Set Name OLA18

User Name RVSA

Current Date 18/10/00

Current Time 02:29:43

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	OLA	2,683	126856	15667	7,239	mg/kg

blind blank

(33)

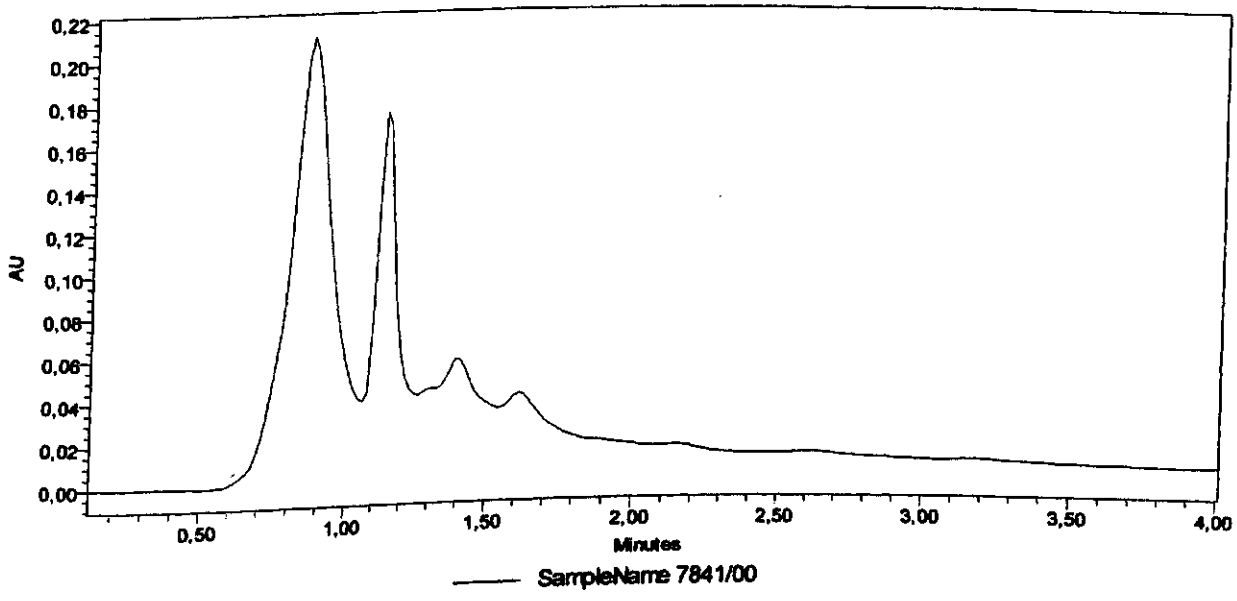
Sample Set Name OLA18

User Name RVSA

Current Date 18/10/00

Current Time 02:29:38

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	OLA	2,744				

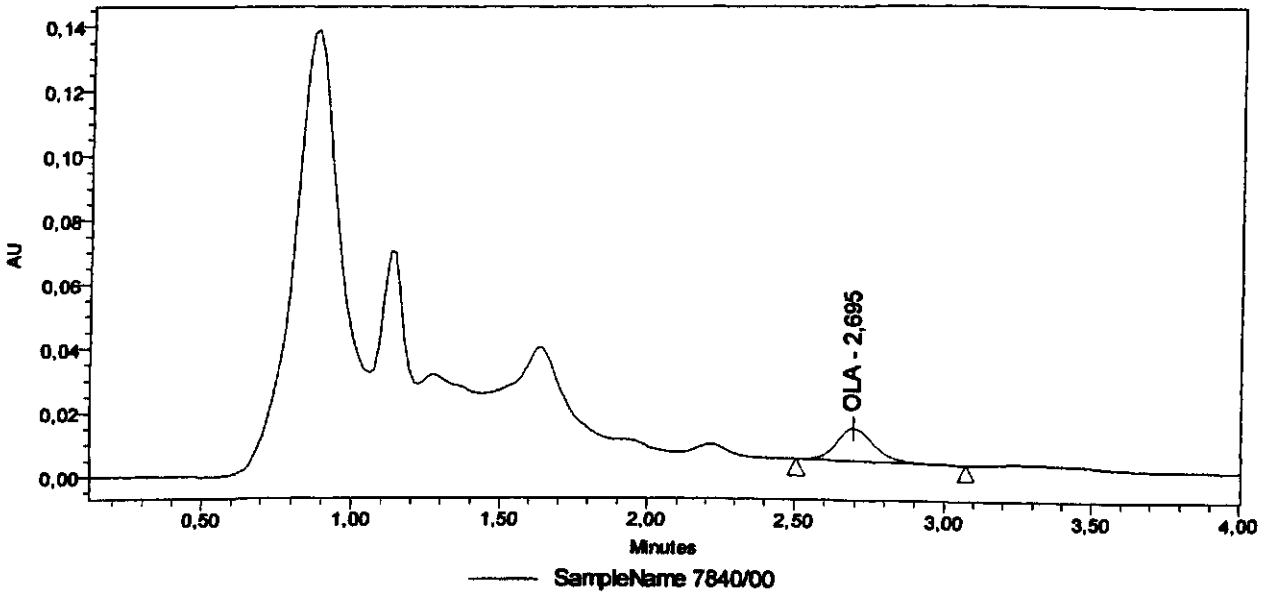
Sample Set Name OLA18

User Name RVSA

Current Date 18/10/00

Current Time 02:29:34

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	OLA	2,695	88628	10323	2,495	mg/kg

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 34

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**
Lab-name:
Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
345698	0	0
345717	1,5	1,5
345750	4,7	4,8
345752	1,5	1,5
345778	4,8	4,9
345826	0	0

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

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

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: ..20..µl
- Retention time of olaquindox: ..7.8.. 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: ..84.. %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ..82.. % and ..86.. %
- Spiking level: ..5..... mg/kg

