Projectnr.: 71.316.24
Development and Validation of HPLC-methods for the official control of Coccidiostatics and Antibiotics used as Feed AdditiveS (SMT4-CT98-2216)

Coordinator: Dr. J. de Jong

FINAL REPORT

Report 2002.013 August 2002

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

J.J.M. Driessen, M.J.H. Tomassen, J. de Jong

Business Unit: A&O (Analysis and Development)

State Institute for Quality Control of Agricultural Products (RIKILT)
Bornsesteeg 45, 6708 PD Wageningen
P.O.box 6700 AE Wageningen
Phone +31 317-475400
Fax +31 317-417717

MAILING LIST

INTERNAL:
director
authors
program leaders (4x)
Marketing and Communication (2x)
library (3x)
J.A. van Rhijn

EXTERNAL:
Participants
Mrs. D. Ramaekers, European Commission, M&T program, DG Research
F. Verstraete, European Commission, DG SANCO
A. Thalmann, LUFA Augustenberg
H.J. Keukens, LRVV
Secr. CEN/TC 327 Animal Feedingstuffs; ISO/TC34/SC10, O.J.M. Kolsteren, NEN
AOAC - Methods Committee on Feeds, Fertilisers and Related Agricultural Topics, M.R. Coleman (chair) and L. Wetzler (secretary)
AAFCO Laboratory Methods and Services Committee, N. Thiex
H. Campbell, Canadian Food Inspection Agency
P. de Vries, Pre-Mervo
H. van der Voet, Biometris, Wageningen UR

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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 carbadox in piglet feeds

Results / Product	Declared content (mg/kg)	Measured content (mg/kg)	Homogeneity results	
			Between sample CV (%)	Within sample CV (%)
Piglet feed	2,5	1,5	6,8	5,3
Piglet feed	10	9,1	3,8	5,2

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 carbadox 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 equilibrated with water and extracted with methanol-acetonitrile. An aliquot portion of the filtered extract is subjected to clean-up on an aluminium oxide column. The content of carbadox is determined by reversed-phase high performance liquid chromatography (HPLC) with UV-detection at 365 nm.

The samples that were tested in the collaborative study were 2 piglet feeds with declared carbadox contents of 2,5 and 10 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 20 laboratories. Statistical evaluation was performed according to ISO 5725.

The results of the collaborative study were evaluated in a meeting attended by the participants. The panel has accepted the results of the statistical evaluation. One laboratory is a Cochran outlier for the 2,5 mg/kg sample. The repeatability and reproducibility of the method is acceptable. The results obtained for the blind blank feeds and for the recovery are also acceptable.

The panel agreed that the method can be recommended for adoption as an official 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 carbadox (Directive 1999/27/EC) has been validated for low contents in feeds. Carbadox is a growth promoter that was registered for use in feeds for piglets with contents ranging from 20 - 50 mg/kg. Since September 1999, the use of carbadox 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 2 mg/kg.
The method was validated by LUFA - Augustenberg, Karlsruhe, Germany. Compared with the original method, only the concentration of the standard solutions was adapted (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 sometimes lower than 80 % (down to 72 %) but, while the use of carbadox 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 carbadox. 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 carbadox 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 carbadox contents of 2,5 and 10 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 carbadox 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.

- 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, K. Needman
- 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, Barth
- Masterlab, Putten, The Netherlands; K. van Schalm, A. Schaaf.
- Plant Production Inspection Centre Agricultural Chemistry Department, Vantaa, Finland; R. Muhonen, T.Heikkinen
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Bral, R. van San
- RIKILT, Wageningen, The Netherlands; H.J. van der Kamp, H.C.H. Kleijnen
- State Laboratory Dublin, Ireland, P. Shearan, A. Cunningham.
- 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	2,5	mg/kg	IPC – Dier, Barneveld (NL)	05/09/2000
Piglet feed	10	mg/kg	IPC – Dier, Barneveld (NL)	05/09/2000

The feed sample with 2,5 mg/kg carbadox also contained 7,5 mg/kg olaquindox, the feed sample with 10 mg/kg carbadox also contained 2 mg/kg olaquindox. 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 carbadox, 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 which contained carbadox, a blind blank feed was sent to the participants as well as a blank feed labelled "blank feed for carbadox 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 no detectable amounts of carbadox or interfering substances. The blank feed for carbadox 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 carbadox 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 carbadox in piglet feeds

Results / Product	Declared content (mg/kg)	Measured content (mg/kg)	Homogeneity results	
			Between sample CV (%)	Within sample CV (%)
Piglet feed	2,5	1,5	4,8	5,3
Piglet feed	10	9,1	2,7	5,2

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. 3E121-84QCS) is 99,3 %. 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 January 2000. For this reason 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 column, 250 mm x 4,6 mm with a particle size of 5 µm).
The mobile phase described in the method is acetate buffer 0,01M, pH 6 : acetonitrile (825:175 (v/v)). Four 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

a) Graphical consistency techniques: Mandel's h plot for between-laboratory variability, Mandel's k plot for within-laboratory variability
b) 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 C18	Sodium acetate buffer 0,01M, pH 6 : acetonitrile = 86:14 (v/v)
16	Spherisorb ODS 1, 5 µm, 250 x 4 mm	As described in the method
17	Spherisorb S10 ODS-1 10 µ	As described in the method
18	250 x 4,6 mm; 5 µm; Sperisorb ODS 2 C18	As described in the method
20	Alltech Alltima C18, 250x4,6 mm 5 µm	As described in the method
21	Supercosil LC 18 25 cm x 4,6 mm (5 µm) + supelguard LC18	Gradient elution of acetonitrile and ammoniumacetate buffer 0,01M, pH 4,6
23	Not reported	
24	250 mm x 4,6 mm C18 5µm	As described in the method
26	Luna ODS-2 250x4,6 mm, 5µm	As described in the method
27	As described in the method	As described in the method
29	Nova Pack, 250 x 4,6 mm; C18; 4 µ	As described in the method
31	As described the method	Acetate buffer:acetonitrile = 850:150 (v/v)
32	Waters symmetry C18, 5 µm, 4,6x250 mm	As described in the method
33	As described in the method	Other mixing ratio: Buffer : acetonitrile = 80:20 (v/v)
34	As described in the method	As described in the method
35	Lichrosper RP-18 5 mm (125 x 40 mm)	As described in the method
37	Lichrosper RP-18 5 endcapped	As described in the method
38	Hypersil ODS C-18, 250 x 4,6 mm, 5 µm	As described in the method
41	Lichrosorb RP 8, 5 µm, 250 mm x 4mm	As described in the method

5 RESULTS

5.1 Statistical evaluation

The results reported by the participants are given in Table 6. The corresponding Mandel h and k plots are shown in Figure 1.
Statistical analysis of the results showed that lab 38 is a Cochran outlier for the 2.5 mg/kg sample. The resulting 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 carbadox collaborative study

Mean after elimination of outliers[1] (mg/kg)	Predicted rsd_R	Established rsd_R	Horrat[2]	Conclusion
1,821	14,620	22,910	1,57	Reproducibility OK
7,646	11,780	14,230	1,21	Reproducibility OK

[1] = lab 38/sample 2.5 mg/kg

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

The Mandel h and k plots, excl. lab 38 (2,5 mg/kg) are shown in Figure 2.

Table 6: Carbadox in two piglet feeds

Table 6. Carbadox in two piglet feeds

	Result (mg/kg)							
Sample	CARB 2,5 mg/kg	CARB 2,5 mg/kg	CARB 2,5 mg/kg	CARB 2,5 mg/kg	CARB 10 mg/kg	CARB 10 mg/kg	CARB 10 mg/kg	CARB 10 mg/kg
Lab								
12	2,50	2,45	2,30	2,48	8,62	8,93	8,48	8,53
15	1,73	1,66	1,65	1,54	7,18	7,24	6,76	6,91
16	1,89	1,82	1,82	1,83	8,32	8,40	7,99	8,26
17	2,33	2,19	2,21	2,24	8,99	8,86	8,50	8,60
18	2,40	2,22	2,40	2,58	9,42	9,52	9,15	9,76
20	1,91	1,94	1,86	1,84	7,15	7,14	7,16	6,74
21	2,40	2,30	2,20	2,20	8,60	8,80	8,70	8,50
23	1,40	1,34	1,61	1,50	6,99	6,63	6,99	6,84
24	2,00	2,00	2,10	2,10	7,40	7,30	7,40	7,50
26	1,50	1,70	1,80	1,80	8,90	8,00	8,00	8,30
27	1,77	1,80	1,97	2,06	8,18	8,24	8,26	8,21
29	1,80	1,90	1,60	1,70	7,10	7,00	7,20	7,00
31	1,24	1,19	1,28	1,27	6,96	7,14	7,22	7,15
32	1,46	1,62	1,59	1,56	8,50	8,62	8,84	8,92
33	1,30	1,50	1,20	1,30	5,10	4,70	5,60	5,80
34	1,20	1,10	1,10	1,10	6,20	6,20	6,20	6,30
35	1,70	1,70	1,70	1,60	7,90	7,80	7,40	7,40
37	2,40	2,56	2,51	2,57	8,36	8,76	9,27	8,67
38	*1,40 Co*	*1,53 Co*	*2,01 Co*	*1,95 Co*	7,17	7,24	7,10	7,55
41	1,55	1,58	1,58	1,61	6,44	6,55	6,00	6,00

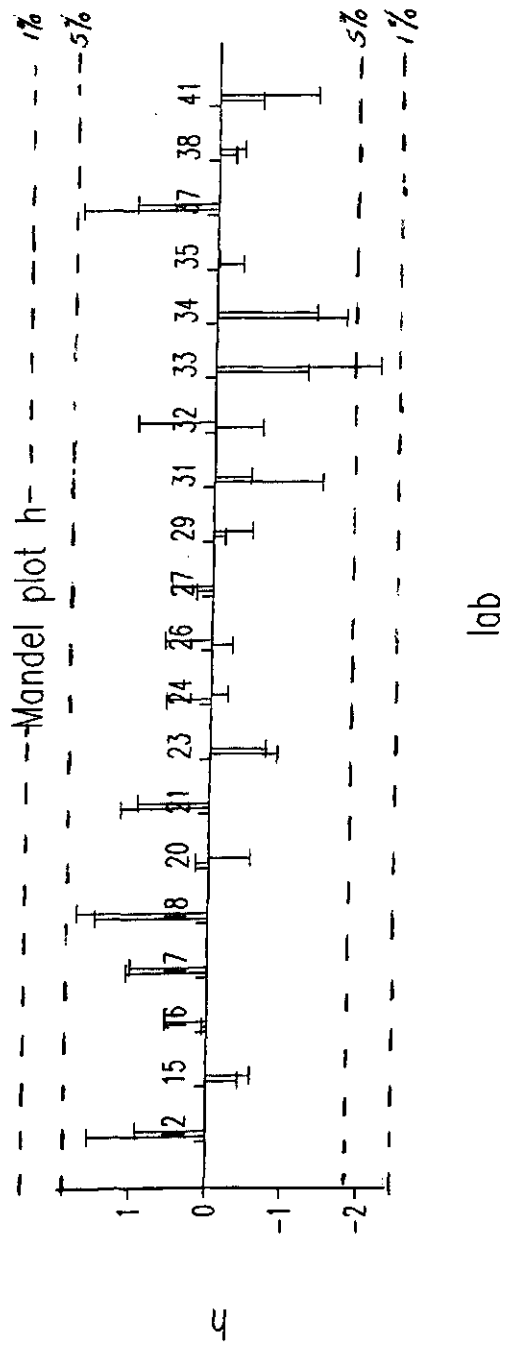
number of labs	19	20
m (mg/kg)	1,821	7,646
rsd_r (%)	5,03	3,15
rsd_R (%)	22,9	14,2

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

Key to symbols :

result^{Co} = Cochran outlier

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



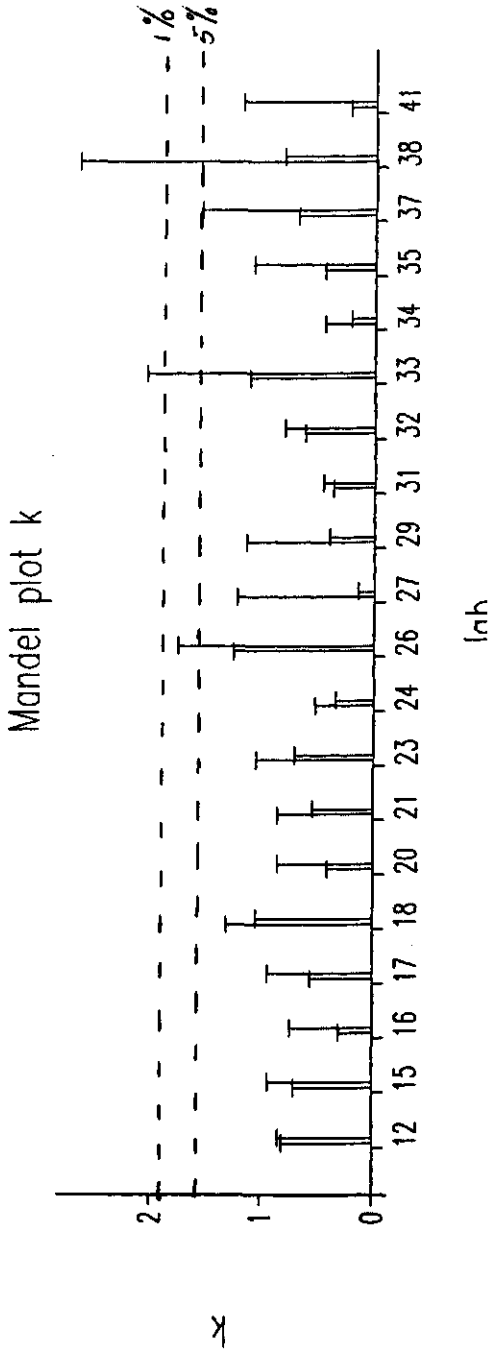
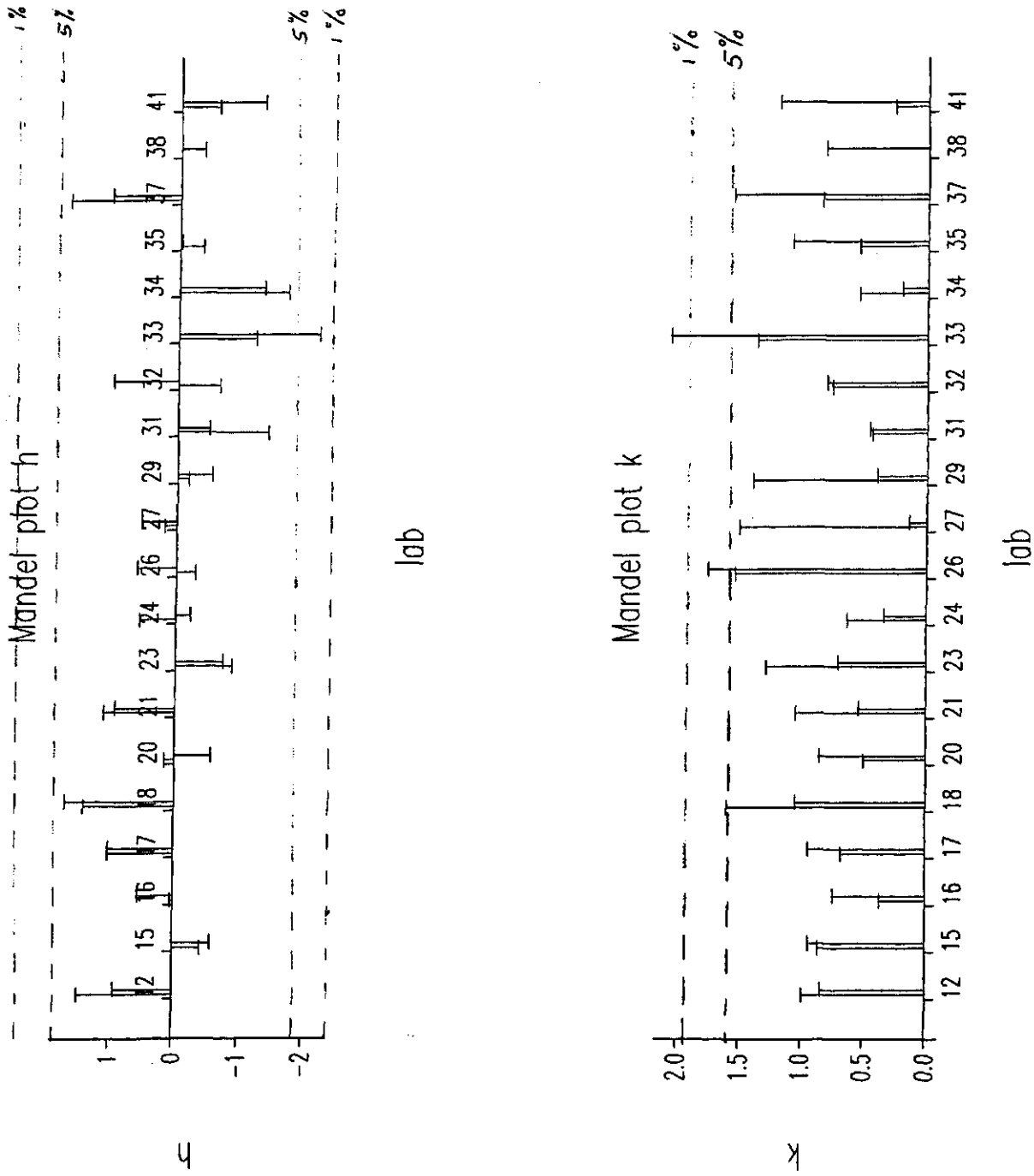


Figure 2: Mandel h and k plots after elimination of laboratory 38, sample 2,5 mg/kg



5.2 Blank samples

Table 7: Reported results of the participants for the blank samples

Partner	Blank sample 1		Blank sample 2	
	Result 1	Result 2	Result 1	Result 2
12	N.S.D.	N.S.D.	N.S.D.	N.S.D.
15	Blank	blank	blank	Blank
16	Not found	Not found	Not found	Not found
17	0	0	0	0
18	Not detected LOD<0,4	Not detected LOD<0,4	Not detected LOD<0,4	Not detected LOD<0,4
20	Neg	neg	neg	Neg
21	0,6 (no spectrum)	0,6 (no spectrum)	0,6 (no spectrum)	0,6 (no spectrum)
23	<0,1	<0,1	<0,1	<0,1
24	Not detectable	Not detectable	Not detectable	Not detectable
26	0	0	0	0
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
35	<0,1	<0,1	<0,1	<0,1
37	ND	ND	ND	ND
38	0	0	0	0
41	0	0	0	0

Only lab 21 measured a small peak in the chromatogram, see also Remarks, Table 9.

5.3 Recoveries

Table 8: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	Recovery in %
12	99		99
15	95	94	95
16	95	96	95
17	96	100	98
18	100	103	102
20	100	100	100
21	99	98	99
23	Not reported		
24	85	85	85
26	89	80	84
27	90	92	91
29	94	97	96
31	81	83	82
32	101	100	101
33	84	86	85
34	86	88	87
35	101	99	100
37	109	109	109
38	99	97	98
41	100	102	101

The recoveries were consistently higher than 80%. These results are better than the results of the between-lab validation where in some cases recoveries between 70% and 80% were measured.

5.4 Remarks

Table 9: Remarks made by the partners

Partner	Remarks
12	No remarks
15	No remarks
16	Using mentioned HPLC conditions, we observed 'negative peaks' in the chromatogram after about 41 minutes (see chromatograms). This negative peaks might be caused by different composition of HPLC mobile phase (with acetate buffer) and extraction solvent
17	No remarks
18	HPLC System: Thermo Quest System (pump; autosampler; dual wavelength PC 1000 logicial; detector) Difference(s) with CANFAS/CARB/2209200: • par 5.2 extraction: The solution was not filtered through a folded filter MN619 G ¼ but centrifuged before purification step. • Calibration graph without the 2 µg/ml point because of an error of dilution in our laboratory • Receive of package of samples on October 5, 2000; storage of samples until analysis at < 8 °C in a refrigerated room • 3.11.1 stock standard solution: 25 mg/250 ml weigh to the nearest 1 mg Results • result 1 = average (result 1 height + result 1 area)/2 • result 2 = average (result 2 height + result 2 area)/2 • calibration from the height and the area (8 points, forced through origin) DAD detection The same extracts from the CANFAS clean-up were reinjected on a DAD-HPLC system (with same column), after 1 night in freezer. ⇒ loss of carbadox contents in sample extracts (-2 to -80 %) ⇒ identify confirmation OK (see Appendix 6)
20	No remarks

Partner	Remarks
21	We used a gradient elution because using your chromatographic conditions (as described in the familiarisation method) the retention time of carbadox wasn't stable (from 7,9 min to 8,4 min). Instead of glass columns with an internal diameter of 10 mm, we used plastic reservoirs with internal diameters of 12 mm. Also in the blank feed you sent us, we noted a peak with the same RT of carbadox We tried to quantify this peak and the amount was less than 1 mg/kg. We couldn't have a significant spectrum with this small amount we noted these peaks also in the blind blank samples.
23	Not reported
24	In the case of the analysis of the sample unit number 241162 and 241173 the background effect due to the matrix did not enable any quantification. Is this a specific problem of this sample, or could it occur in other circumstances when the feed has a similar composition?
26	We experienced no problems with the method
27	1. If you want to quantify carbadox concentrations of 2 mg/kg in feedingstuffs, the calibration curve should begin with a carbadox solution containing 0,25 µg/ml 2. As to the description of the procedure of the method the numbers of the reagents often do not correspond to the numbers in the paragraph "Reagents" 3. As to the sample-code number 271115, there additionally exist two results from two single determinations made on two different days (2,03 mg/kg and 1,78 mg/kg), this is why they have not been taken into consideration.
29	To quantify the feed samples 29108 and 29222, it was necessary to do a calibration on solutions lower than 0,5 µg/ml (0,25 µg/ml) (3.11.2) So, we made another calibration curve: 0,25; 0,5; 1 and 2 µg/ml.
31	Because of the limits of the deuteriumlamp in the UV-detector, the wavelength used was 360 in stead of 365 nm. Runtimes was 1 hour because of matrix interference. Al_2O_3 of ICN (article 02090) was used.
32	No remarks
33	The flow-rate for the method was too high with regards to the back pressure on the column
34	No remarks
35	No remarks
37	No remarks
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. Note from co-ordinator: detection at 365 nm was applied, so the method was followed
41	No remarks

5.5 Special requests

5.5.1 SPE clean-up

The following partners performed the clean up step on SPE Neutral Alumina column:

- Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy; G. Brambilla, C. Cartoni, M. Fiori.
- LNIV, Lisbon, Portugal; J.M. Nunes da Costa, M.B. Casqueira.