KromaSystem 2000

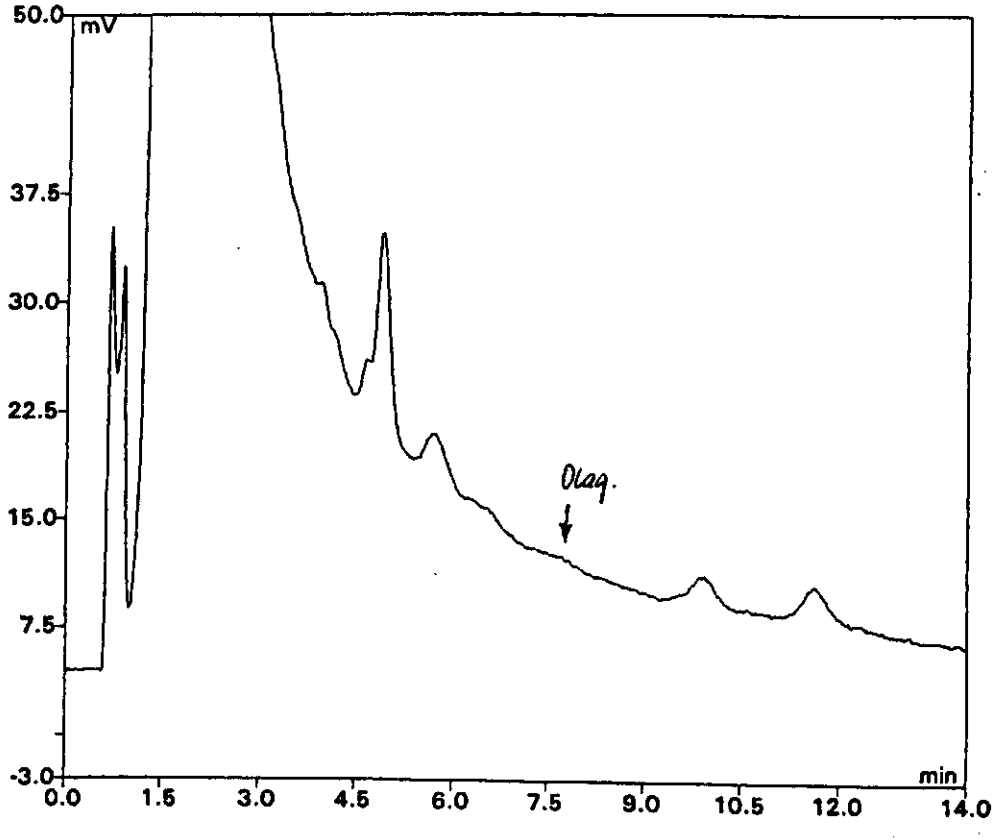
blind blank

Channel 2

KromaSystem 2000 Version 1.83 RESULT REPORT: INTEGRATION

SYS2 - OLAQ025.SMP: Olaquindox- Enquete
No. 07: C1908" 25g/50ml Acquired : 30.10.00 13:01:45
Channel 2: DETECT 332 Processed: 15.11.00 11:06
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 %
					0.0000e+000	0.0000e+000	0.00

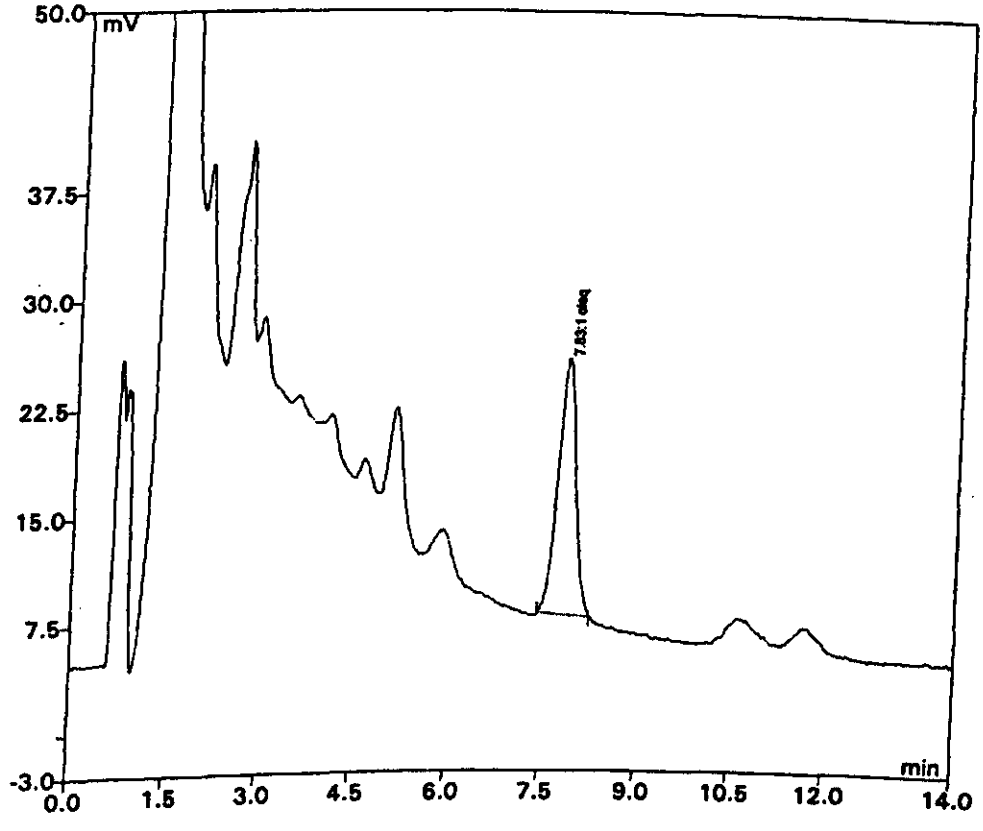
KromaSystem 2000

Channel 2

KromaSystem 2000 Version 1.83 RESULT REPORT: INTEGRATION

SYS2 - OLAQ025.SMP (modified): Olaquindox Enquete
No. 13: C1910" 25g/50ml Acquired : 30.10.00 14:32:18
Channel 2: DETECT 332 Processed: 15.11.00 11:07
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	7.83	MOD	olaquindox	5.7859e+000	4.7822e+000	100.00
					5.7859e+000	4.7822e+000	100.00

mg/kg

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 37

Olaquindox in Feed
Mode: Reprocessed Data
Original Results: C:\TSP\SYSTEM1\Data\ola011200AM.RES
Reprocessed Results: C:\TSP\SYSTEM1\Data\ola011200AM.RMS

Reported On: 07-12-00 10:04:54

Analysis Report

Name: 8
Description: 8
Type: Sample
Injection Volume: 60.0 µL

Vial: A28

Injection: 1 of 1

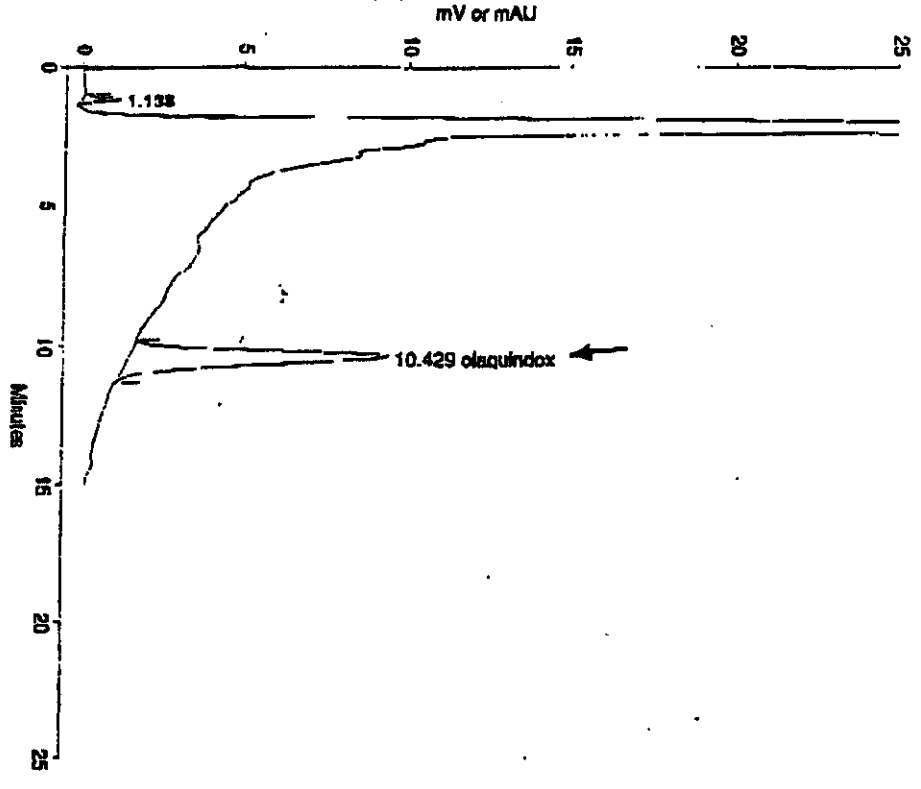
Injected On: 02-12-00 02:34:50

Acquisition Log
Column Pressure (PSI): 2590
Noise (microAU): 1e+02
Run-Time Messages: None

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

Pump Flow Stability: 2.2

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



Typical chromatograms for Olaquindox

Blind Positive Feed (375792)

Component	RT(min)	Area	Height	ug/ml	Peak Type
olaquindox	10.429	260545	7962	1.7086	Modified
Totals		260545	7962	1.7936	

System: Reprocess
Acquisition Method: C:\TSP\SYSTEM1\Methods\olaquindox.AOM
Calculation Method: C:\TSP\Methods\olaq.CAM
Report Method: C:\TSP\Methods\olaq.RPM

Analyst: AM

PC1000 Ver 3.5.1
29-11-00 18:31:34
04-12-00 18:10:48
06-12-00 12:57:28

(37)

Olaquindox in Feed
Mode: Reprocessed Data
Original Results: C:\TSP\SYSTEM1\Data\ola011200AM.RES
Reprocessed Results: C:\TSP\SYSTEM1\Data\ola011200AM.RMS