5.5.1.1 HPLC conditions

Table 10: HPLC conditions

Partner	HPLC column	Mobile phase
Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy	As described in the method	Acetate buffer: acetonitrile = 80:20 (v/v)
LNIV, Lisbon, Portugal	Waters symmetry C18, 5 µm, 4,6x250 mm	As described in the method

5.5.1.2 Recoveries

Table 11: Recoveries

Partner	Recovery 1 in %	Recovery 2 in %	Recovery in %
Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy	97		97
LNIV, Lisbon, Portugal	98	95	97

5.5.1.3 Remarks

Table 12: Remarks made by the partners

Partner	Remarks
Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy	We performed the clean up step on SPE Neutral Alumina columns (1g) from Mallincrodt-J.T. Baker. To this purpose, the amount of extract loaded on such columns have been reduced of 75% (3,75 ml loaded instead of 15,00 ml; discharged the first 0,50 ml; collected the next 1,25 ml, filtered on 0,45 µm disposable.
LNIV, Lisbon, Portugal	The samples were purified by clean-up with solid phase extraction (SPE) columns: J.T. Baker, Ref: 7214-07. After extraction, the solution obtained under 5.2 was purified on SPE Alumina Neutra columns and 1 ml was collected and filtered through a 0,45 µm filter for further HPLC determination.

5.5.1.4 Samples

Table 13: Reported results of the analysed samples

Partner	Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy		LNIV, Lisbon, Portugal	
Sample content (mg/kg)	Result 1	Result 2	Result 1	Result 2
0	Nd	Nd	negative	Negative
0	Nd	Nd	negative	Negative
2,5	2,40	2,40	3,74	3,83
2,5	2,40	2,40	3,81	3,81
10	8,10	8,00	7,98	8,39
10	8,00	7,95	8,31	8,43

The results for the sample with a declared content of 10 mg/kg are in line with the results obtained with the CANFAS-method. The results obtained by LNIV for the sample with a declared content of 2,5 mg/kg are too high. For this reason the applicability of SPE columns should be studied in more depth before the use of these SPE columns can be recommended in the method.

Representative chromatograms of the analysed samples and the questionnaires are presented in Appendix 7.

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. Laboratory 38 is a Cochran outlier for the 2,5 mg/kg sample.
Consequently it can be concluded that the repeatability and reproducibility of the method is acceptable. The results obtained for the blind blank feeds and for the recovery are also acceptable.
The panel agreed that the method can be recommended for adoption as an official method.
Minor problems were encountered with interfering peaks in the blind blank feed. Due to these interfering peaks, the limit of detection described in the method (0,1 mg/kg) may not be realistic for all types of samples. The results of task 1, where different types of samples have been analysed, will be reviewed.
The use of a gradient system cannot be recommended because this gives rise to many interfering peaks (see results of lab 21). For this reason the isocratic mode will be prescribed in the method.
The results obtained for the blind blank feed indicate that different columns lead to differences in interfering peaks (large peak eluting prior to carbadox). The following columns will be recommended in the method: Spherisorb ODS 1, 5 µm, 250 x 4 mm and Spherisorb S10 ODS-1, 10 µm, 250 x 4 mm.
Based on the results, the use of SPE alumina columns cannot be recommended in the method as an alternative to the use of the handpacked alumina columns.
The following remarks, related to the method description have been accepted (see par. 5.4 of this report):

- Lab 27, remarks 1 and 2

The method description will be modified and the final method, together with the results of the collaborative study will be sent to the European Commission (CEMA), CEN and ISO.

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 carbadox 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
carbadox 71.316.24

ENCLOSURE(S)
5

OUR REFERENCE
00/0022092

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 carbadox:

- 6 feed samples, with the text "additive: CARBADOX" and with a sample code; these samples constitute 2 blind duplicates of feed samples containing carbadox (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 carbadox 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.

Annex 5 contains information about special requests. We hope that, next to the regular determinations, you are prepared to volunteer to do some extra work.

The reference standard of carbadox which has to be used (3E 121-84 QCS) 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

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

RIKILT
State Institute for Quality Contr of Agricultural Products

DATE
2 October 2000

OUR REFERENCE
00/0022092

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 1 - Description of the method

CANFAS/CARB/22092000/K.MICHELS

SMT4-CT98-2216

Determination of low level contents of Carbadox in feeding stuffs

1. Purpose and scope

This method is for the determination of low contents of carbadox in feedingstuffs. The limit of determination (=quantification) is 2 mg/kg. The limit of detection (=qualification) is 0,1 mg/kg.

2. Principle

The sample is equilibrated with water and extracted with methanol-acetonitrile. For feedingstuffs, an aliquot portion of the filtered extract is subjected to clean-up on an aluminium oxide column. The content of carbadox is determined by reversed-phase high performance liquid chromatography (HPLC) using a UV detector.

3. Reagents

3.1 Methanol

3.2 Acetonitrile, HPLC grade

3.3 Acetic acid, w = 100 %

3.5 Aluminium oxide: neutral, activity grade I, 70 – 230 mesh or 0,063 – 0,2 mm ASTM, e.g. Merck, Art.-No. 1077

3.6 Methanol-acetonitrile 1 + 1 (v + v):
Mix 500 ml of methanol (3.1) with 500 ml of acetonitrile (3.2).

3.7 Acetic acid σ = 10 %:
Dilute 10 ml of acetic acid (3.3) to 100 ml with water.

3.8 Sodium acetate, CH_3COONa

3.9 Water, HPLC grade

3.10 Acetate buffer solution c =0.01 mol/l, pH 6.0:
Dissolve 0.82 g of sodium acetate (3.7) in 700 ml of water (3.8) and adjust the pH to 6.0 with acetic acid (3.6). Transfer to a 1000 ml graduated flask, make up to the mark with water (3.8) and mix

3.11 Mobile phase for HPLC:
Mix 825 ml of acetate buffer solution (3.9) with 175 ml of acetonitrile (3.2). Degas the solution (e.g. by ultrasonification for 10 minutes).

3.12 Standard substance

Pure carbadox: Methyl-3-(2-quinoxalinylmethylene)carbazate - N^1,N^4-dioxide, E 850

3.11.1. Carbadox stock standard solution, 100 µg/ml (see Note 5. Procedure);

Weigh to the nearest 0.1 mg, 25 mg of carbadox standard substance (3.11) into a 250 ml graduated flask. Dissolve in methanol-acetonitrile (3.5) by ultrasonification (4.7). After ultrasonic treatment bring the solution to room temperature, make up to the mark with methanol-acetonitrile (3.5) and mix. Wrap the flask with aluminium foil or use amber glassware and store in a refrigerator. At this temperature of ≤ 4°C the solution is stable for 1 month.

3.11.2. Calibration solutions

Transfer 0.5, 1.0, 2.0, 5.0 and 10.0 ml of the stock standard solution (3.11.1) into a series of 100 ml calibrated flasks. Add 30 ml of water, make up to the mark with methanol-acetonitrile (3.5) and mix. Wrap the flask with aluminium foil. These solutions correspond to 0.5, 1.0, 2.0, 5.0 and 10.0 µg/ml of carbadox respectively. Calibration solutions must be freshly prepared before use.

4. Apparatus

4.1 Laboratory shaker or magnetic stirrer

4.2 Folded Filter (MN 619 G 1/4 or equivalent)

4.3 Glass column (length 300 to 400 mm, internal diameter approximately 10 mm) with sintered glass frit and draw-off valve.

Note: a glass column fitted with a stopcock or a glass column with a tapered end may also be used; in this case, a small glass-wool plug is inserted into the lower end and it is tamped down using a glass rod

4.4. HPLC equipment with injection system, suitable for injection volumes of 20 – 100 µl

4.4.1. Liquid chromatographic column: 250 mm x 4 mm, C18, 5 µm packing or equivalent

4.4.2. UV detector with variable wavelength adjustment or diode array detector operating in the range of 225 to 400nm

4.5. Ultrasonic bath

4.6. Membrane filter, 0.45 µm

5. Procedure

Note: Carbadox is light-sensitive. Carry out all procedures under subdued light or use amber glassware or glassware wrapped with aluminium foil.

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 carbadox nor interfering substances are present. The blank feed should be similar in type to that of the sample and on analysis carbadox or interfering substances should not be detected.

5.1.2. Recovery test

A recovery test should be carried out by analysing the blank feed (5.1.1) which has been fortified by the addition of a quantity of carbadox, similar to that present in the sample. To fortify at a level of 5 mg/kg, transfer 0.5 ml of the stock standard solution (3.11.1) to a 200 ml conical flask. Add 10 g of the blank feed, mix and wait for 10 minutes 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 carbadox, similar to that already present in the sample. Both samples are analysed. Calculate the analyte content in sample A and B and calculate the recovery by subtraction.

5.2. Extraction

Weigh to the nearest 0.01 g approximately 10 g of the sample and transfer to a 200 ml conical flask. Add 15.0 ml of water, mix, and equilibrate for 5 min. Add 35.0 ml of methanol-acetonitrile (3.5),stopper and shake for 30 min on the shaker or stir on the magnetic stirrer (4.1). Filter the solution through a folded filter (4.2). Retain this solution for the purification step (5.3).

5.3. Purification

5.3.1. Preparation of the aluminium oxide column

Weigh 4 g of aluminium oxide (3.4) and transfer it to the glass column (4.3).

5.3.2. Sample purification

Apply 15 ml of the filtered extract (5.2) to the aluminium oxide column and discard the first 2 ml of eluate. Collect the next 5 ml and filter an aliquot through a 0.45 µm filter (4.6).

Proceed to the HPLC determination (5.4).

5.4. HPLC determination

5.4.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided they yield equivalent results:

Liquid chromatographic column (4.4.1): 250 mm x 4 mm, C18, 5 µm packing or equivalent

Mobile phase (3.10): Mixture of acetate buffer solution (3.9) and acetonitrile (3.2), 825 + 175 (v+v)

Flow rate: 1.5-2 ml/min

Detection wavelength: 365 nm

Injection volume: 20 µl

Check the stability of the chromatographic system, injecting the calibration solution (3.11.2) containing 5.0 µg/ml several times, until constant peak heights (areas) and retention times are achieved.

5.4.2. Calibration graph

Inject each calibration solution (3.11.2) several times and measure the peak heights (areas) for each concentration. Plot a calibration curve using the mean peak heights or areas of the calibration solutions as the ordinates and corresponding concentrations in µg/ml as the abscisses.

5.4.3. Sample solution

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

6. Calculation of the results

From the height (area) of the carbadox peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.4.2).

6.1. Feedingstuffs

The content of carbadox w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times 50}{m} \quad [mg/kg]$$

in which:

c carbadox concentration of the sample extract (5.3.2) in µg/ml,

m mass of the test portion in g

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: CARBADOX

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
311153		
311154		
311181		
311183		
311201		
311203		

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

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 - CARBADOX

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 carbadox: ..... min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

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

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 !

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 5 - Special requests

Volunteers are asked to do the following *additional* work:

Clean-up of the feed samples with solid phase extraction (SPE)
Apply the following procedure in stead of par. 5.3:
Extracts are purified on SPE Alumina Neutra Columns (J.T. Baker).
1 ml is collected and filtered through a 0.45 µm filter.

Please report the results in a copy of annex 4 and give additional information on conditions. Please also include representative chromatograms.

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. : 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 3.00 sec
M.D. : ..73. l/h

Meel temp : ..35. °C koeldemp 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 Rikilt 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)		(7)	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 Rikilt		(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. : 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 : 15 °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 : **Carbadox**
Product : **Feed sample: 2.5 ppm**

Date of determination : September 22th, 2000

Sample	Content mg/kg	Duplicate average mg/kg
341015 A	1,5	1,5
341015 B	1,5	
341019 A	1,4	1,4
341019 B	1,4	
341014 A	1,6	1,5
341014 B	1,3	
341016 A	1,6	1,6
341016 B	1,6	
341012 A	1,5	1,5
341012 B	1,4	
341013 A	1,5	1,5
341013 B	1,4	
341017 A	1,4	1,4
341017 B	1,3	
341020 A	1,4	1,4
341020 B	1,4	
341018 A	1,5	1,5
341018 B	1,5	
341011 A	1,5	1,5
341011 B	1,5	

Homogeneity OK Criterion : $CV_{between}$ = < 20%		
Average	**1,5**	
SD (between samples)	**0,07**	
CV (between samples)	**4,8**	Result Grubb's test
Grubb's test, single lower	**1,573**	**no outlier**
Grubb's test, single upper	**2,002**	**no outlier**
Grubb's test, double lower	**0,5611**	**no outliers**
Grubb's test, double upper	**0,4261**	**no outliers**

Repeatability		
SD (within samples)	(sd_r)	**0,08**
CV (within samples)	(CV (%))	**5,3**

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 : **Carbadox**
Product : **Feed sample: 10 ppm**

Date of determination : September 22th, 2000

Sample	Content mg/kg	Duplicate average mg/kg
341025 A	8,6	8,9
341025 B	9,1	
341021 A	8,7	8,9
341021 B	9,0	
341029 A	9,5	9,0
341029 B	8,5	
341030 A	9,7	9,6
341030 B	9,5	
341026 A	8,4	9,2
341026 B	10,0	
341027 A	9,2	9,3
341027 B	9,3	
341022 A	9,6	9,3
341022 B	9,0	
341023 A	9,3	9,2
341023 B	9,1	
341024 A	8,8	8,9
341024 B	8,9	
341028 A	9,0	9,2
341028 B	9,4	

Homogeneity OK Criterion : $CV_{between}$ = < 10%		
Average	**9,1**	
SD (between samples)	**0,24**	
CV (between samples)	**2,7**	Result Grubb's test
Grubb's test, single lower	**1,153**	**no outlier**
Grubb's test, single upper	**1,935**	**no outlier**
Grubb's test, double lower	**0,6309**	**no outliers**
Grubb's test, double upper	**0,4331**	**no outliers**

Repeatability		
SD (within samples)	(sd_r)	**0,48**
CV (within samples)	(CV (%))	**5,2**

APPENDIX 4

Sample codes

Sample codes supplied to the participants in the carbadox collaborative study

Carbadox number of participants 22	CARB piglet 2,5ppm CARB 1a	CARB piglet 2,5ppm CARB 1b	CARB piglet 10ppm CARB 2a	CARB piglet 10ppm CARB 2b	VIRG bull 5ppm CARB blank 1a	VIRG bull 5ppm CARB blank 1b
Participant code						
12	121182	121152	121158	121204	121166	121219
14	141135	141220	141124	141126	141122	141227
15	151155	151117	151137	151192	151187	151207
16	161196	161118	161133	161163	161197	161157
17	171205	171128	171120	171161	171134	171186
18	181214	181198	181178	181123	181139	181143
20	201172	201194	201146	201232	201114	201229
21	211225	211145	211170	211107	211151	211105
23	231148	231195	231230	231144	231176	231199
24	241142	241218	241138	241106	241173	241162
25	251212	251168	251102	251121	251129	251164
26	261111	261184	261175	261228	261231	261165
27	271136	271115	271119	271109	271185	271209
29	291108	291222	291208	291159	291210	291132
31	311203	311153	311154	311181	311201	311183
32	321150	321149	321104	321190	321217	321103
33	331224	331130	331174	331140	331226	331206
34	341216	341221	341180	341147	341125	341167
35	351177	351113	351193	351200	351213	351188
37	371191	371169	371101	371223	371189	371179
38	381156	381160	381110	381116	381127	381202
41	411211	411215	411131	411171	411141	411112

APPENDIX 5

Carbadox reference standard profile, identity and purity

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

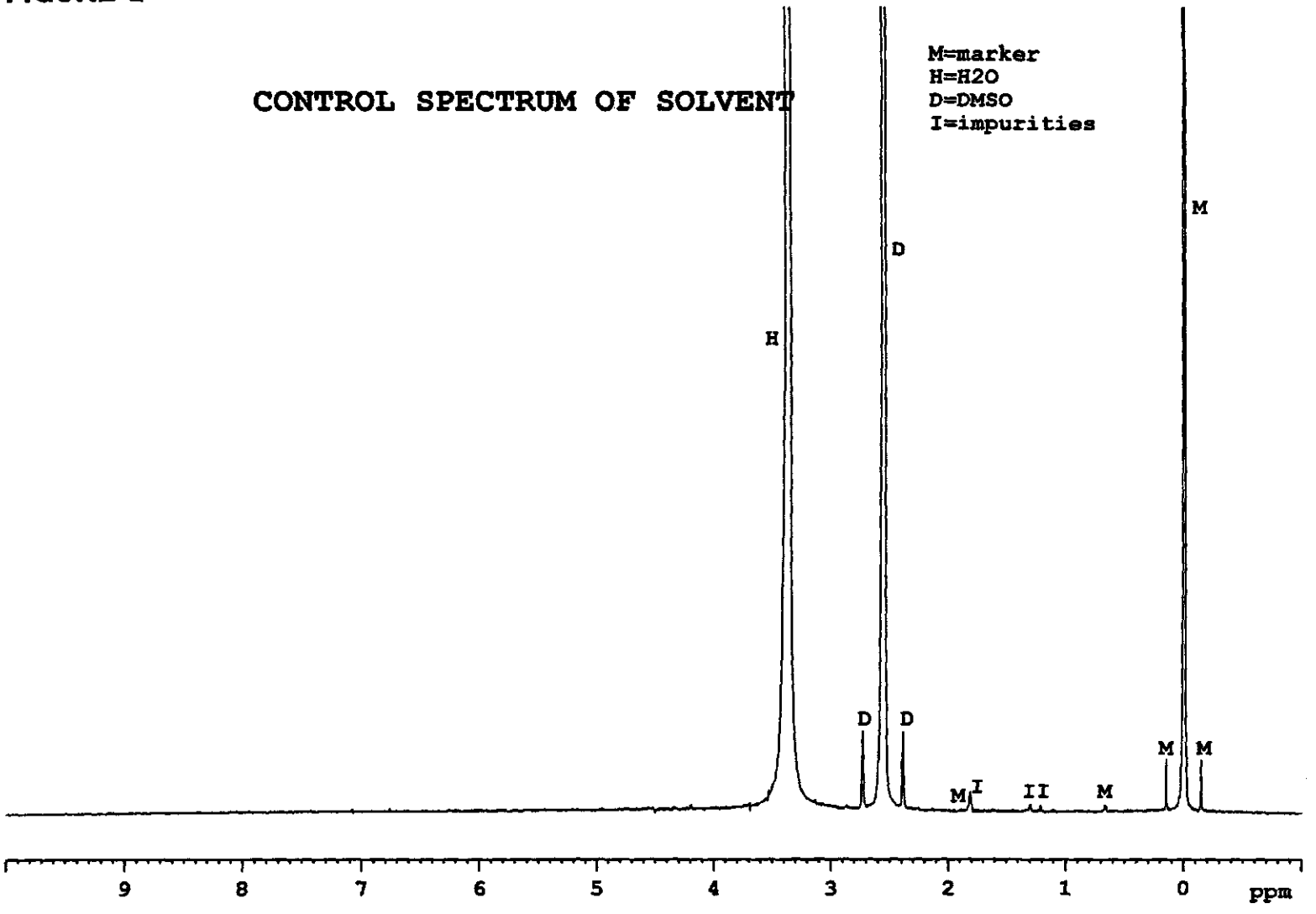

CONTROL SPECTRUM OF SOLVENT
M=marker
H=H2O
D=DMSO
I=impurities
H
D
D
D
M
M
M
M
I
II
M
9
8
7
6
5
4
3
2
1
0
ppm