Reported On: 07-12-00 16:34:54

Analysis Report

Name: 10
Description: 10
Type: Sample
Injection Volume: 50.0 µL

Vial: A32

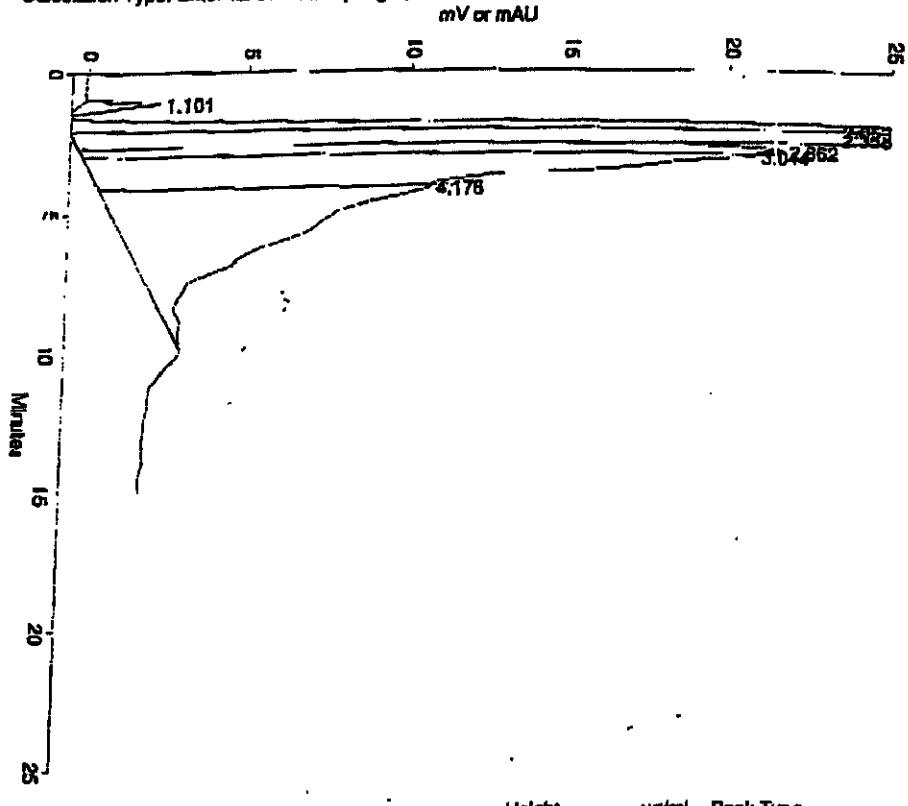
Injection: 1 of 1
Injected On: 02-12-00 03:40:06

Acquisition Log
Column Pressure (PSI): 2575
Noise (microAU): 2e+02
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): -4e+02

Pump Flow Stability: 2.2

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



Typical chromatograms for Olaquindox

Blind Blank Feed (375722)

Component	RT(min)	Area	Height	ug/ml	Peak Type
Totals		0	0	0.0	

Analyst: AM

System: Reprocess
Acquisition Method: C:\TSP\SYSTEM1\Methods\olaquindox.ACM
Calculation Method: C:\TSP\Methods\olaq.CAM
Report Method: C:\TSP\Methods\olaq.RPM

PC1000 Ver 3.5.1
29-11-00 18:31:34
04-12-00 18:10:48
06-12-00 12:57:28

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 38

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**

Lab-name:

Contact person:

e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
385726	6,00	5,52
385737	0	0
385783	1,72	1,76
385789	2,01	1,94
385803	0	0
385815	5,53	5,38

CANFAS COLLABORATIVE STUDIES - OLAQUINDOX

Annex 4 – Questionnaire

Date(s) of analysis: **11/27/00**

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - **X** Other: **Hypersil ODS C-18, 250 x 4,6 mm, 5 µm**
- Mobile phase:
  - **X** As described in the method
  - ☐ Other:
- Flow-rate: **1** ml/min
- Injection volume: **20** µl
- Retention time of Carbadox: **6** min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank samples

Please indicate the olaquindox peak with an arrow

Recovery results:

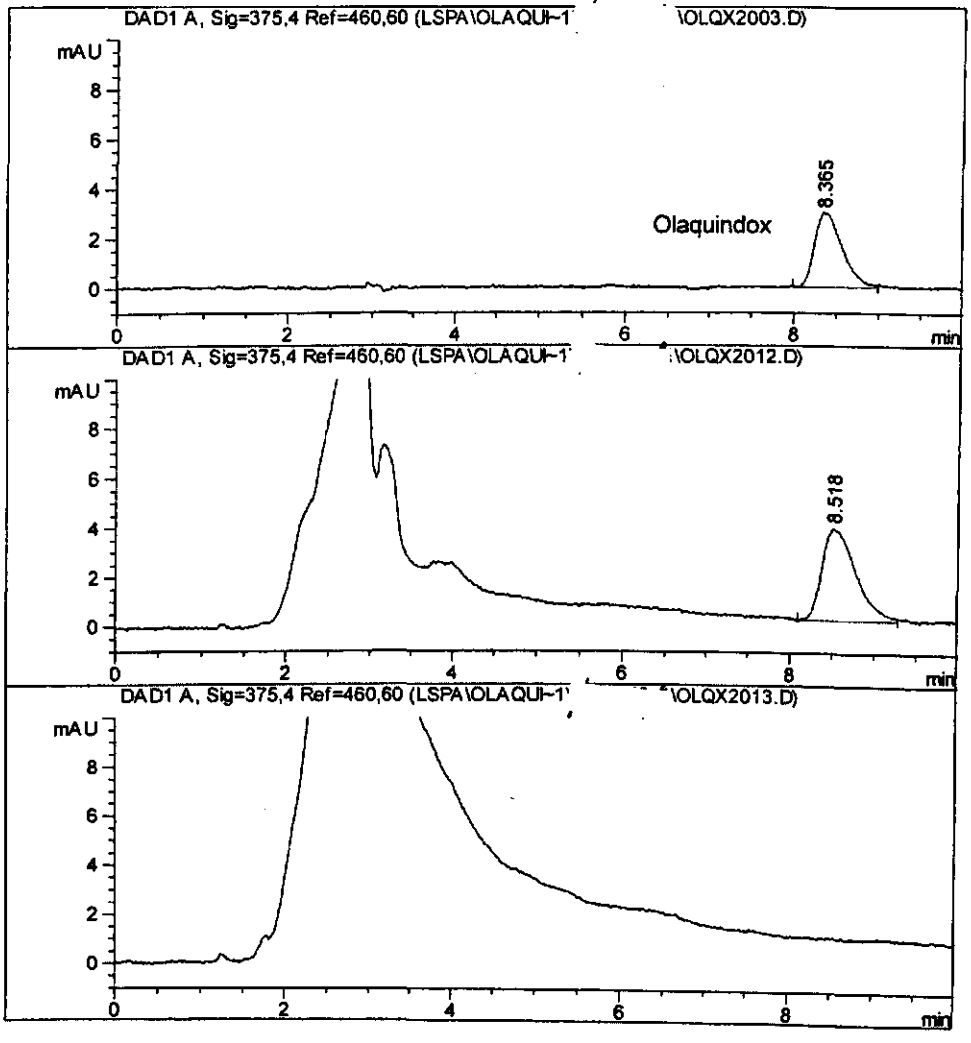
- Percentage recovery: **81 %**
- Single / duplicate determinations: **X** single ☐ duplicate
- If duplicate, please give both percentages: ...% and ... %
- Speaking level: **2** mg/kg

CANFAS COLLABORATIVE STUDIES - OLAQUINDOX

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

Please note that our detection system has been DAD; not a single wavelength UV-detection (as it has been indicated at particulary instruction) because we have not it.

Chromatograms for standard (3 ppm), sample (385726) and blank



APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 40

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**

Lab-name:
Contact person:
e-mail:
fax:
telephone:

Date of analysis:

Analyte: **OLAQUINDOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
405697	blanco	blanco
405701	blanco	blanco
405734	1,50	1,80
405779	4,75	4,56
405799	2,21	1,62
405800	5,20	5,74

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 8 - 10 NOVEMBER 2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: C18 spherical 5µm 3.9 x 15 cm. WATERS
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other:
- Flow-rate: 1.3 ml/min
- Injection volume: 25 µl
- Retention time of olaquindox: 4.8 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

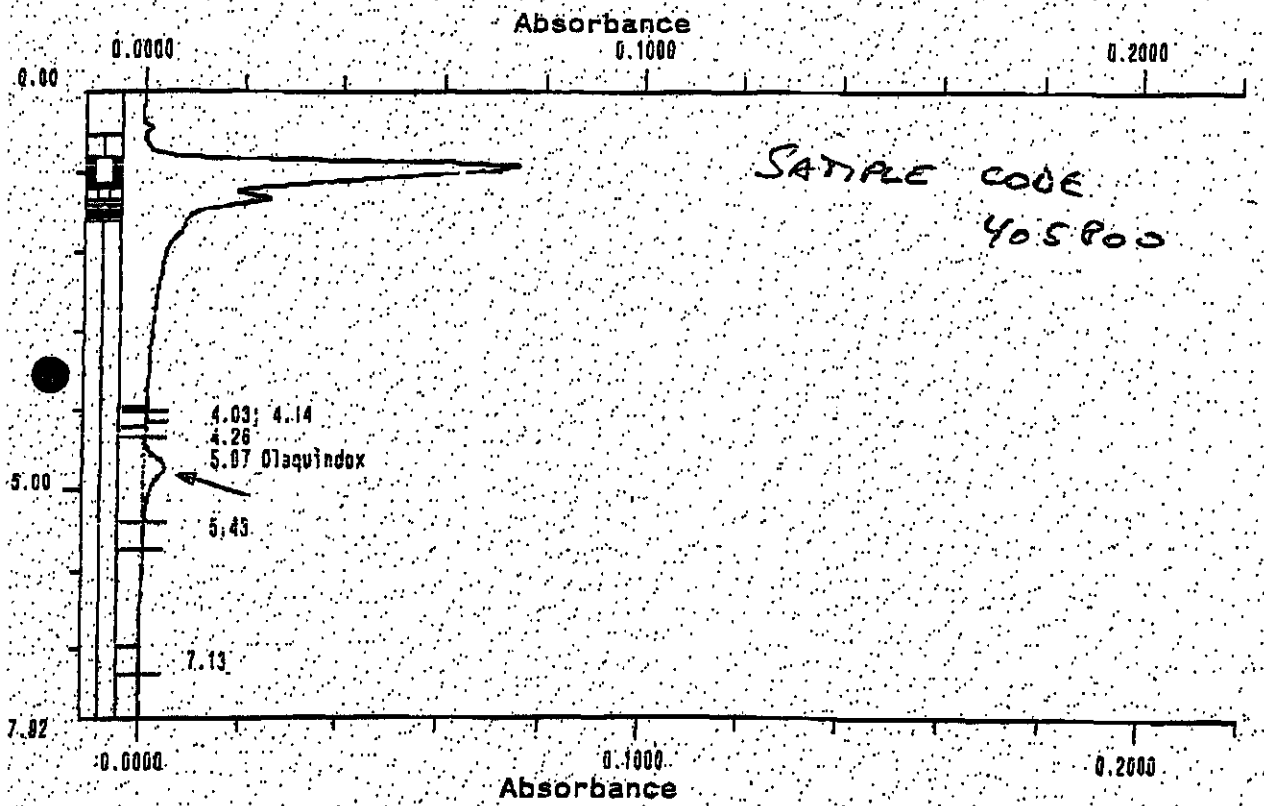
Recovery results:

- Percentage recovery: 83 %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: 1.25 mg/kg

	NAME	CHAN	LEV	REP	TYPE	DIRECTORY		TIME	DATE
OLLECTION	DATA 13_C7	A	1	2	Orig	C:\GOLD\SYS1DATA\OLAQUIND\	INJECTION	13:20:35	13 NOV 2000
	METHOD OLAQUIND					C:\GOLD\SYS1METH\	ANALYSIS	14:28:13	13 NOV 2000
							REPORT	11:39:08	21 DEC 2000