FIGURE 2

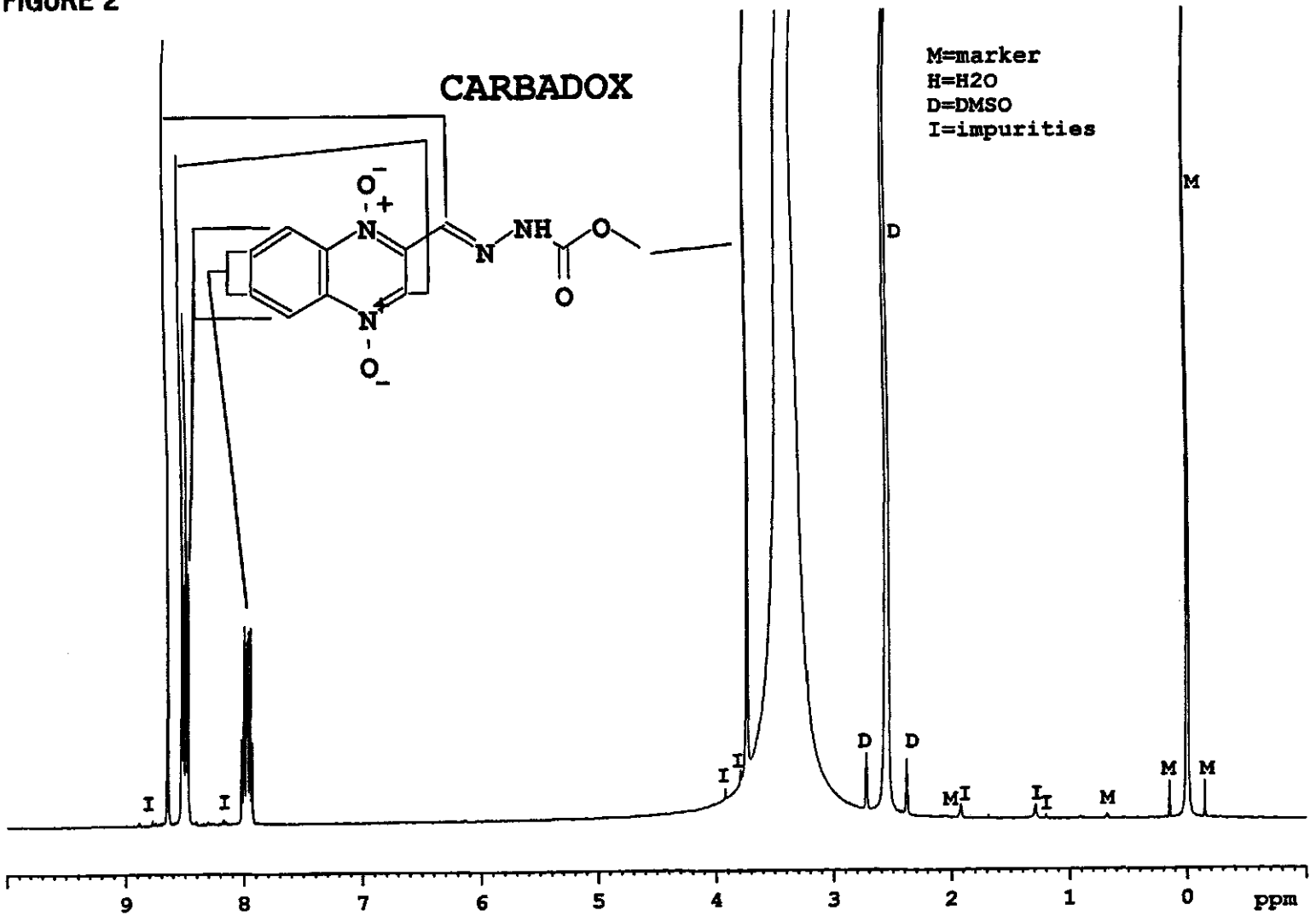
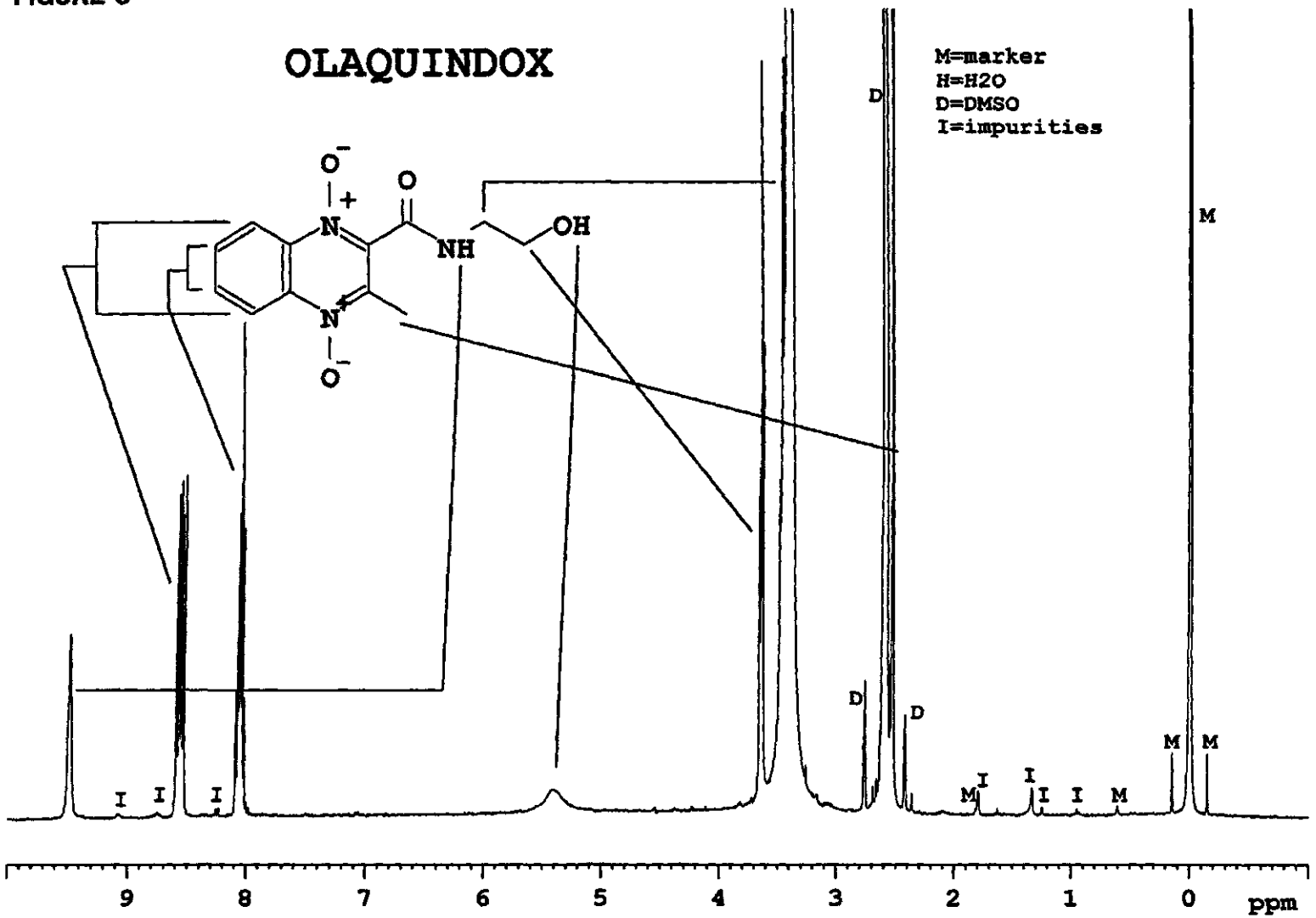


FIGURE 3


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

Carbadox

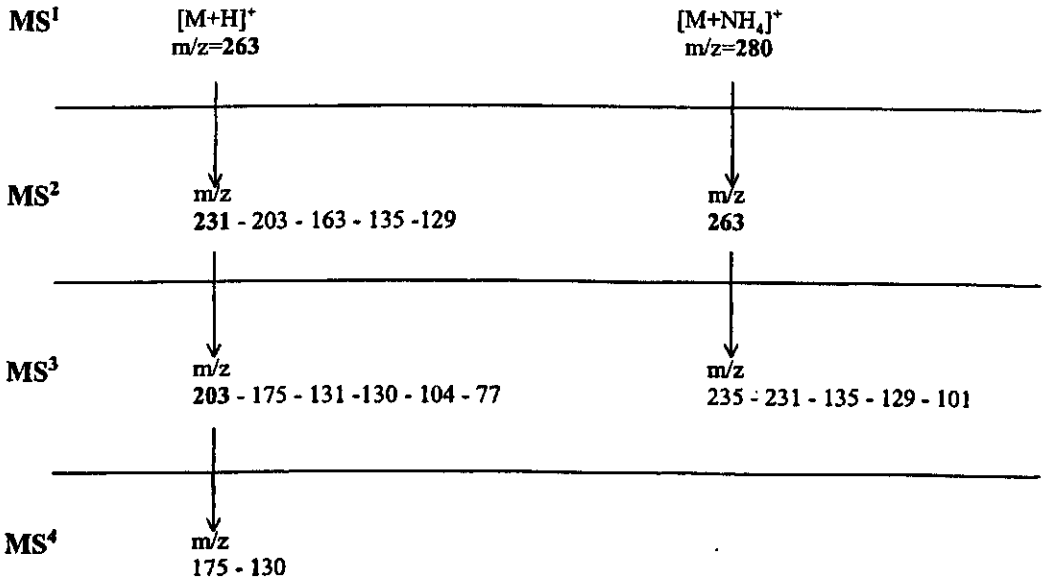


Figure 4 Schematic representation of the fragmentations observed for Carbadox in an MS^n experiment.

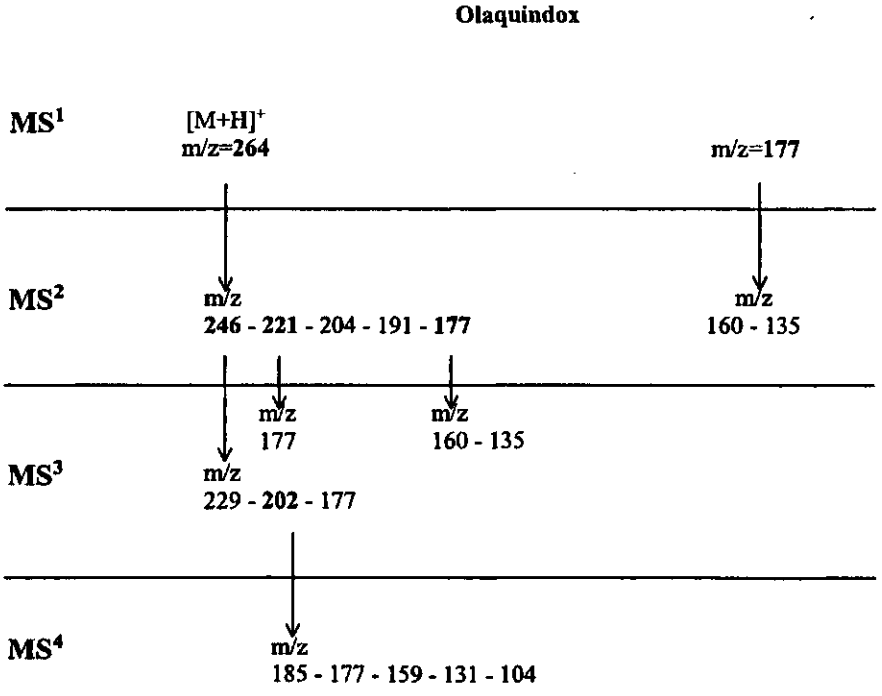


Figure 5 Schematic representation of the fragmentations observed for Olaquindox in an MS^n experiment.

Quality Control Division
Pfizer Inc
Eastern Point Road
Groton, CT 06340

CERTIFICATE OF ANALYSIS
REFERENCE STANDARD

November 16, 1995

CARBADOX
LOT 3E121-84QCS

Purity: 99.3% Carbadox when used as is.

PARAMETER	RESULT
Appearance	Yellow Powder
Identity (IR)	Conforms to prior standard
Total TLC Impurities	~0.7%
Residue-On-Ignition	<0.1%
Water Content	0.02%
UV Assay (versus prior standard)	101.4%
HPLC Assay (versus prior standard)	99.8%

Purity assignment is based on composition mass balance.

Note: Bottles are labeled 3E121-84QCS -XX, where XX represents the subdivision.

J. W. Thomas
Reference Standards Administrator

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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
121152	2,30	2,48
121158	8,62	8,93
121166	N.S.D.	N.S.D.
121182	2,50	2,45
121204	8,48	8,53
121219	N.S.D.	N.S.D.

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 28 Nov : 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: 30 µl
- Retencion time of carbadox: 5°30'

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

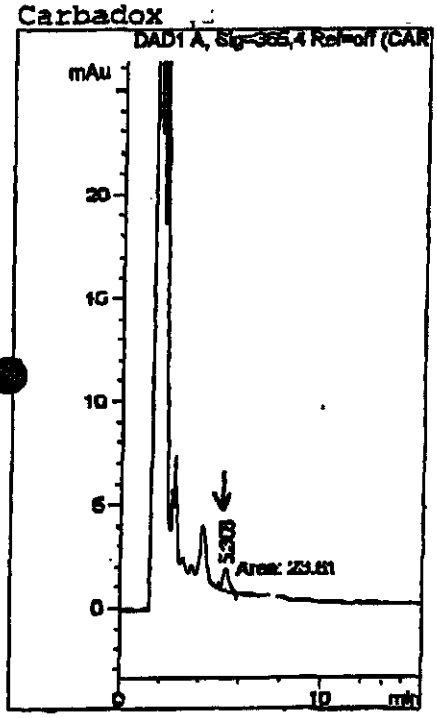
- Percentage recovery: 99%
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: .....% and .....%
- Spiking level: 5 mg/kg

Injection Date : 28/11/00 14:07:11
Sample Name : 00-8424a
Acq. Operator :

Seq. Line : 1
Vial : 6
Inj : 1
Inj Volume : 30 µl

Acq. Method : C:\HPCHEM\1\
Last changed : 28/11/00 14:05:04 by
Analysis Method : C:\HPCHEM\1\
Last changed : 29/11/00 11:41:18 by
(modified after loading)

Carbadox



CARBADOX 121152

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,4 Ref=off
Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	5.308	MM	0.3633	23.61002	1.08315	100.0000
Totals :				23.61002	1.08315	

*** End of Report ***

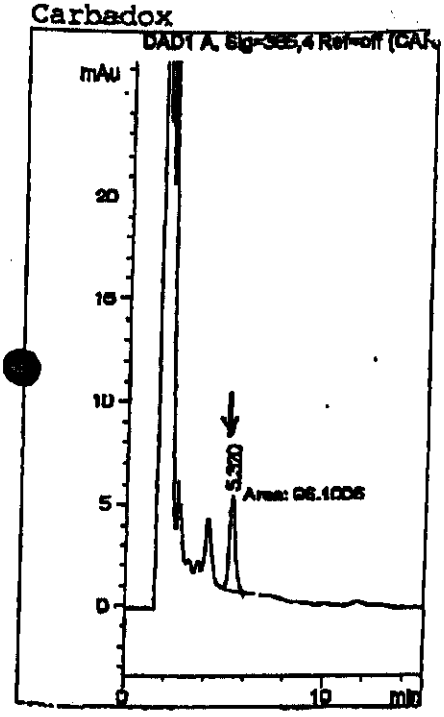
(12)

Injection Date : 28/11/00 14:40:14
Sample Name : 00-8425a
Acq. Operator :

Seq. Line : 3
Vial : 8
Inj : 1
Inj Volume : 30 µl

Acq. Method : C:\HPCHEM\1\
Last changed : 28/11/00 14:05:04 by a
Analysis Method : C:\HPCHEM\1\
Last changed : 29/11/00 11:41:18 by
(modified after loading)

Carbadox



CARBADOX 121158

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,4 Ref=off
Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	5.320	MM	0.3432	96.10062	4.66754	100.0000
Totals :				96.10062	4.66754	

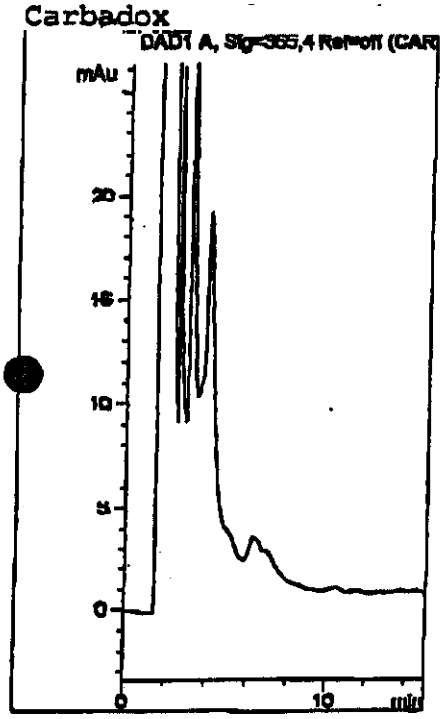
*** End of Report ***

Injection Date : 28/11/00 15:13:16
Sample Name : 00-8426a
Acq. Operator :

Seq. Line : 5
Vial : 10
Inj : 1
Inj Volume : 30 µl

Acq. Method : C:\HPCHEM\1\
Last changed : 28/11/00 14:05:04 by
Analysis Method : C:\HPCHEM\1\
Last changed : 29/11/00 11:41:18 by
(modified after loading)

Carbadox



CARBADOX 121166

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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
151117	1,65	1,54
151137	7,18	7,24
151155	1,73	1,66
151187	blank	blank
151192	6,76	6,91
151207	blank	blank

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 24-28 NOVEMBER 2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: HYPERSIL ODS 5µm 200 x 4.6 mm + GUARD COLUMN C18
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: SODIUM ACETATE BUFFER pH 6.0 0.01M / ACETONITRILE 86% / 14%
- Flow-rate: 1.5 ml/min
- Injection volume: 20 µl
- Retention time of carbadox: 4.8 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

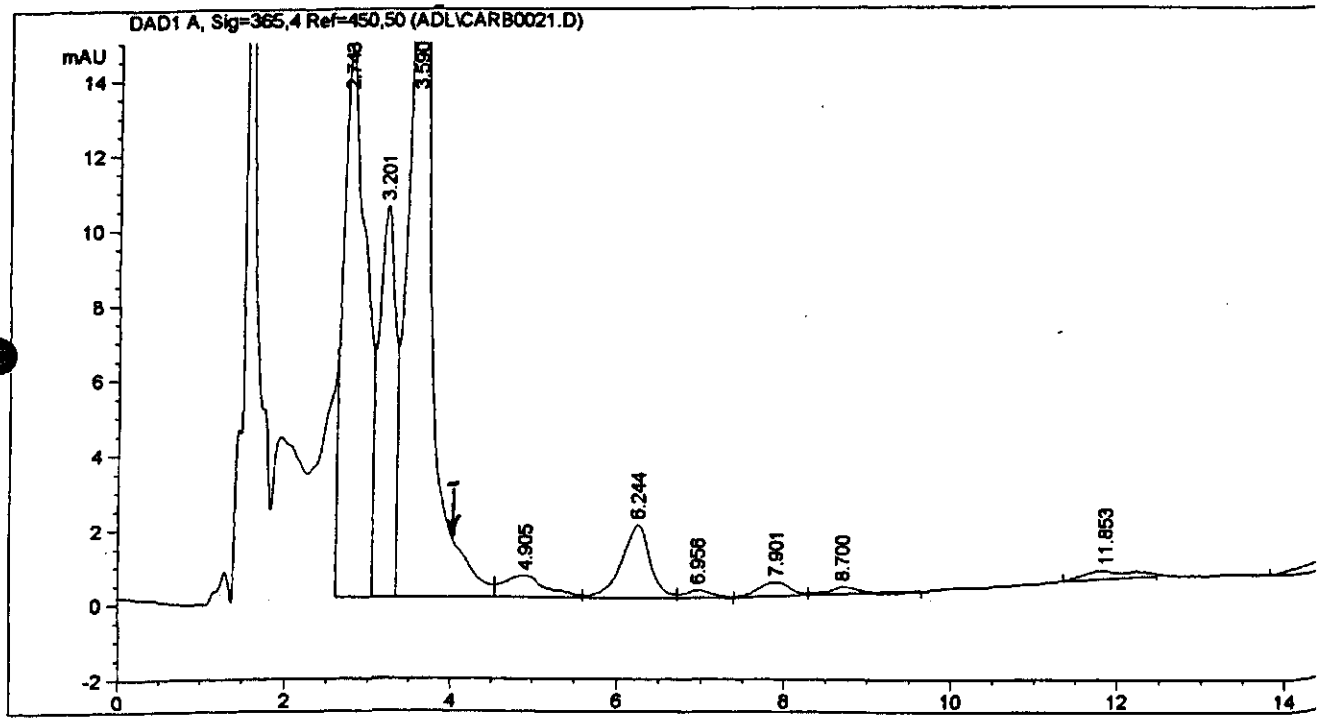
Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 85 % and 84 %
- Spiking level: 5.0 mg/kg

Data File C:\HPCHEM\1\DATA\ADL\carb0021.D Sample Name: 151

Injection Date	: 11/28/00 9:58:00 PM	Seq. Line	: 22
Sample Name	: 151207	Vial	: 17
Acq. Operator	: adl	Inj	: 1
		Inj Volume	: 20 µl
Sequence File	: C:\HPCHEM\1\SEQUENCE\MCARBVAL.S		
Acq. Method	: C:\HPCHEM\1\METHODS\MCARBVAL.M		
Last changed	: 11/28/00 4:15:40 PM by adl		
Analysis Method	: C:\HPCHEM\1\METHODS\MCARBVAL.M		
Last changed	: 11/29/00 1:54:41 PM by adl		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Wednesday, November 29, 2000 1:54:41 PM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=365,4 Ref=450,50