AMPLE TABLE SAMPLES C:\GOLD\SAMPLIBL\
YSTEM 1: SYSTEM1

Chart Speed 1.00 cm/min

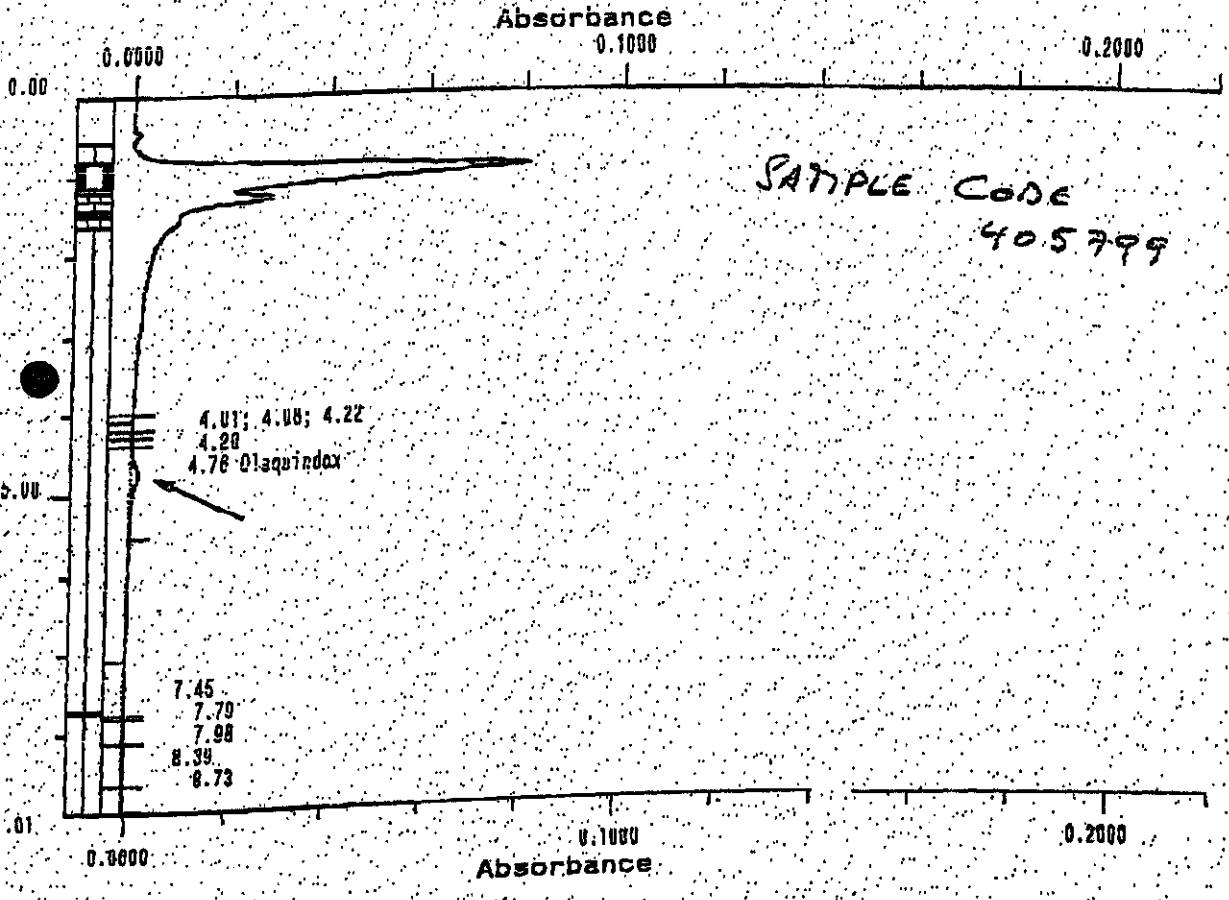


SAMPLE CODE
405800

	NAME	CHAN	LEV	REP	TYPE	DIRECTORY		TIME	DATE
OLLECTION	DATA 13_C6	A	1	2	Orig	C:\GOLD\SYSTDATA\OLAQUIND\	INJECTION	12:09:27	13 NOV 2000
	METHOD OLAQUIND					C:\GOLD\SYSTMETH\	ANALYSIS	12:18:40	13 NOV 2000
							REPORT	11:39:38	21 DEC 2000

AMPLE TABLE SAMPLES C:\GOLD\SAMPLTBL\
YSTEM 1: SYSTEM1

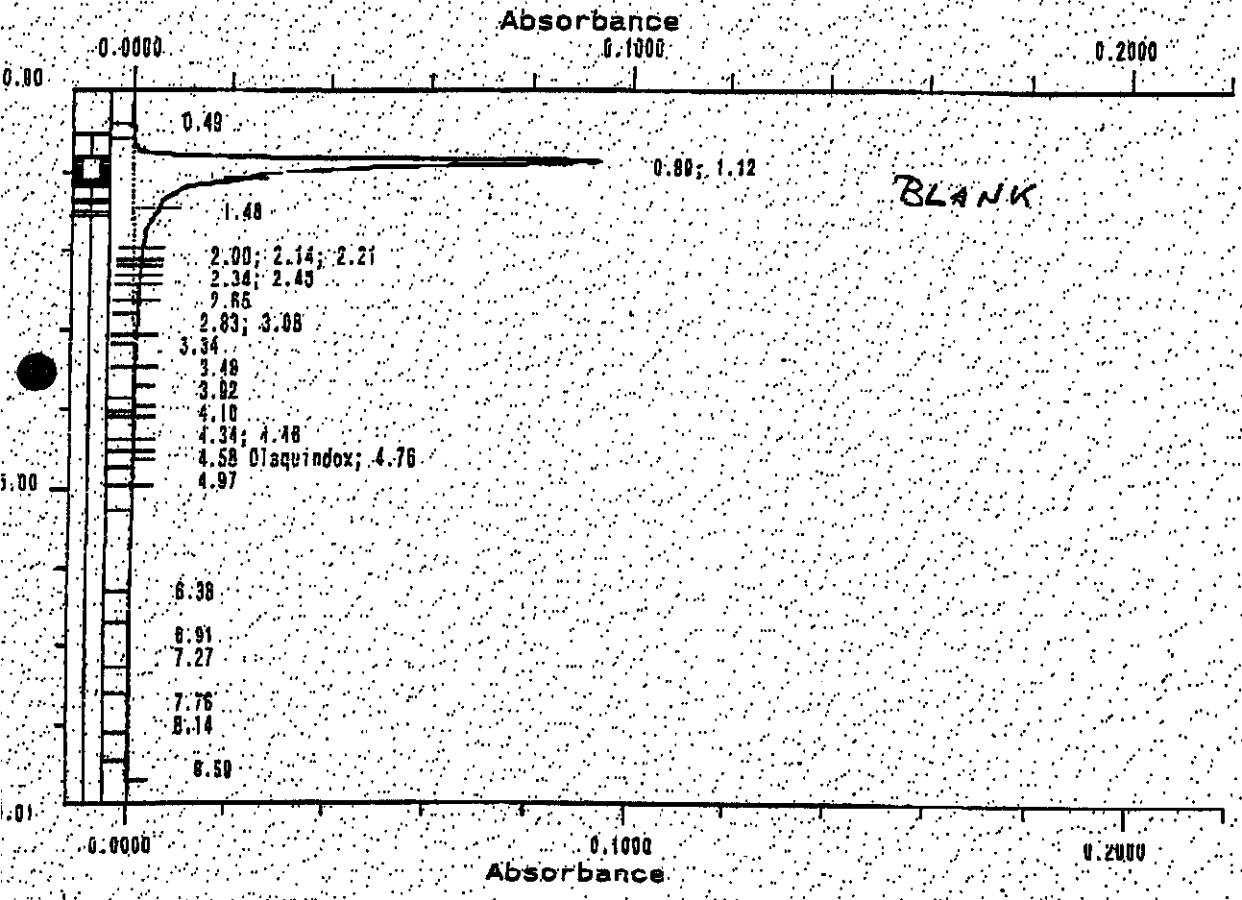
Chart Speed 1.00 cm/min



	NAME	CHAN	LEV	REP	TYPE	DIRECTORY		TIME	DATE
LLECTION DATA	BL9	A	1	1	Orig	C:\GOLD\SYS1DATA\OLAQUIND\	INJECTION	13:44:02	9 NOV 2000
METHOD	OLAQUIND					C:\GOLD\SYS1METH\	ANALYSIS	13:53:19	9 NOV 2000
							REPORT	11:37:47	21 DEC 2000

MPLE TABLE SAMPLES C:\GOLD\SAMPLTBL\
STEM 1: SYSTEM1

Chart Speed 1.00 cm/min



APPENDIX 7

Result of special requests

of

Masterlab, Putten, The Netherlands

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 01-12-2000

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: 20 µl
- Retention time of olaquindox: 6,3 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: 89,8%
- Single / duplicate determinations: □ single ■ duplicate
- If duplicate, please give both percentages: 97,7% and 81,8 %
- Spiking level: 4 mg/kg

CANFAS code 5714

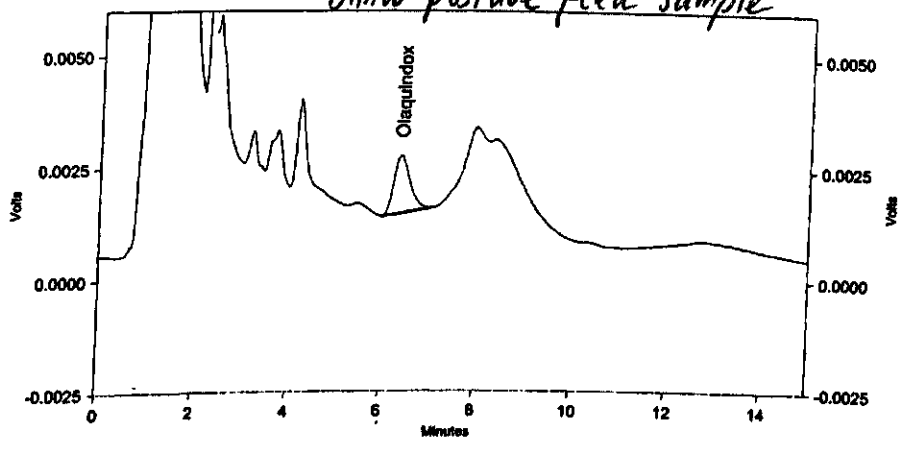
Olaquindox

Monster: 6 450558

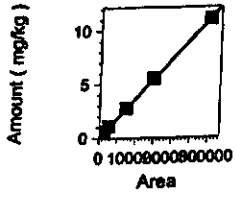
Gebruiker : asc
Runtijdstip: 12-01-2000 18:52:21
Inweeg : 24.9698
Verdunning: 100

Instrument : UV_5
Methode : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Olaquindox\Method\Olaquindox.met
File : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Olaquindox\Data\olaquindox_011200_015
Sequence : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Olaquindox\Sequence\Olaquindox.seq

Blind positive feed sample



sak: Olaquindox -- ESTD -- UV-Detect



UV-Detector Results

	Pk #	Retention Time	Area	Height	ESTD concentration	Units
Olaquindox	1	6.37	30387	1294	4.46817	mg/kg