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

Totals : 0.00000

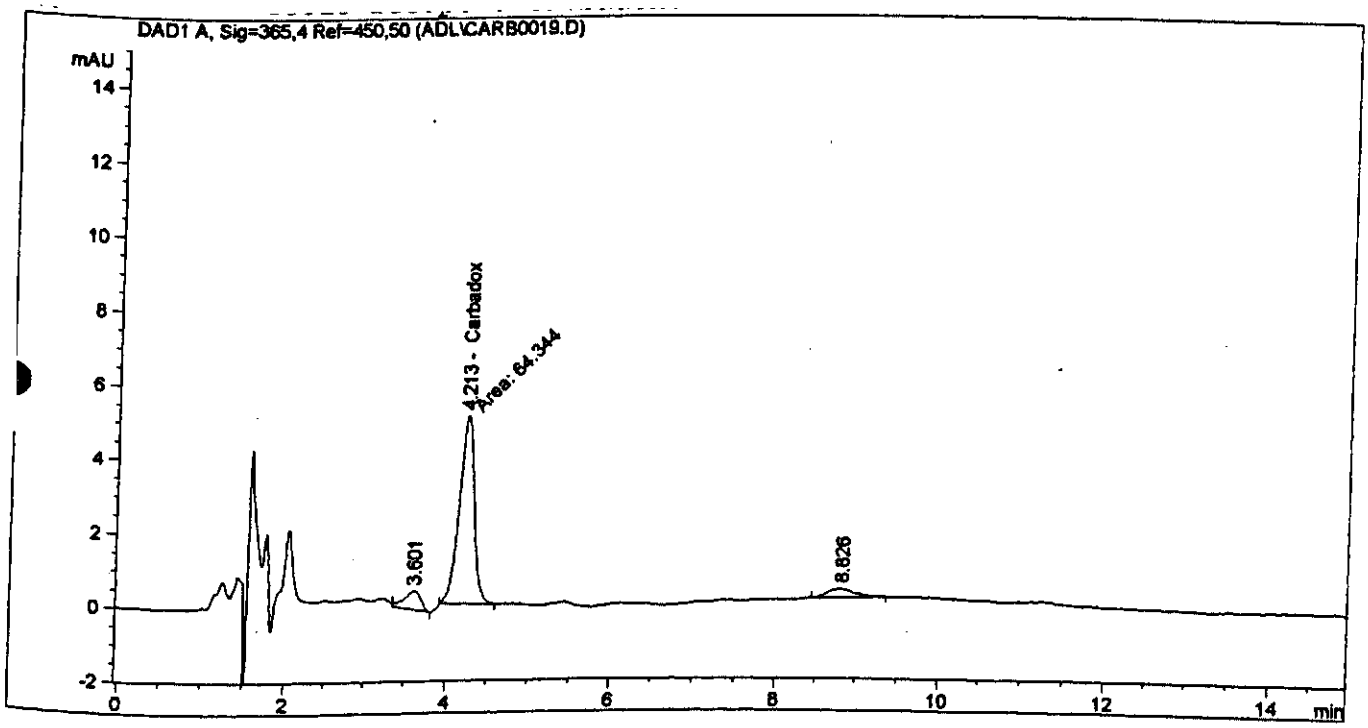
Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibrated compound(s) not found

15

Injection Date : 11/28/00 9:25:33 PM
Sample Name : 151192
Acq. Operator : adl
Seq. Line : 20
Vial : 15
Inj : 1
Inj Volume : 20 µl

Acq. Method : C:\HPCHEM\1\METHODS\MCARBVAL.M
Last changed : 11/28/00 4:15:40 PM by adl
Analysis Method : C:\HPCHEM\1\METHODS\MCARBVAL.M
Last changed : 11/29/00 2:52:03 PM by adl



External Standard Report

Sorted By : Signal
Calib. Data Modified : Wednesday, November 29, 2000 1:57:10 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,4 Ref=450,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
4.213	MM	64.34398	2.14969e-2	1.38319 ×5		Carbadox 6 9 1 ug/kg

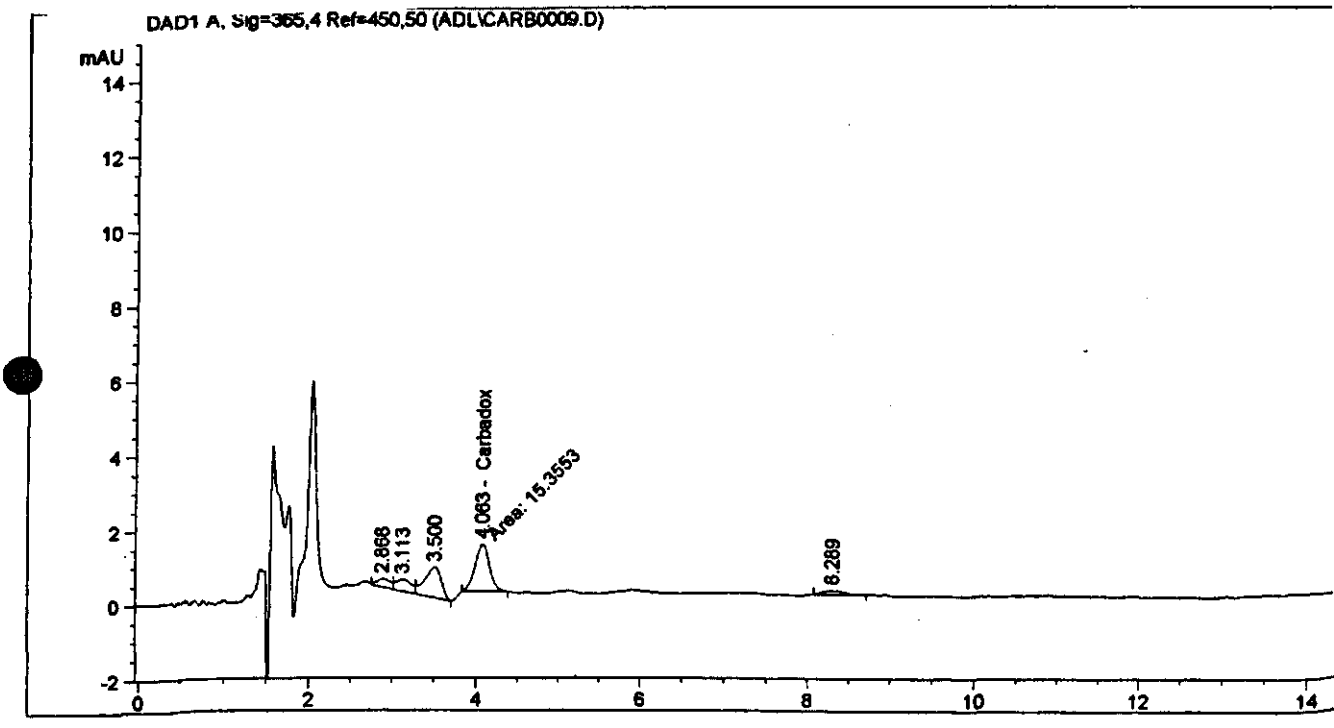
Totals : 1.38319

Results obtained with enhanced integrator!

*** End of Report ***

Data File C:\HPCHEM\1\DATA\ADL\CARB0009.D Sample Name: 151

Injection Date	: 11/28/00 6:43:15 PM	Seq. Line	: 10
Sample Name	: 151155	Vial	: 10
Acq. Operator	: adl	Inj	: 1
		Inj Volume	: 20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\MCARBVAL.M		
Last changed	: 11/28/00 4:15:40 PM by adl		
Analysis Method	: C:\HPCHEM\1\METHODS\MCARBVAL.M		
Last changed	: 11/29/00 2:52:03 PM by adl		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Wednesday, November 29, 2000 1:57:10 PM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=365,4 Ref=450,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
4.063	MM	15.35532	2.25336e-2	3.46010e-1	x5	Carbadox 1.73 ug/g

Totals : 3.46010e-1

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: **CARBADOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
161118	1,82	1,83
161133	8,32	8,40
161157	not found	not found
161163	7,99	8,26
161196	1,89	1,82
161197	not found	not found

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 2000-11-16, 2000-11-20, 2000-11-21 (each assay one day)

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - x Other: Spherisorb ODS 1, 5 µm, 250 x 4 mm
- Mobile phase:
  - x As described in the method
  - ☐ Other:

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

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

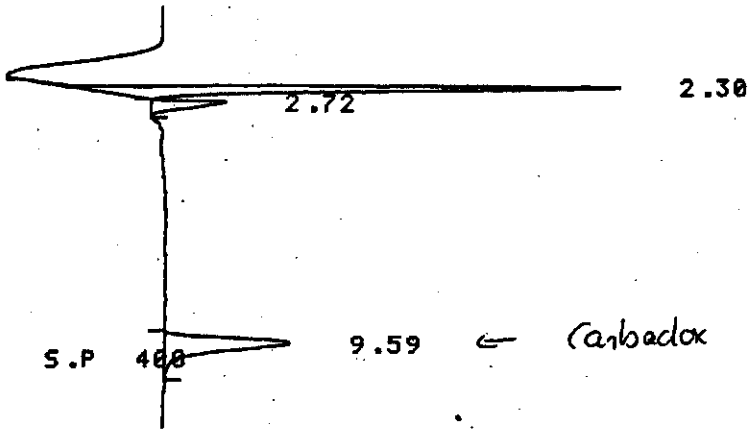
Recovery results:

- Percentage recovery: 95.3 %
- Single / duplicate determinations: ☐ single x duplicate
- If duplicate, please give both percentages: 94.6 and 96.0 %
- Spiking level: 5.65 mg/kg

Carbadox

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/20/00 11:03

Standard 1,130 µg/ml



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

D-2500 11/20/00 11:03

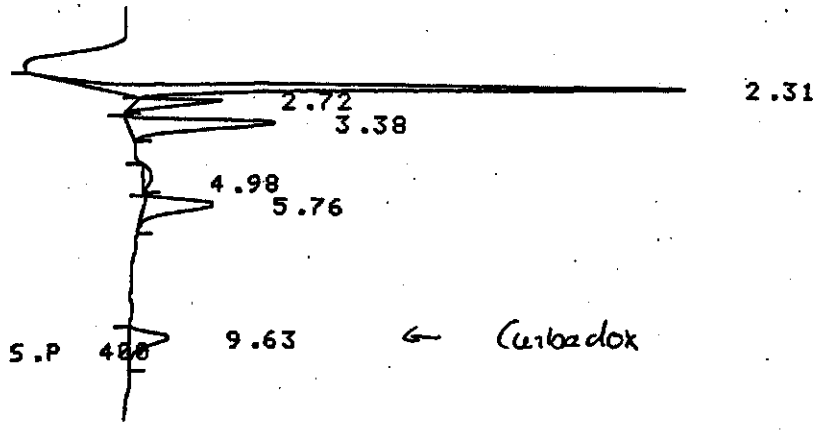
METHOD: 1 TAG: 531 CH: 1

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME
3	9.59	21433	884	1.130	CARBAD

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/20/00 11:17

Sample code 161118



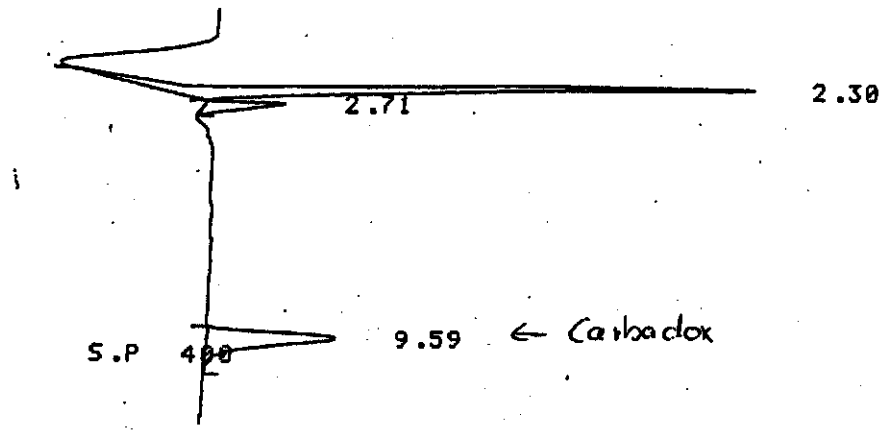
D-2500 11/20/00 11:17

METHOD:

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/20/00 11:46

(16)

Standard 1,130 µg/ml



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

D-2500 11/20/00 11:46

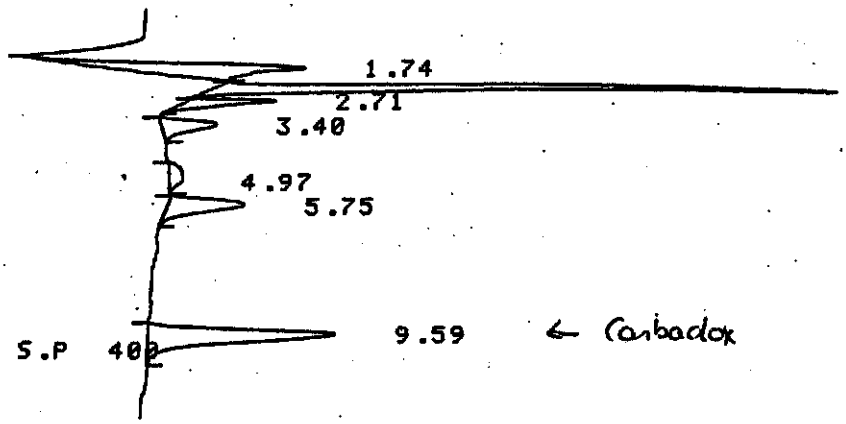
METHC : TAG: 534 CH: 1

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME
3	9.59	22365	911	1.130	CARBAD

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/20/00 11:59

Sample code 161133



D-2500 11/20/00 11:59

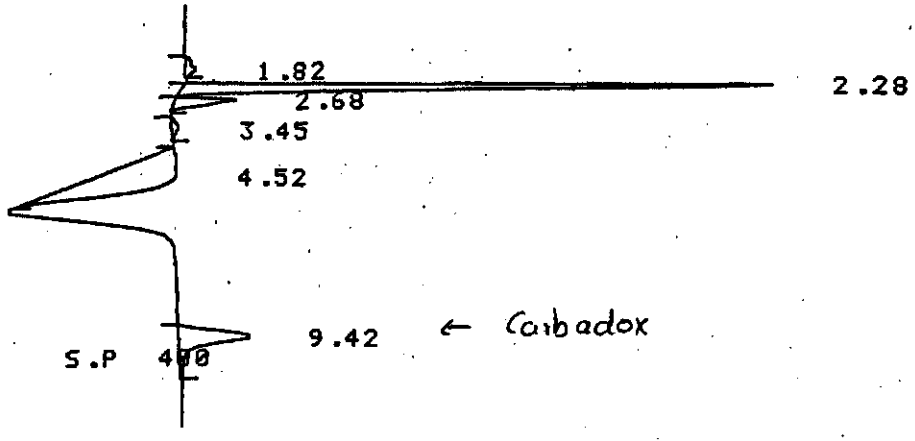
METHOD:

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME
1	1.74	27706	1306	0.001	
2	2.29	42388	4360	0.004	
3	2.71	7595	657	0.001	

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/20/00 15:41

Standard 0,565 µg/l



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

D-2500 11/20/00 15:41

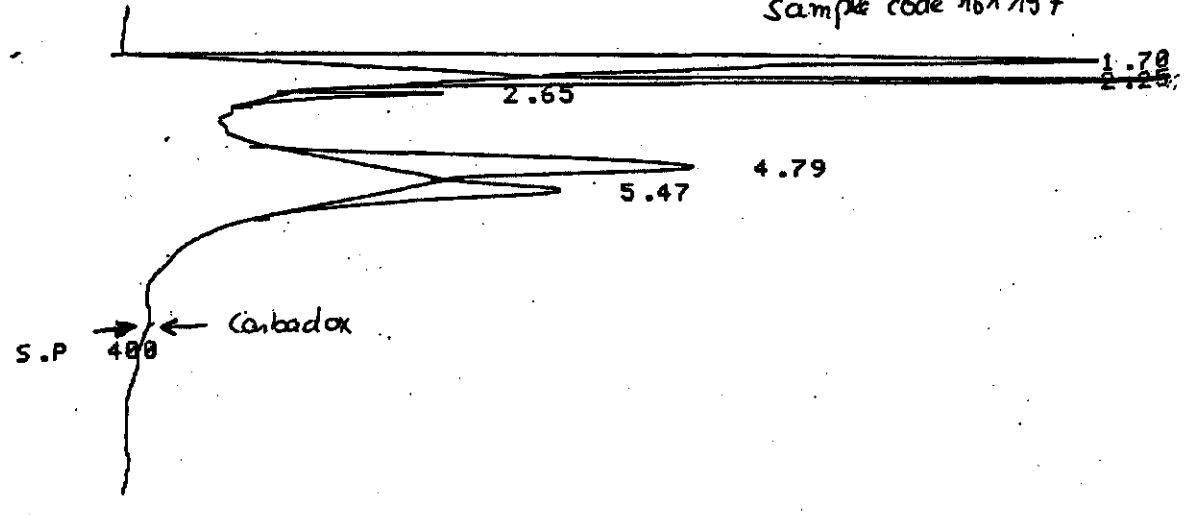
METHOD: TAG: 548 CH: 1

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME
6	9.42	12391	492	0,565	CARBAD

CH. 1 C.S 5.00 ATT 3 OFFS 10 11/20/00 16:08

Sample code 161197



D-2500 11/20/00 16:08

METHOD:

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

NO.	RT	AREA	HEIGHT	UG/ML	NAME

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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
171120	8,99	8,86
171128	2,21	2,24
171134	0	0
171161	8,50	8,60
171186	0	0
171205	2,33	2,19

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 20.10.2000 / 28.10.2000

Chromatographic conditions:

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

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: 98 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 96 % and 100 %
- Spiking level: 2,5 / 5,0 mg/kg

D-7000 HSM: CARBADOX Series: 0013

Sample Name: Standard 1µg/ml

Analyzed: 28.10.00 12:08

Reported: 14.11.00 11:09
Processed: 14.11.00 11:08

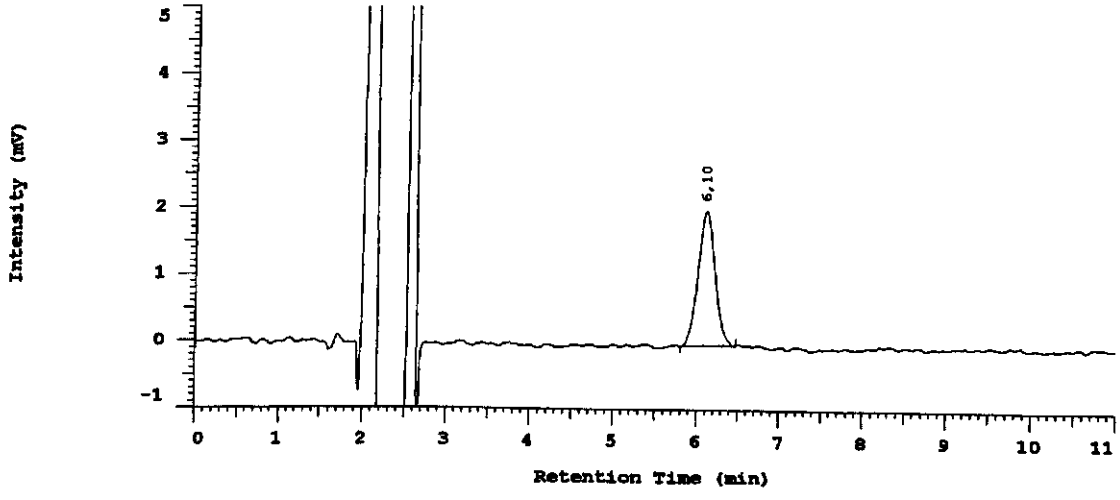
Data Path: C:\win32app\HSM\CARBADOX\DATA\0013\
Processing Method: Carbadox

System(acquisition): Sys 1
Application: CARBADOX
Injection from this vial: 1 of 1

Series:0013
Vial Number: 2
Vial Type: UNK
Volume: 20,0 ul

Sample Description:

Chrom Type: Fixed WL Chromatogram, 365 nm



Acquisition Method: Carbadox
Column Type: Spherisorb S10 ODS-1
Pump A Type: L-7100
Solvent A: Ac/Pu
Solvent C: Ac/Pu
Peak Quantitation: AREA
Calculation Method: EXT-STD
Scale Factor 1: 1,000

Developed by: --

Solvent B: Ac/Pu
Solvent D: Ac/Pu
Sample Amount: 1,000

Name	RT	Area	Conc 1	BC
Carbadox	6,10	29048	0,000	BB
		29048	0,000	

Peak rejection level: 0

D-7000 HSM: CARBADOX Series: 0008

Sample Name: 171120

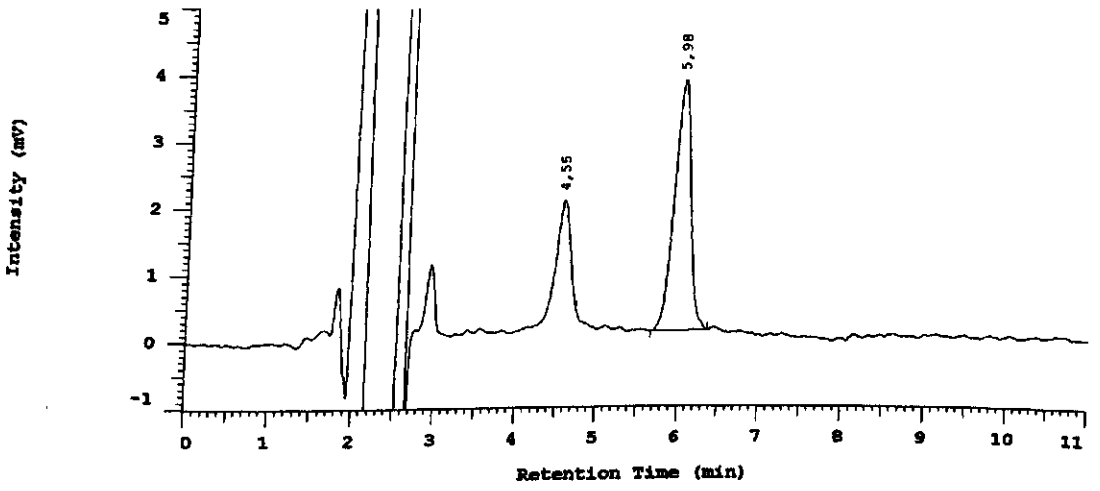
Analyzed: 20.10.00 14:09 Reported: 14.11.00 10:56
Processed: 14.11.00 10:55

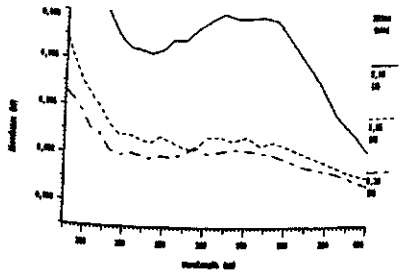
Data Path: C:\win32app\HSM\CARBADOX\DATA\0008\
Processing Method: Carbadox

System(acquisition): Sys 1 Series:0008
Application: CARBADOX Vial Number: 3
Injection from this vial: 1 of 1 Vial Type: UNK
Volume: 20,0 ul

Sample Description: 1.EW

Chrom Type: Fixed WL Chromatogram, 365 nm





Acquisition Method: Carbadox
Column Type: Spherisorb S10 ODS-1 Developed by:
Pump A Type: L-7100
Solvent A: Ac/Pu Solvent B: Ac/Pu
Solvent C: Ac/Pu Solvent D: Ac/Pu
Peak Quantitation: AREA Sample Amount: 1,000
Calculation Method: EXT-STD
Scale Factor 1: 1,000

Name	RT	Area	Conc 1	BC
	4,55	0	0,000	
Carbadox	5,98	52457	0,000	MC
		52457	0,000	

D-7000 HSM: CARBADOX Series: 0009

Sample Name: 171128

Analyzed: 20.10.00 15:58

Reported: 14.11.00 11:00
Processed: 14.11.00 10:58

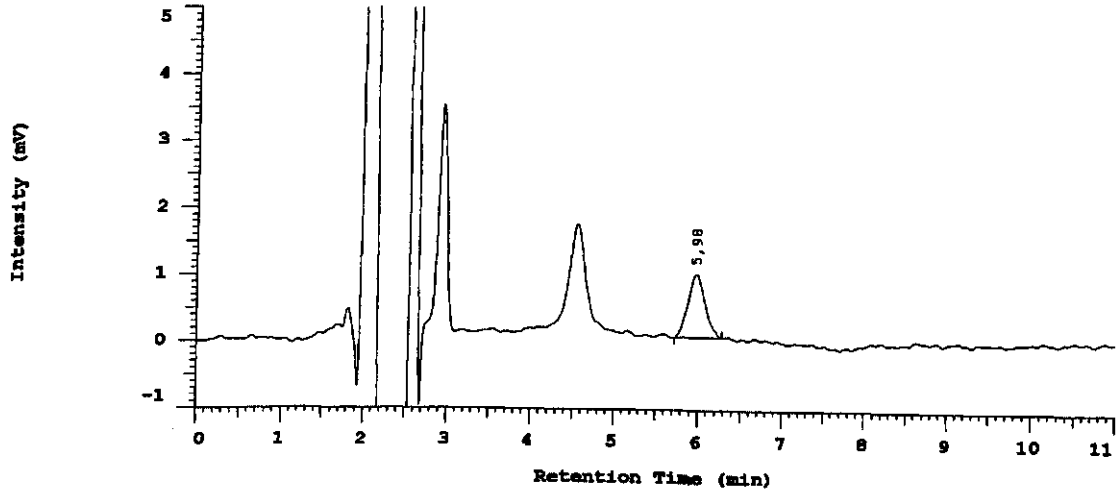
Data Path: C:\win32app\HSM\CARBADOX\DATA\0009\
Processing Method: Carbadox

System(acquisition): Sys 1
Application: CARBADOX
Injection from this vial: 1 of 1

Series:0009
Vial Number: 4
Vial Type: UNK
Volume: 20,0 ul

Sample Description:

Chrom Type: Fixed WL Chromatogram, 365 nm



Acquisition Method: Carbadox
Column Type: Spherisorb S10 ODS-1
Pump A Type: L-7100
Solvent A: Ac/Pu
Solvent C: Ac/Pu
Peak Quantitation: AREA
Calculation Method: EXT-STD
Scale Factor 1: 1,000

Developed by: r

Solvent B: Ac/Pu
Solvent D: Ac/Pu
Sample Amount: 1,000

Name	RT	Area	Conc 1	BC
Carbadox	5,98	13208	0,000	MC
		13208	0,000	

Peak rejection level: 0

D-7000 HSM: CARBADOX Series: 0008

Sample Name: 171134

Analyzed: 20.10.00 14:32

Reported: 14.11.00 10:55
Processed: 14.11.00 10:54

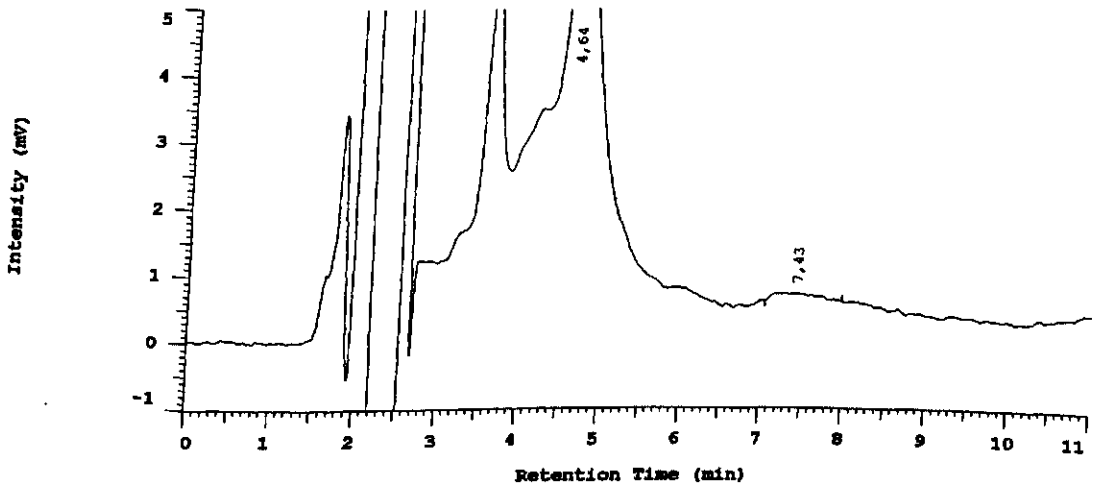
Data Path: C:\win32app\HSM\CARBADOX\DATA\0008\
Processing Method: Carbadox

System(acquisition): Sys 1
Application: CARBADOX
Injection from this vial: 1 of 1

Series:0008
Vial Number: 5
Vial Type: UNK
Volume: 20,0 ul

Sample Description: 1.EW

Chrom Type: Fixed WL Chromatogram, 365 nm



Acquisition Method: Carbadox
Column Type: Spherisorb S10 ODS-1 , Developed by:
Pump A Type: L-7100
Solvent A: Ac/Pu
Solvent B: Ac/Pu
Solvent C: Ac/Pu
Solvent D: Ac/Pu
Peak Quantitation: AREA
Sample Amount: 1,000
Calculation Method: EXT-STD
Scale Factor 1: 1,000

Name	RT	Area	Conc 1	BC
	4,64	0	0,000	
	7,43	0	0,000	
		0	0,000	

Peak rejection level: 0

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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
181123	9,15	9,76
181139	Not detected; LOD<0,4	Not detected; LOD<0,4
181143	Not detected; LOD<0,4	Not detected; LOD<0,4
181178	9,42	9,52
181198	2,40	2,58
181214	2,40	2,22

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 13/11/00

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: 250 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: 20 µl
- Retention time of carbadox: 6,1 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: 101,7 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 100,1 % and 103,2%
- Spiking level: 5 mg/kg

Reported On: 14/11/00 09:57:36

Raw Results: C:\TSP\ Data\carba 001113-2.RES
Processed Results: C:\TSP\ Data\carba 001113-2.RMS

Combined Analysis Report

Name: 7807-2
Description: 10 +0.5*100ug/ml /50
Type: Sample
Sample Amount: 10.000 g
Scale Factor: 50.000 ml
Injection Volume: 20.0 uL

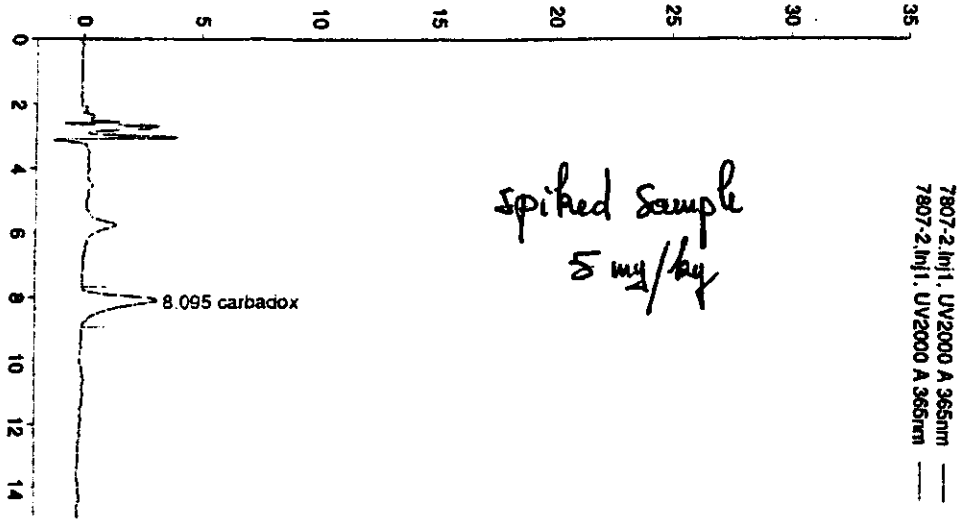
Vial: A07

Injection: 1 of 1

Injected On: 13/11/00 17:25:54

Acquisition Log
Column Pressure (PSI): 2949
Column Temperature (C): N/A
Pump Flow Stability: 10.6
Noise (microAU): 3e+001
Drift (microAU/min): 2e+001
Run-Time Messages: None

Signal 1: UV2000 A 365 nm
Calculation Type: External Standard (Height)
Signal 2: UV2000 A 365 nm
Calculation Type: External Standard (Area)
Signal 3: Unused
Calculation Type: External Standard (Height)



Reported On: 14/11/00 09:57:40

Reprocessed Data
Original Results: C:\TSP\ Data\carba 001113-2.RES
Reprocessed Results: C:\TSP' Data\carba 001113-2.RMS

Combined Analysis Report

Name: 7796-1
Description: 10/50
Type: Sample
Sample Amount: 10.000
Scale Factor: 50.000
Injection Volume: 20.0 uL

Vial: A10

Injection: 1 of 1

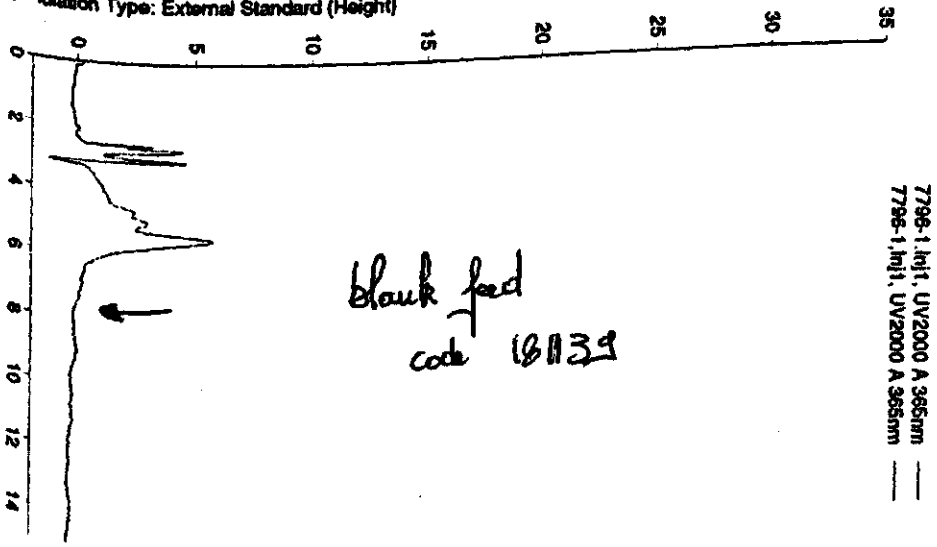
Injected On: 13/11/00 18:14:18

Acquisition Log
Column Pressure (PSI): 3061
Noise (microAU): 8e+001
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): -7e+002

Pump Flow Stability: 8.6

Signal 1: UV2000 A 365 nm
Calculation Type: External Standard (Height)
Signal 2: UV2000 A 365 nm
Calculation Type: External Standard (Area)
Signal 3: Unused
Calculation Type: External Standard (Height)



Processed Data
Results: C:\TSP\ .\Data\carba 001113-2.RES
Processed Results: C:\TSP\ \Data\carba 001113-2.RMS

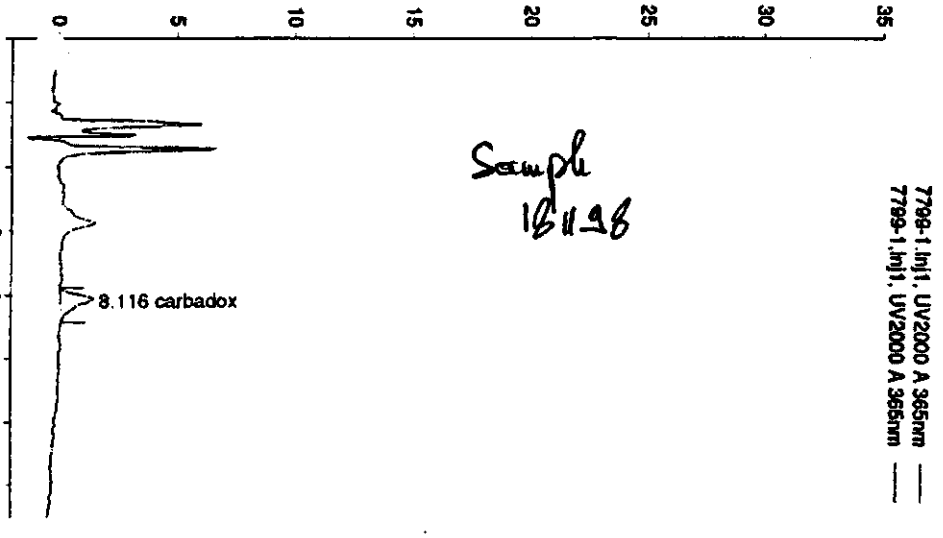
Reported On: 14/11/00 09:57:51

Combined Analysis Report

ıe: 7799-1 Vial: A16 Injection: 1 of 1
cription: 10/50
s: Sample Injected On: 13/11/00 19:50:56
ıple Amount: 10.000 g
le Factor: 50.000 mg
ction Volume: 20.0 uL

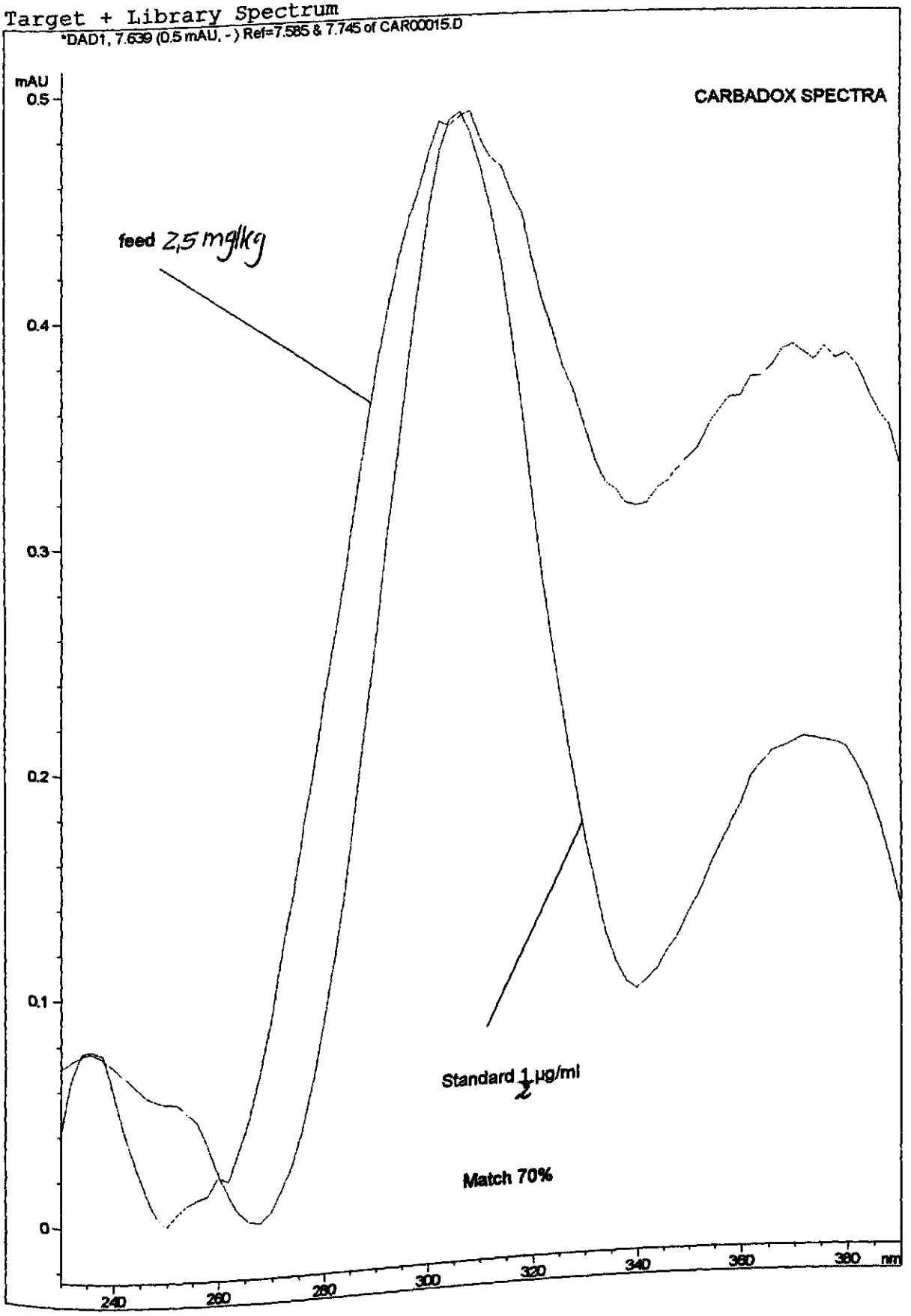
uisition Log
umn Pressure (PSI): 3104 Column Temperature (C): N/A Pump Flow Stability: 11.1
se (microAU): 4e+001 Drift (microAU/min): 6e+001
Time Messages: None

nal 1: UV2000 A 365 nm
iculation Type: External Standard (Height)
nal 2: UV2000 A 365 nm
iculation Type: External Standard (Area)
nal 3: Unused
iculation Type: External Standard (Height)



Target + Library Spectrum

*DAD1, 7.639 (0.5 mAU, -) Ref=7.585 & 7.745 of CAR00015.D



10/11/00 15:25:34 mcr

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: **CARBADOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
201114	neg	neg
201146	7,15	7,14
201172	1,86	1,84
201194	1,91	1,94
201229	neg	neg
201232	7,16	6,74

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 14/11/2000

Chromatographic conditions:

- Column:
  - ☒ As described in the method AllTech Alltima C18 250 x 4,6 mm 5µm
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1,5 ml/min
- Injection volume: 20 µl
- Retention time of carbadox: 3,4 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