Olaquindox

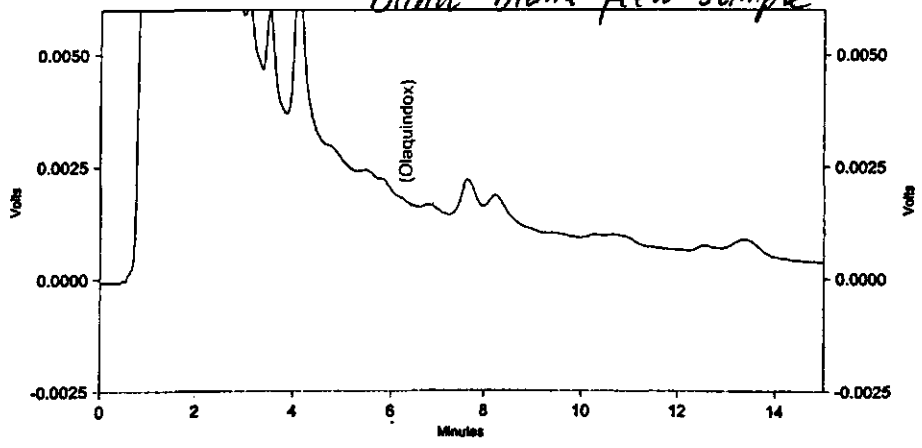
Monster: 1 450556

Gebruiker : asc
Runtijdstip: 12-01-2000 17:34:56
Inweeg : 25.4734
Verdunning: 100

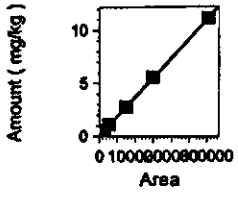
Instrument : UV_5
Methode : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Olaquindox\Method\Olaquindox.met
File : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Olaquindox\Data\olaquindox_011200_010
Sequence : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Olaquindox\Sequence\Olaquindox.seq

7,5 PPM

Blind blank feed sample



Peak: Olaquindox -- ESTD -- UV-Detect



UV-Detector Results

	Pk #	Retention Time	Area	Height	ESTD concentration	Units
Olaquindox					0.00000 BDL	mg/kg

APPENDIX 7

Result of special requests

of

National Veterinary Institute, Uppsala, Sweden

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 001117 / 001120

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: Hypersil C18 ODS BDS 250×4.6 mm ; 5µ
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.3 ml/min
- Injection volume: 50 µl
- Retention time of olaquindox: 7.8 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: 96 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 95 % and 96 %
- Spiking level: 3.3 mg/kg

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

********** 11-20-2000 09:27:10 Version 5.1 **********

Data File: D:BAYO051

* Sample Name: prov nr 1
* Date: 11-19-2000 15:09:24 Method: D:BAYONOX 11-20-2000 09:26:09 # 222*
* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *
* Starting Peak Width: 15 Threshold: 1 Area Threshold: 200 *

Starting Delay: 0.00
Area reject: 200
Amount injected: 50.00
Sample Weight: 1.00000

Ending retention time: 15.00
One sample per 0.200 sec.
Dilution factor: 1.00

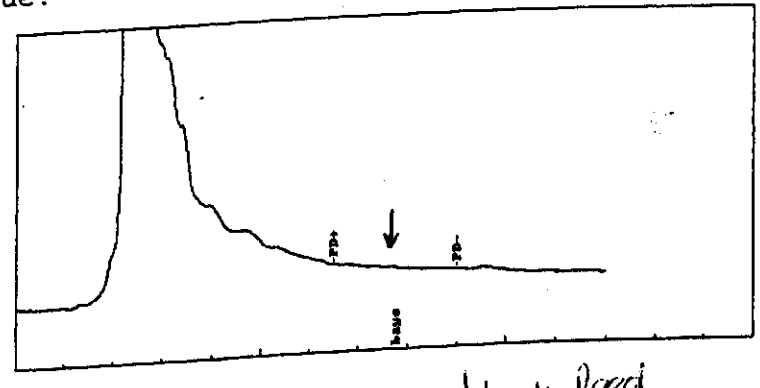
PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in ug/ml	NORMALIZED CONC	AREA	AREA/ HEIGHT	HEIGHT BL	REF PEAK	% DELTA RET TIME	CONC/AREA

TOTAL AMOUNT = 0.0000

PEAKS NOT FOUND IN THIS RUN

NAME	ADJUSTED RET.TIME.	REFERENCE PEAK
bayo	7.77	bayo

Areas, times, and heights stored in: D:BAYO051.ATB
Data File = D:BAYO051.PTS Printed on 11-20-2000 at 09:27:12
Start time: 0.00 min. Stop time: 15.00 min. Offset: 0 mv.
Low Value: 0 uv High Value: 133401 uv Scale factor: 5.0



Hank Peed

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

********** 12-05-2000 14:00:30 Version 5.1 **********

* Sample Name: prov nr 5 Data File: D:BAYO047 *

* Date: 11-19-2000 14:12:10 Method: D:BAYONOX 12-05-2000 13:59:28 # 235*

* 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	AREA/ HEIGHT	HEIGHT BL	REP PEAK	% DELTA RET TIME	CONC/AREA
1	7.774	bayo	0.4249	100.0000%	28933	1689	17.1 1	1	0	1.4684E-05

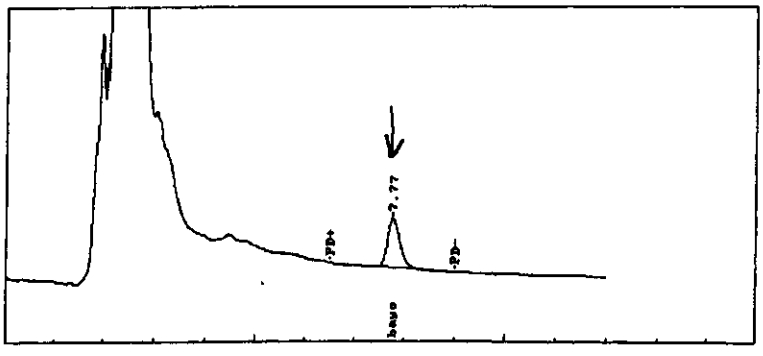
TOTAL AMOUNT = 0.4249

Areas, times, and heights stored in: D:BAYO047.ATB

Data File = D:BAYO047.PTS Printed on 12-05-2000 at 14:00:32

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

Low Value: 0 uv High Value: 47888 uv Scale factor: 5.0



2 ppm sample

omatograms of:

86. %