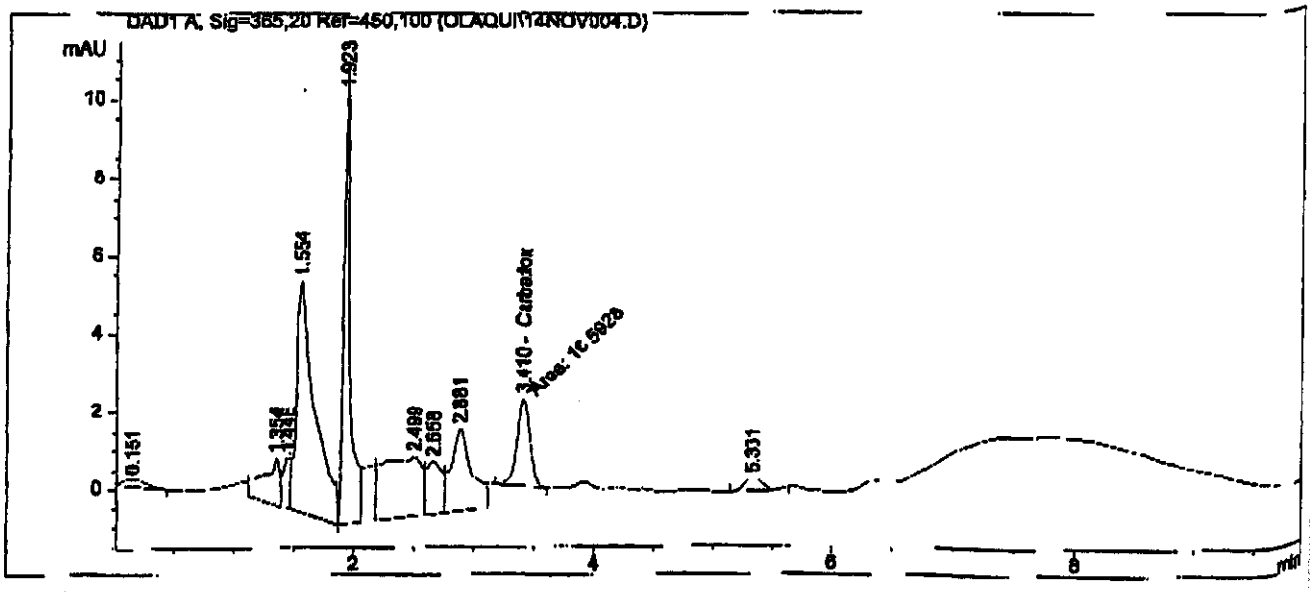
- Percentage recovery: 100 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 100,4% and 99,9 %
- Spiking level: 5,6 mg/kg

Data File D:\... Sample Name: 24/1

Injection Date : 11/14/2000 6:21:17 PM Seq. Line : 4
Sample Name : 24/1 Vial : 3
Acq. Operator : 201172 Inj : 1
Inj Volume : 20 µl

Acq. Method : C:\HPCHEM\1\METHODS\CARB-RK.M
Last changed : 11/8/2000 12:56:34 PM by
Analysis Method : C:\HPCHEM\1\METHODS\CARB-RK.M
Last changed : 11/15/2000 11:47:35 AM by

Col Alltima 250 mm 3-4-98



External Standard Report

Sorted By : Signal
Calib. Data Modified : 11/15/2000 11:47:35 AM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,20 Ref=450,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ug/ml]	Grp	Name
3.410	MM	16.59278	2.27707e-2	3.77829e-1		Carbadox

Totals : 3.77829e-1

Results obtained with enhanced integrator!

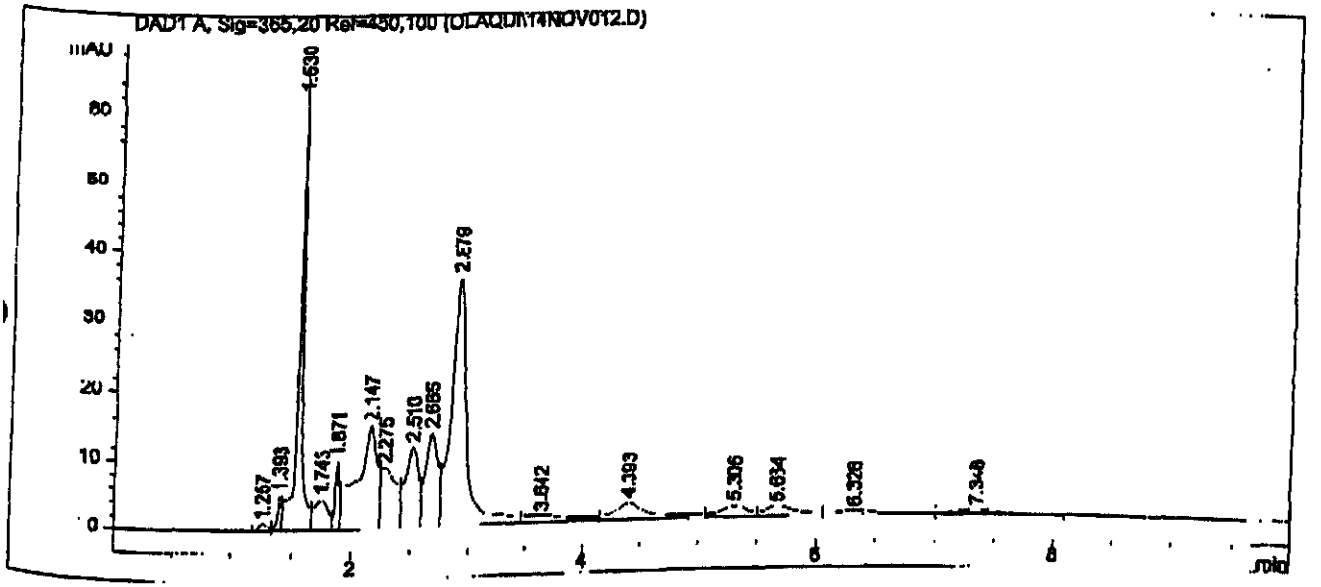
$\frac{0.378 \cdot 50}{10.12\ gr} = 1.863\ mg/gr$

Data File :

Sample Name: 27/1

Injection Date	: 11/14/2000 7:46:49 PM	Seq. Line	: 12
Sample Name	: 27/1 BLANK	Vial	: 11
Acq. Operator	:	Inj	: 1
		Inj Volume	: 20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\CARB-RK.M		
Last changed	: 11/8/2000 12:56:34 PM by		
Analysis Method	: C:\HPCHEM\1\METHODS\CARB-RK.M		
Last changed	: 11/15/2000 11:47:46 AM by		

Col Alltima 250 mm 3-4-00



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	11/15/2000 11:47:35 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=365,20 Ref=450,100

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

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 21

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: CARBADOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
211105	0,6 no spectrum	0,6 no spectrum
211107	8,7	8,5
211145	2,2	2,2
211151	0,6 mg/kg no spectrum	0,6 mg/kg no spectrum
211170	8,6	8,8
211225	2,4	2,3

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 17/10/00

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: SUPELCOSIL LC 18 25cm x 4,6mm (5µm) + SUPELGUARD LC18
- 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 carbadox: 9,53 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

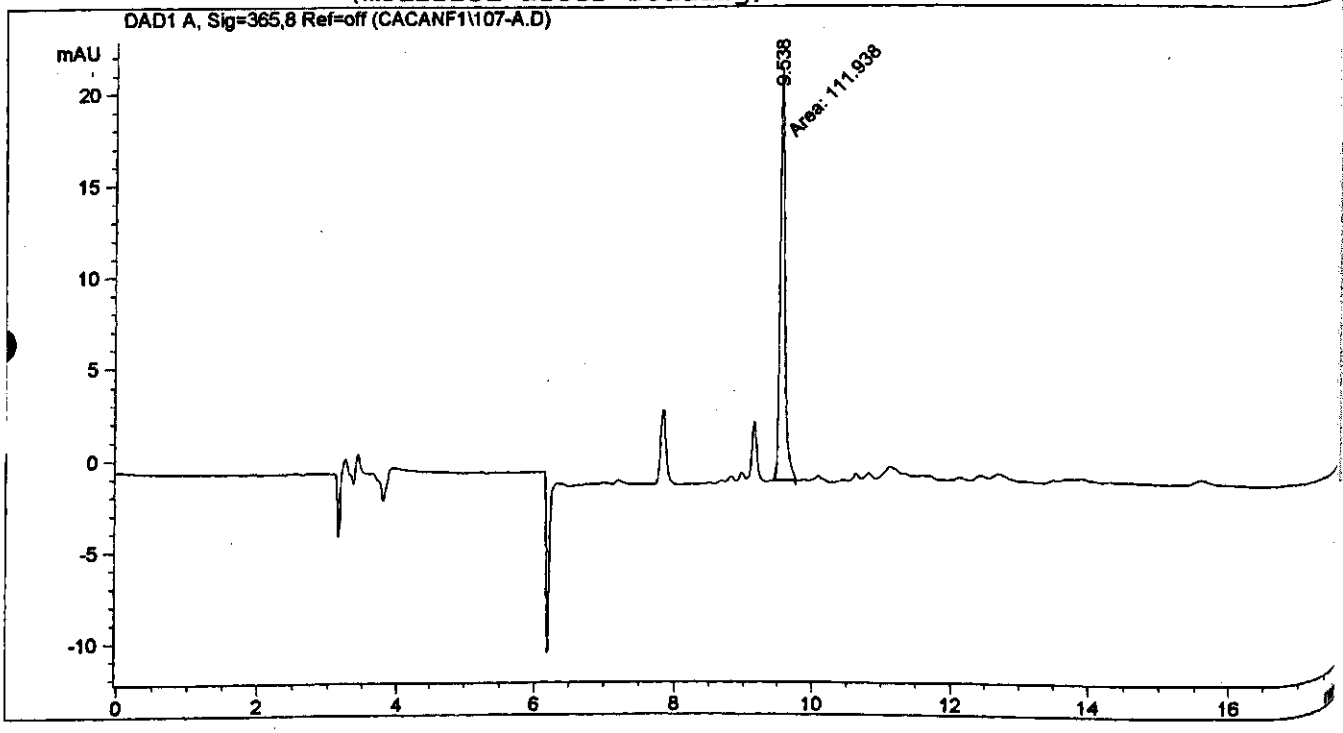
- Percentage recovery: 99 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 99,4% and 98,6%
- Spiking level: 5,0 mg/kg

(21)

Injection Date : 17/10/2000 15.00.33
Sample Name : 107-A
Acq. Operator :

Seq. Line : 4
Vial : 2
Inj : 1
Inj Volume : 20 µl

Acq. Method : C:\HPCHEM\1\
Last changed : 17/10/2000 14.58.40
(modified after loading)
Analysis Method : C:\HPCHEM\1\
Last changed : 18/10/2000 10.08.25
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 18/10/2000 10.08.22
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
9.538	MM	111.93823	3.10813e-1	34.79186		CARB-carbadox

Totals : 34.79186

Results obtained with enhanced integrator!

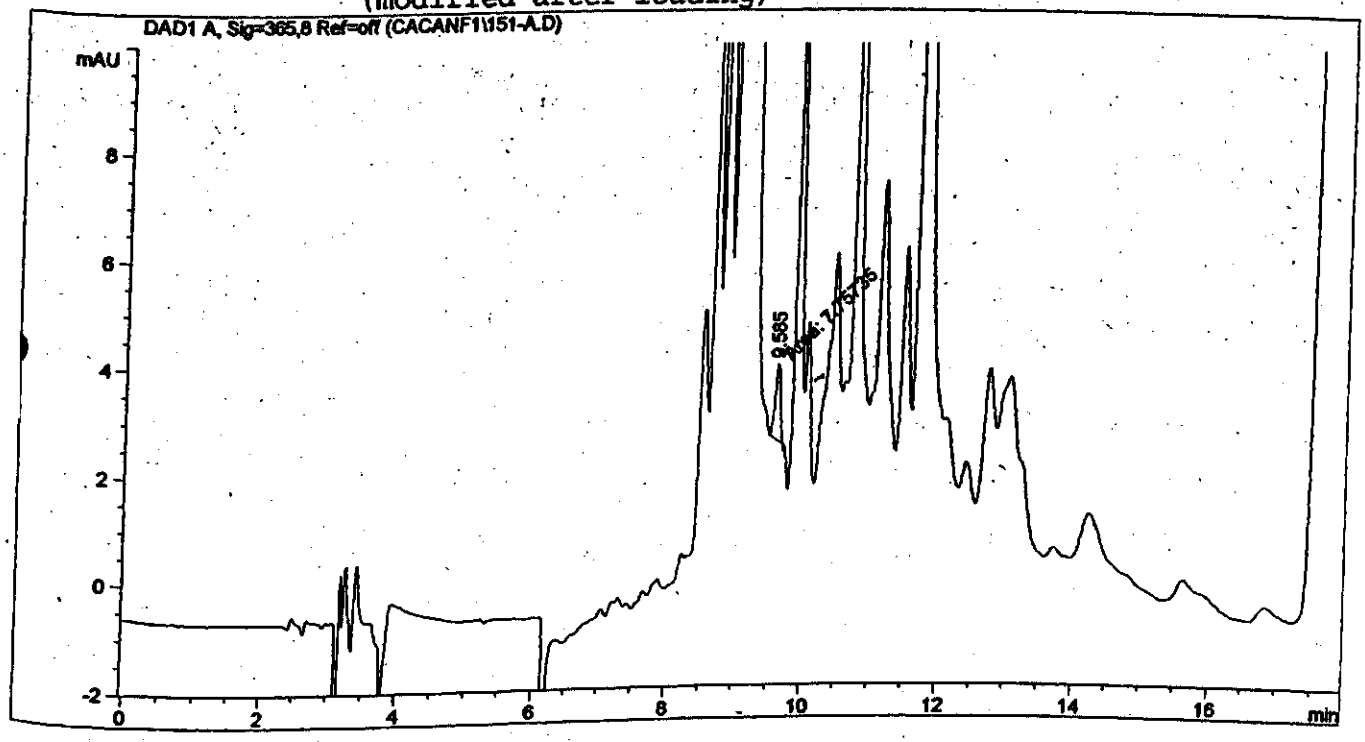
Data File C:\HPCHEM\1\CROMAT\CACANF1\151-A.D

21

Injection Date : 17/10/2000 18.06.26
Sample Name : 151-A
Acq. Operator :

Seq. Line : 12
Vial : 9
Inj : 1
Inj Volume : 20 µl

Acq. Method : C:\HPCHEM\1\
Last changed : 17/10/2000 18.04.28
(modified after loading)
Analysis Method : C:\HPCHEM\1\
Last changed : 18/10/2000 11.40.41
(modified after loading)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 18/10/2000 10.08.22
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
9.585	MM	7.75735	3.01492e-1	2.33878		CARB-carbadox
Totals :				2.33878		

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: **CARBADOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
231144	6,99	6,63
231148	1,61	1,5
231176	< 0,1	< 0,1
231195	1,4	1,34
231199	< 0,1	< 0,1
231230	6,99	6,84

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: **CARBADOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
241106	7,4	7,5
241138	7,4	7,3
241142	2,0	2,0
241162	not detectable	not detectable
241173	not detectable	not detectable
241218	2,1	2,1

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 24th 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 carbadox: 5:1 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

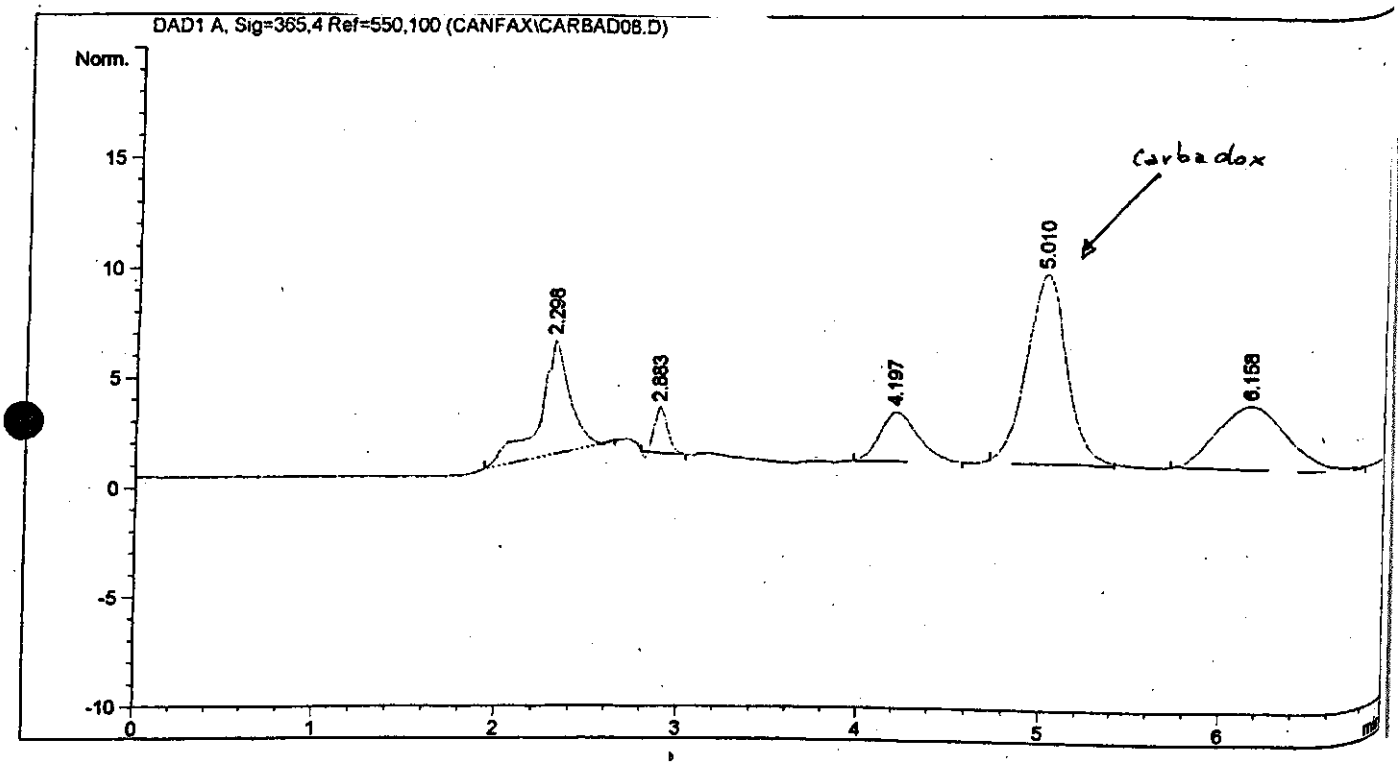
Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: 85 % (average)
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 85 % and 85 %
- Spiking level: 5 mg/kg

1A Sample n° 241106 (24)
(carbadox)

Injection Date : 24/10/00 17.01.45
Sample Name :
Location : Vial 1
Acq. Operator :
Acq. Method : C:\HPCHEM\1\METHODS\IZS_ME~1\CARBADOX.M
Last changed : 24/10/00 17.07.06 by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\IZS ME~1\CARBADOX.M
Last changed : 24/10/00 17.15.30 by
(modified after loading)



External Standard Report

Sorted By : Retention Time
Calib. Data Modified : Tuesday 24 October 2000 17.15.26
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,4 Ref=550,100

RetTime [min]	Sig	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
5.010	1	BB	132.70752	1.11594e-2	1.48094		carbadox

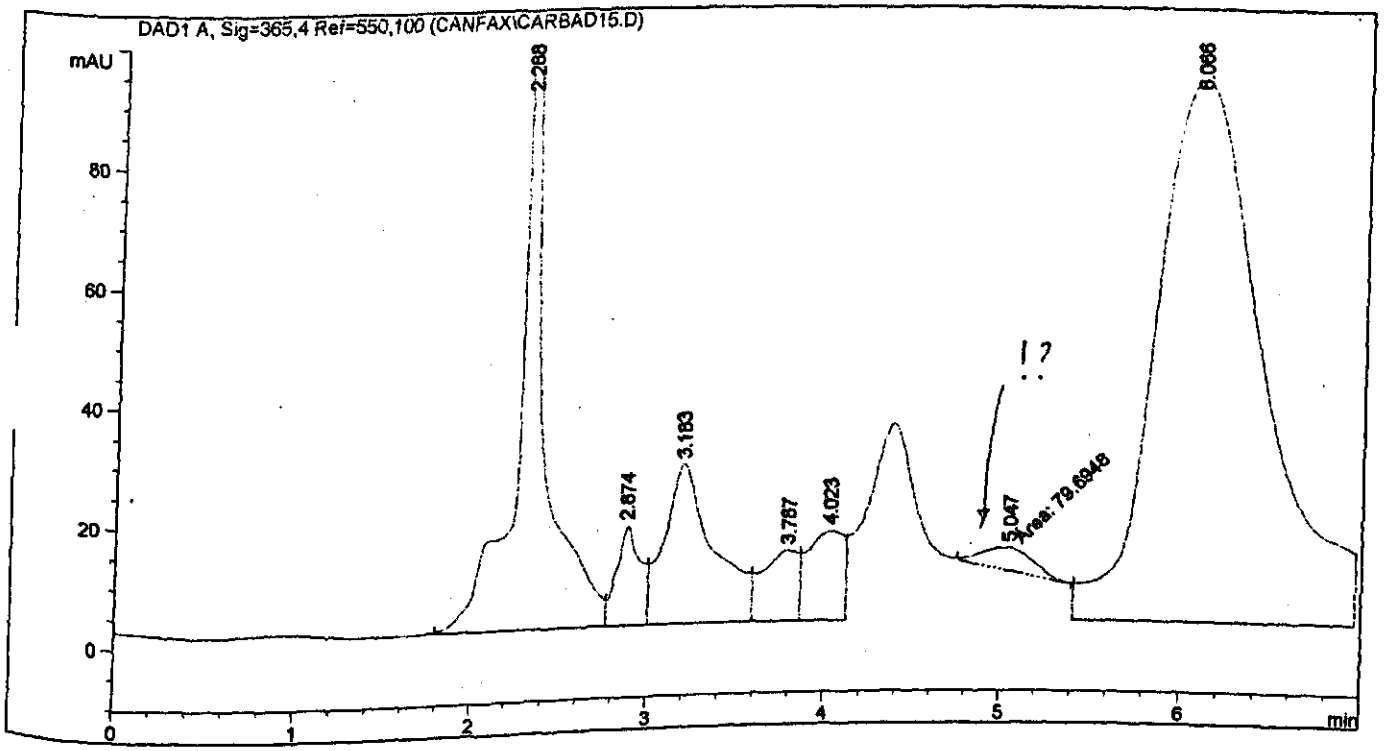
Totals : 1.48094

Results obtained with enhanced integrator!

*** End of Report ***

sample n° 24 11 6 2 (carbadox) (24)

Injection Date : 24/10/00 18.08.47
Sample Name :
Location : Vial 1
Acq. Operator : ------
Acq. Method : C:\HPCHEM\1\METHODS\IZS_ME~1\CARBADOX.M
Last changed : 24/10/00 17.18.15 by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\IZS_ME~1 ---------
Last changed : 25/10/00 9.24.47 by
(modified after loading)
---------, ----------, ---------



External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 25/10/00 9.24.43
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,4 Ref=550,100
Uncalibrated Peaks : compound name not specified

RetTime [min]	Sig	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
2.288	1	BV	1139.95703	0.00000	0.00000	?	
2.874	1	VV	159.25775	0.00000	0.00000	?	
3.183	1	VV	518.27631	0.00000	0.00000	?	
3.787	1	VV	168.36952	0.00000	0.00000	?	
4.023	1	VV	212.84628	0.00000	0.00000	?	
5.047	1	MM	79.69480	1.11013e-2	8.84715e-1		
6.066	1	VBA	3740.10376	0.00000	0.00000	?	
Totals :					8.84715e-1		

Results obtained with enhanced integrator!

rument 1 25/10/00 9.26.02

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:

Analyte: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
261111	1,8	1,8
261165	0	0
261175	8,9	8,0
261184	1,5	1,7
261228	8,0	8,3
261231	0	0

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 1/12/00

Chromatographic conditions:

- Column:
  - ☑ As described in the method
  - ☑ Other: LUNA ODS 2 5µm 25cm x 4.6mm
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other:
- Flow-rate: 1.5 ml/min
- Injection volume: 20 µl
- Retention time of carbadox: 5.3 min

Chromatograms: Please include representative chromatograms of:

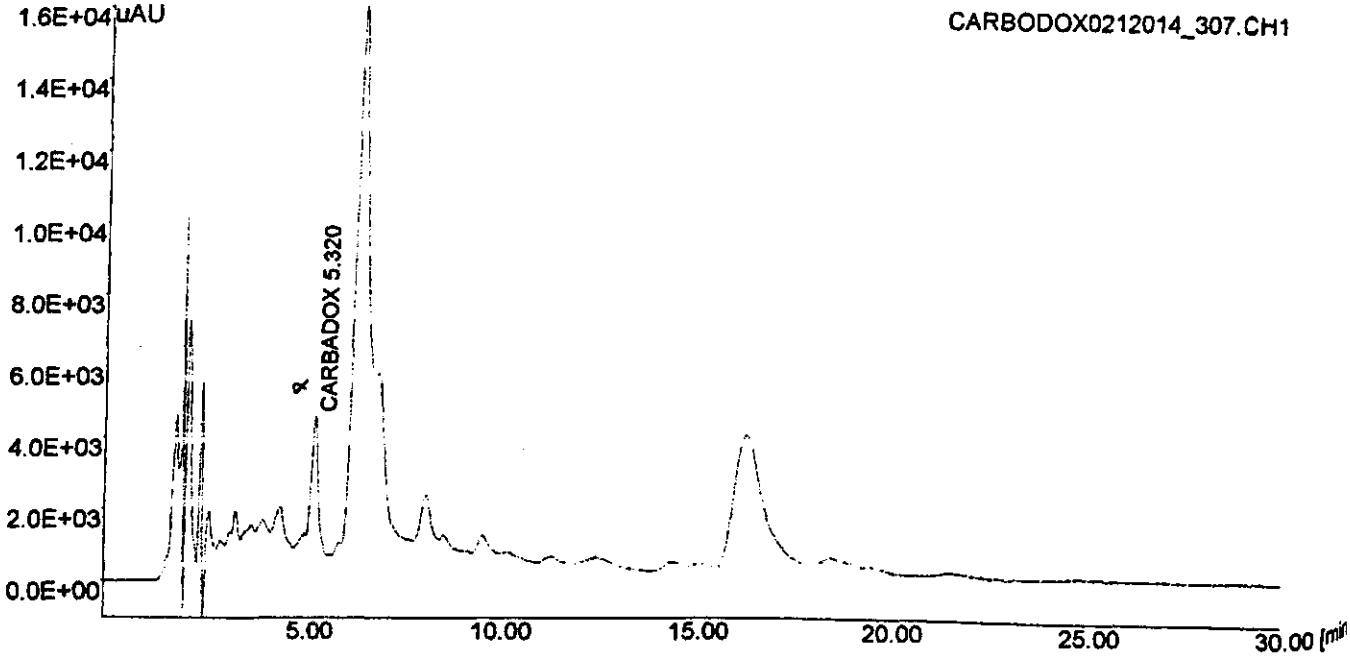
- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: 84 %
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: 89 % and 80 %
- Spiking level: 5 mg/kg

Chromatogram 261111



File name : CARBODOX0212014_307.CH1

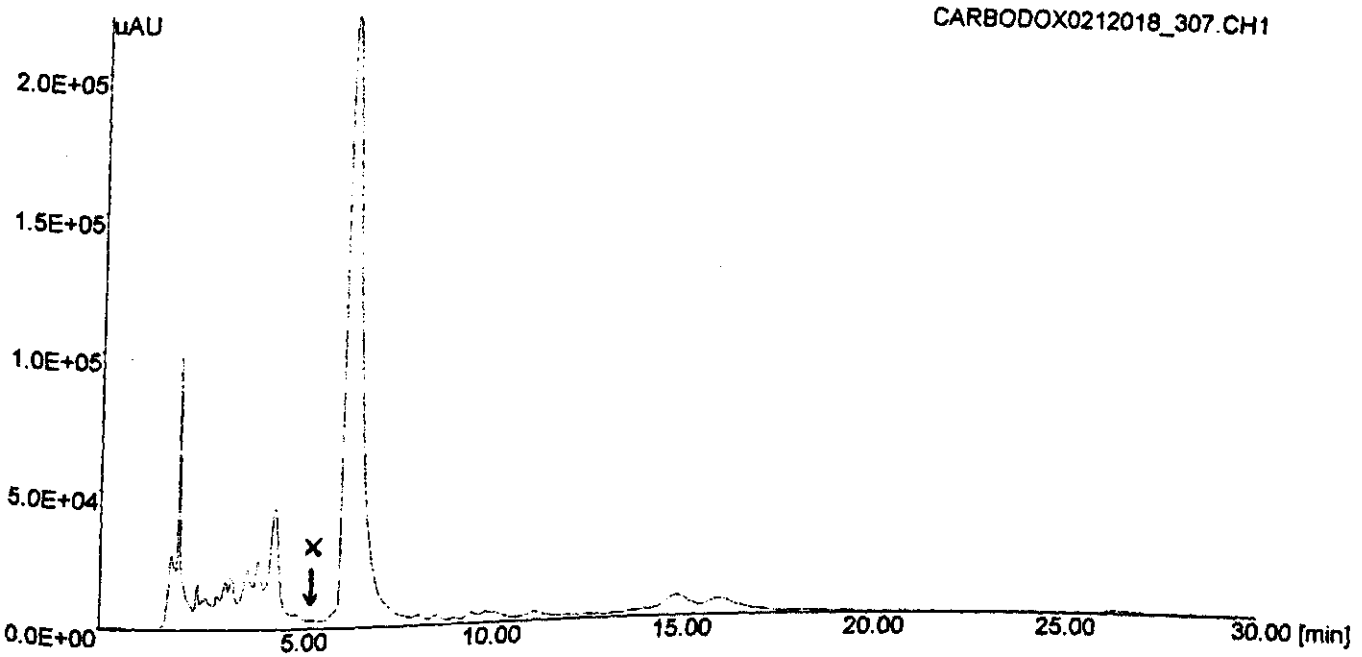
Info :
a3009142a
Wavelength = 307 [nm]
Tacc [Sec] = 0.80 Wacc [nm] = 4.0
Autozero [min] : 0.00

Vial # = 7 Rack # = 0
Injection Date : 4-Dec-2000 21:19:16
Curr. Date : 19-Dec-2000 16:16:18
User : KL
Group : NICARB
Control Method :

#	Name	RT	Height[uAU]	Area[uAU.Sec]	%Area
1	CARBADOX	5.320	3579	41131.086	100.00

Total Area of Peak = 41131.086 [uAU.Sec]

Chromatogram 261165



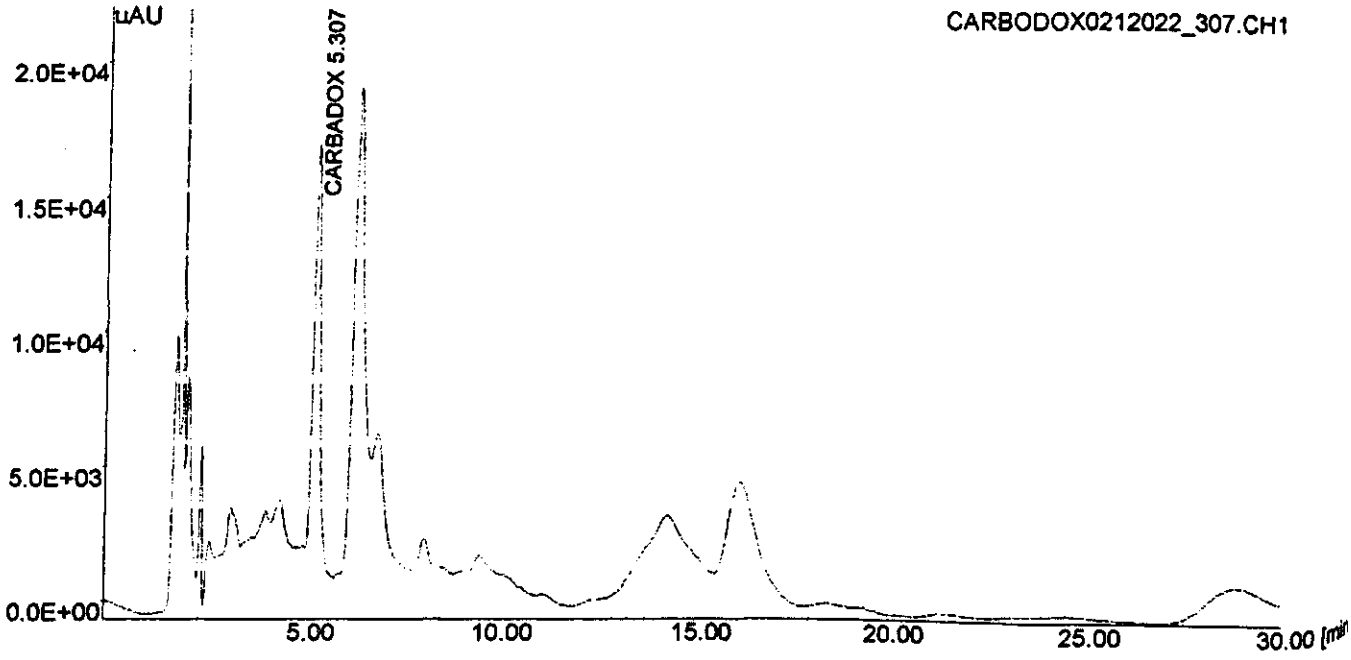
File name : CARBODOX0212018_307.CH1

Info :
a3009143a
Wavelength = 307 [nm]
Tacc [Sec] = 0.80 Wacc [nm] = 4.0
Autozero [min] : 0.00

Vial # = 9 Rack # = 0
Injection Date : 4-Dec-2000 23:21:28
Curr. Date : 19-Dec-2000 16:16:34
User : KL
Group : NICARB
Control Method :

Peak Detection Not Available

Chromatogram 261175



File name : CARBODOX0212022_307.CH1

Info :
a3009144a
Wavelength = 307 [nm]
Tacc [Sec] = 0.80 Wacc [nm] = 4.0
Autozero [min] : 0.00

Vial # = 11 Rack # = 0
Injection Date : 5-Dec-2000 1:23:40
Curr. Date : 19-Dec-2000 16:16:54
User : KL
Group : NICARB
Control Method :

#	Name	RT	Height[uAU]	Area[uAU.Sec]	%Area
1	CARBADOX	5.307	15544	189280.346	100.00

Total Area of Peak = 189280.346 [uAU.Sec]

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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
271109	8,26	8,21
271115	1,97	2,06
271119	8,18	8,24
271136	1,77	1,80
271185	not detectable	not detectable
271209	not detectable	not detectable

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 23.10. - 30.10.2000

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1,1 ml/min
- Injection volume: 20 µl
- Retention time of carbadox: 6,1 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

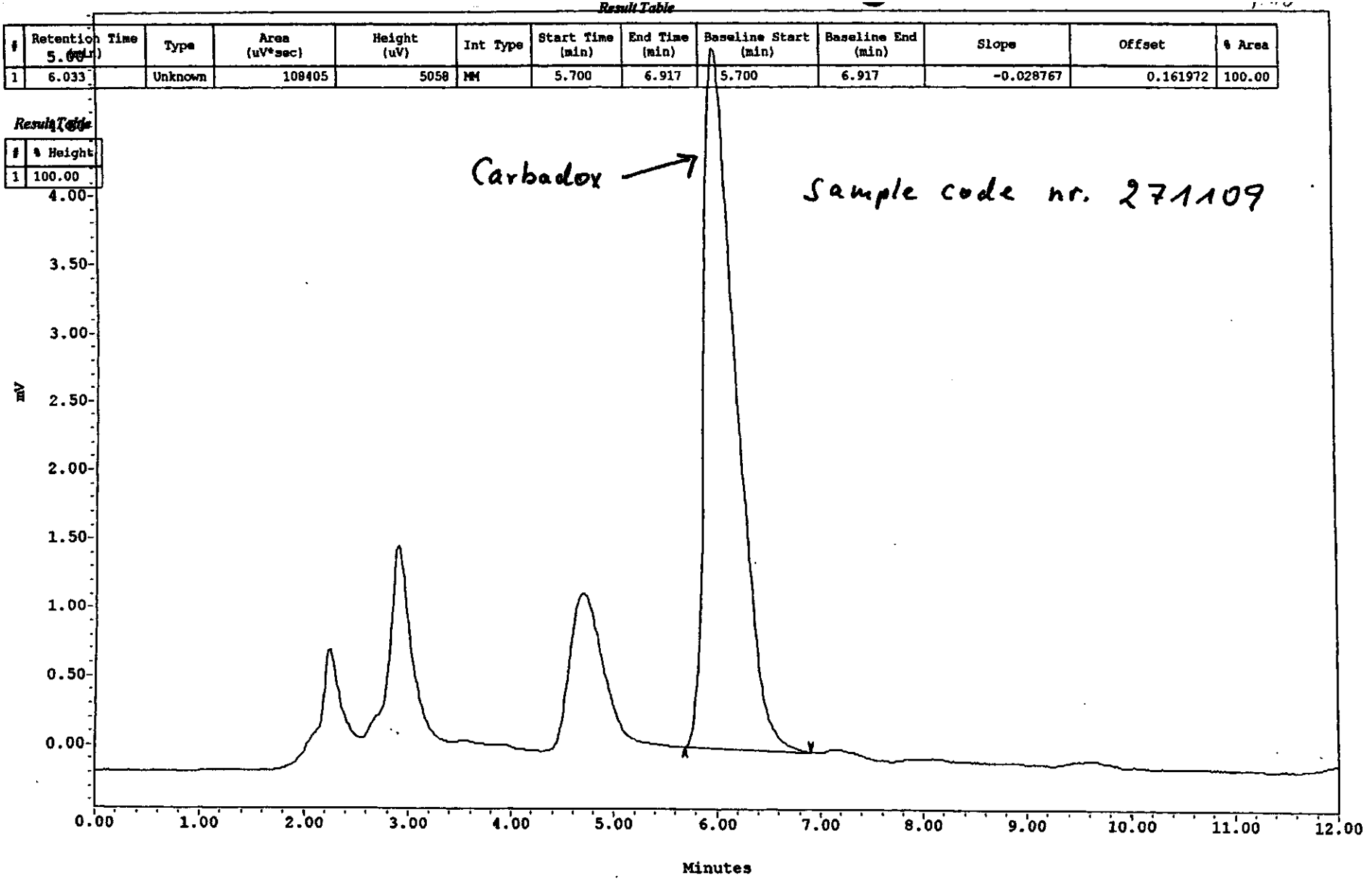
- Percentage recovery: 90,9 %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 89,9% and 91,9%
- Spiking level: 5 mg/kg

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	6.033	Unknown	108405	5058	MM	5.700	6.917	5.700	6.917	-0.028767	0.161972	100.00

Result Table

#	% Height
1	100.00



SampleName: CAR 1 b Vial: 9 Inj: 1 Ch: SATIN Type: Unknown Page: 1 of 1

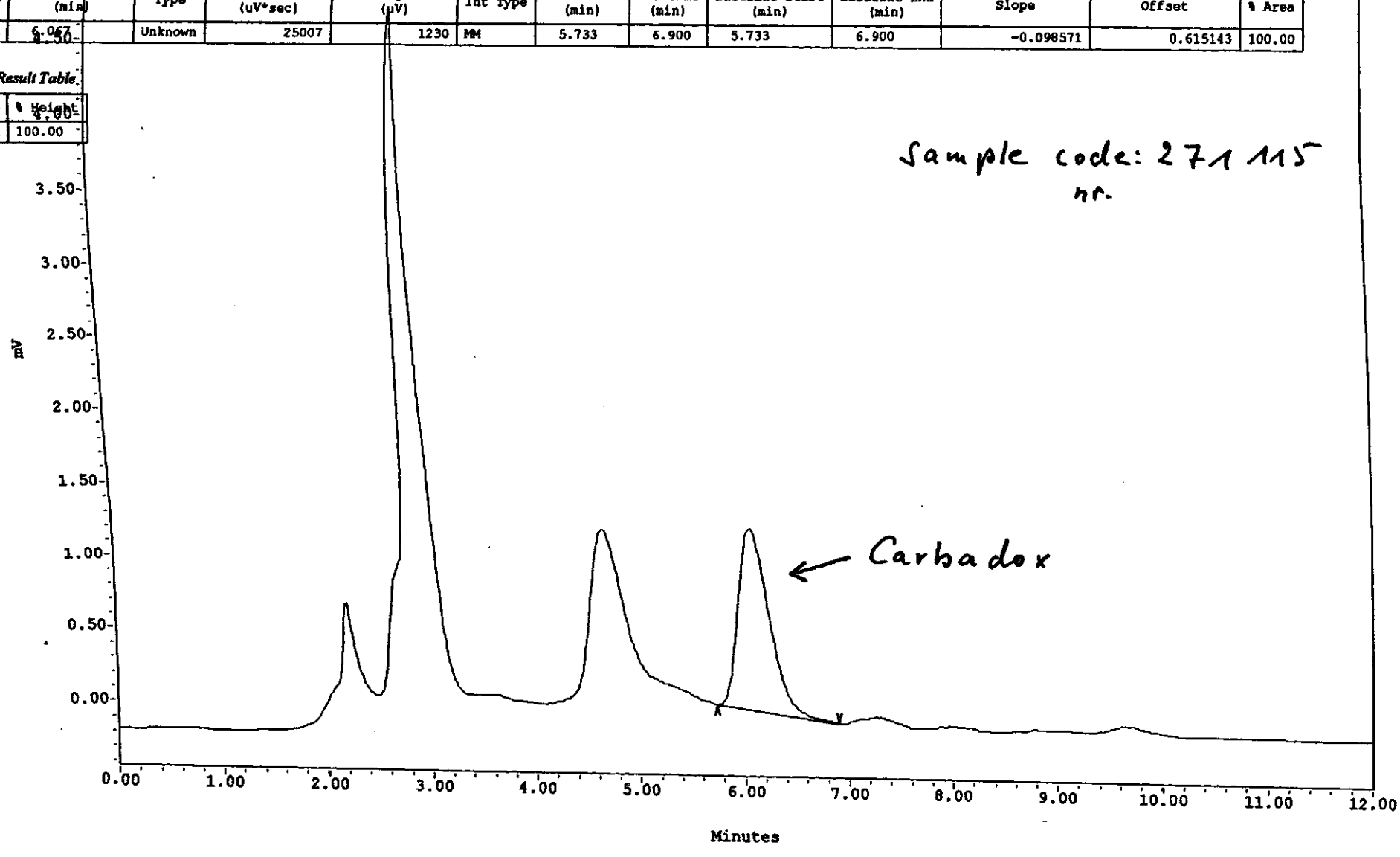
'30. OKT. 2000

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	6.067	Unknown	25007	1230	MM	5.733	6.900	5.733	6.900	-0.098571	0.615143	100.00

Result Table

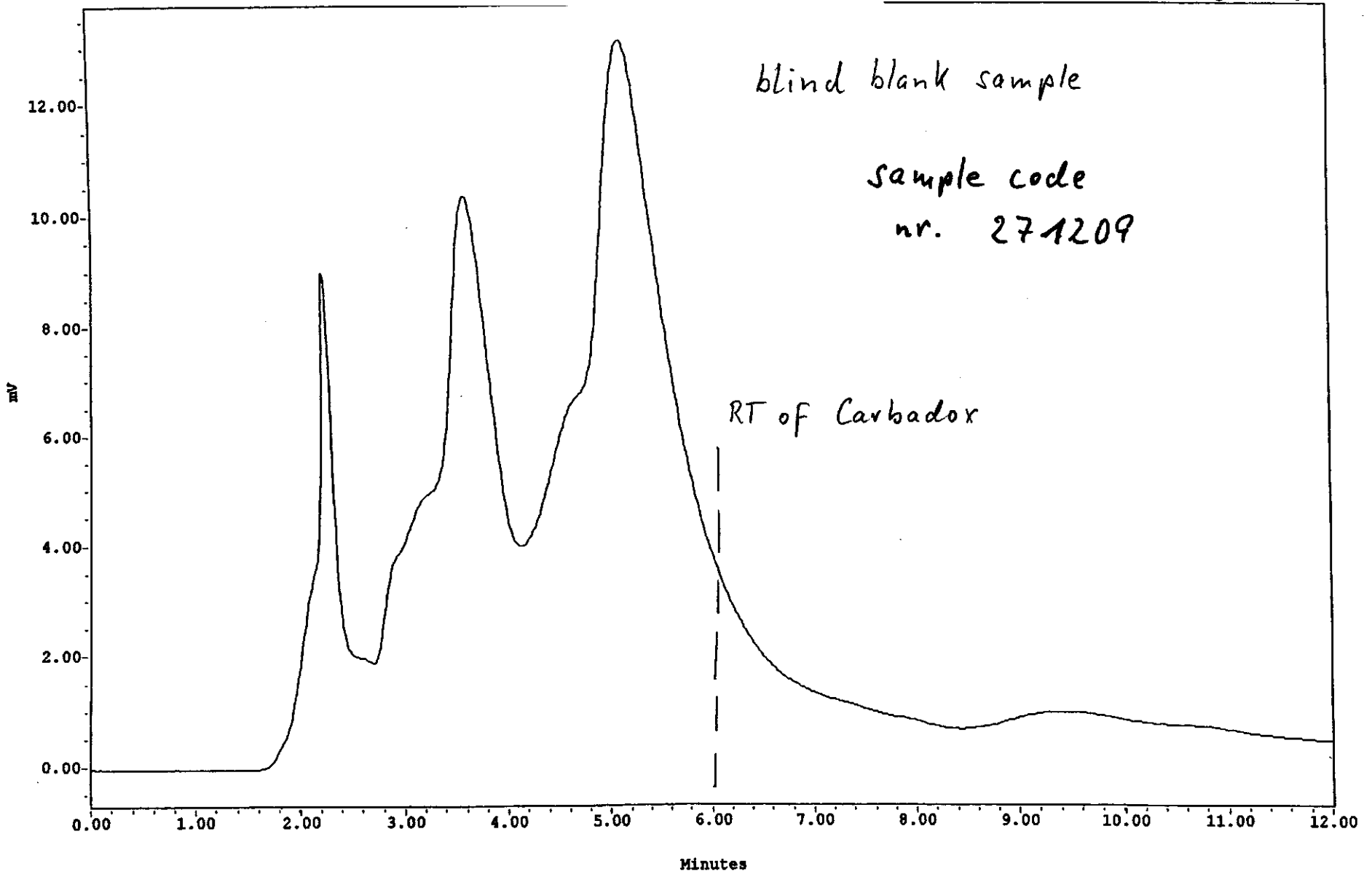
#	% Height
1	100.00



SampleName: Probe 2c Vial: 5 Inj: 1 Ch: SATIN Type: Unknown Page: 1 of 1

(27)

25.10.2007



SampleName: CAR 6a Vial: 9 Inj: 1 Ch: SATIN Type: Unknown

25. OKT. 2000

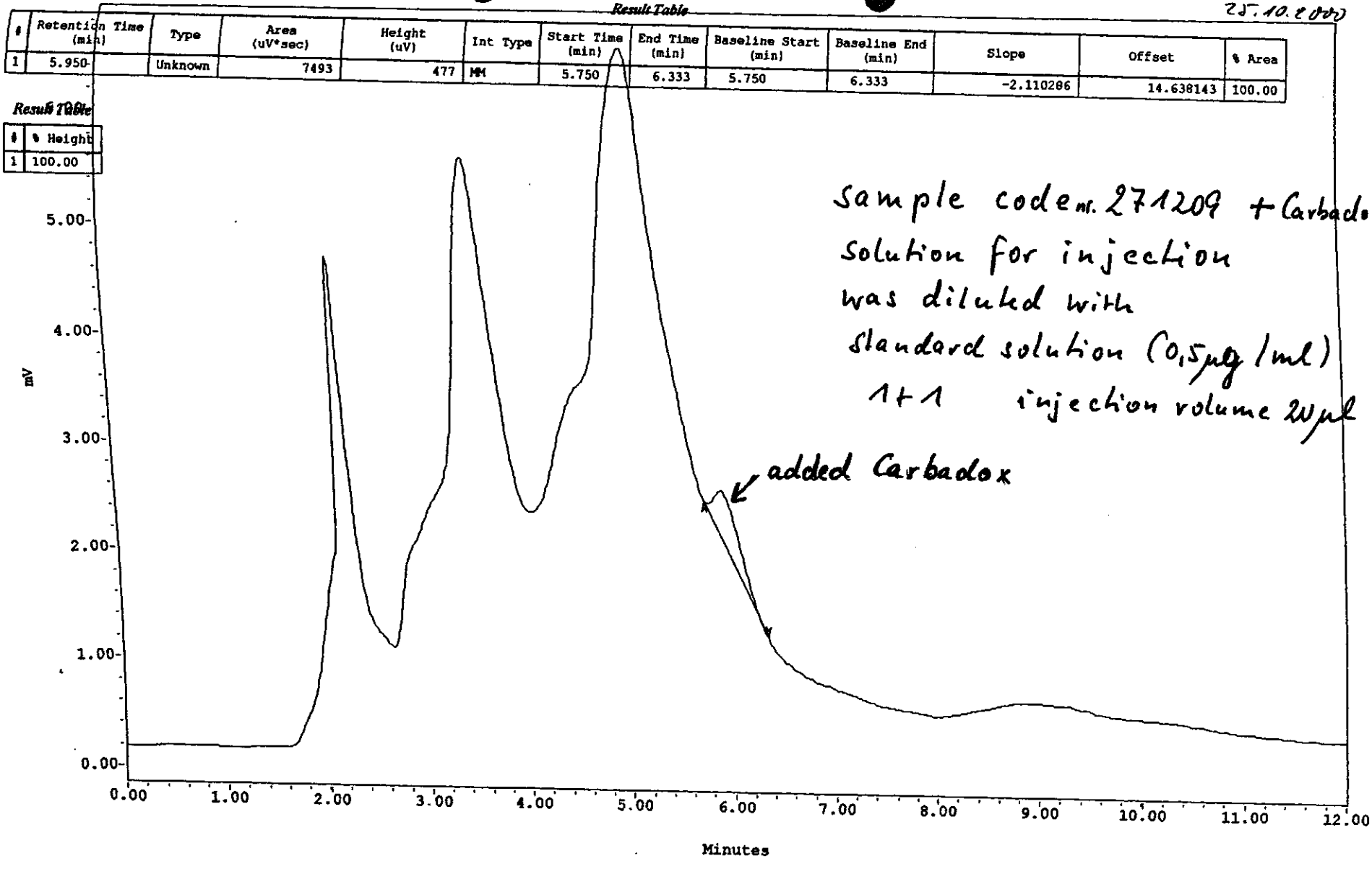
25.10.2000

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	5.950	Unknown	7493	477	MM	5.750	6.333	5.750	6.333	-2.110286	14.638143	100.00

Result Table

#	% Height
1	100.00



sample code nr. 271209 + Carbadox
solution for injection
was diluted with
standard solution (0,5 µg/ml)
1+1 injection volume 20 µl

added Carbadox

SampleName: CAR6a+0.5/1+1 Vial: 10 Inj: 1 Ch: SATIN Type: Unknown Page: 1 of 1

µg/ml

(4)

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: **CARBADOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
291108	1,8	1,9
291132	0	0
291159	7,2	7
291208	7,1	7
291210	0	0
291222	1,6	1,7

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: ...13.:...11.....2000.....

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: Nova Pack, 250x4,6 mm; C18; 4µ
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .....
- Flow-rate: .....1..... ml/min
- Injection volume: ..20...µl
- Retention time of carbadox: 4,4.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: ..94.. % and ..92.. %
- Spiking level: .....5..... mg/kg

Reported by User: System

Project Name: Carbadox

Sample Name:

Vial: 32

Injection #: 1

Injection Volume: 20,00 ul

Run Time: 6,0 Minutes

Sample Set Name: CARBADOX

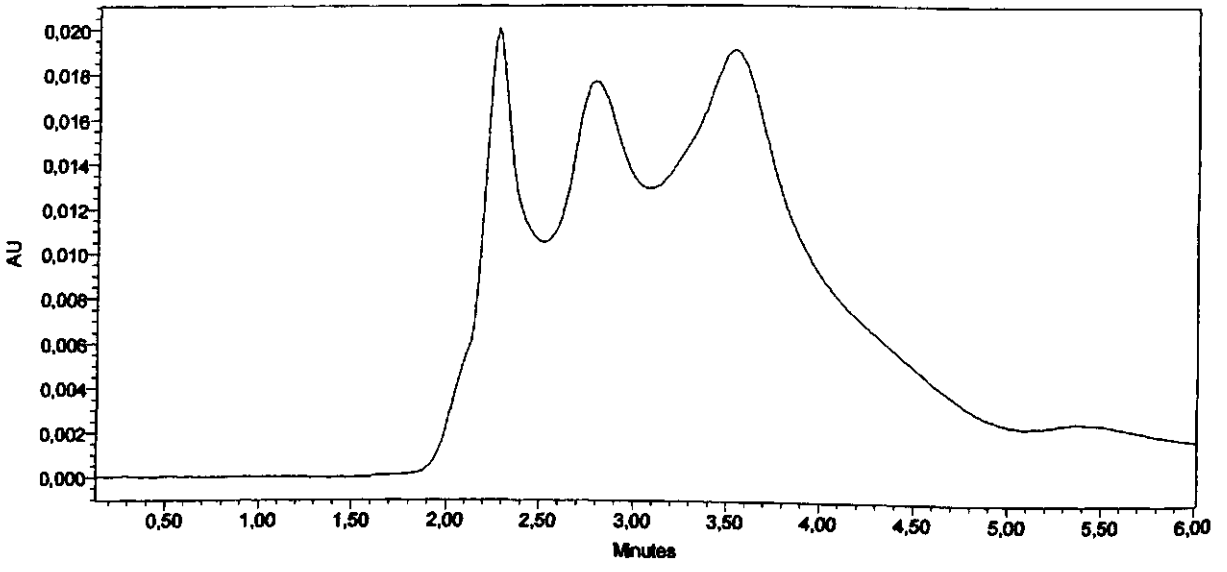
Date Acquired: 17-11-2000 17:16:27

Acq. Method Set: Carbadox

Date Processed: 18-11-2000 16:15:09

Processing Method: proces carbadox 18_11_00

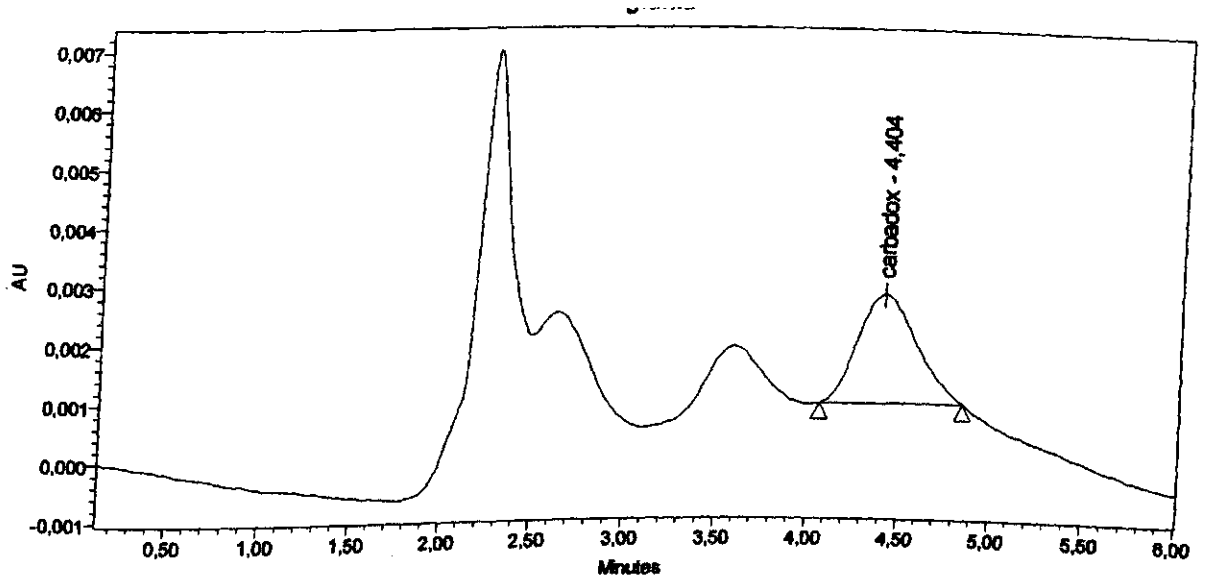
Proc. Chnl. Descr.: PDA 365,0 nm



	Name	RT	Area	Height	Amount	Units
1	carbadox	4,444				

Sample Name:
Vial: 35
Injection #: 1
Injection Volume: 20,00 ul
Run Time: 6,0 Minutes
Sample Set Name: CARBADOX

Date Acquired: 17-11-2000 17:59:30
Acq. Method Set: Carbadox
Date Processed: 18-11-2000 16:15:09
Processing Method: proces carbadox 18_11_00
Proc. Chnl. Descr.: PDA 365,0 nm



	Name	RT	Area	Height	Amount	Units
1	carbadox	4,404	41467	1868	1,436	ug/ml

(7,0 mg/kg)

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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
311153	1,28	1,27
311154	6,96	7,14
311181	7,22	7,15
311183	0	0
311201	0	0
311203	1,24	1,19

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: ....9.-.11.-.2000.....

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: ...850.:.150.(v/v).acetatebuffer.:.acetonitrile
- Flow-rate: .....1,5..... ml/min
- Injection volume: ..20....µl
- Retention time of carbadox: .5,1.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

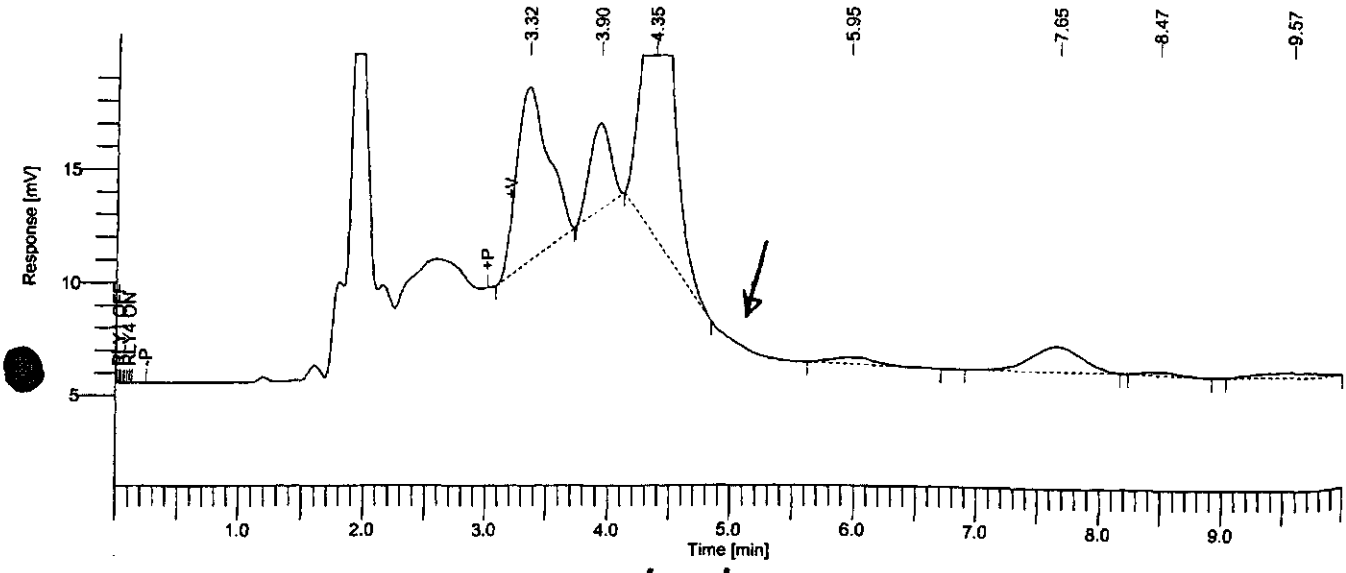
Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: ..... %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: .81.. % and ..83. %
- Spiking level: ....5..... mg/kg

Software Version	: 6.1.1.0.0:K20	Date	: 11/13/00 9:30:13 AM
Sample Name	: 22006-b	Data Acquisition Time	: 11/10/00 11:21:25 AM
Instrument Name	: HPLC-2	Channel	: A
Rack/Vial	: 0/0	Operator	:
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 22		

Result File : \\rik004s\TCdata\. Residue\HPLC-1\carbadox\091100-022.rst
Sequence File : \\rik004s\TCdata\. Residue\HPLC-1\carbadox\carbadox 091100.seq



carbadox

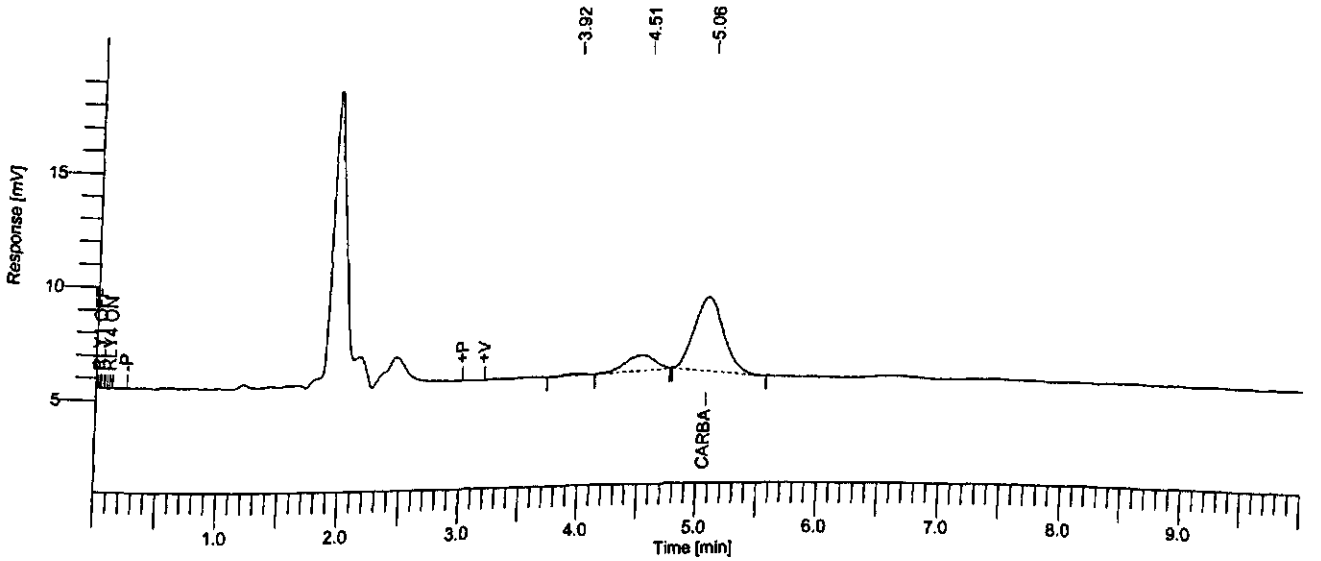
Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	3.32		137102.50	7741.34	28.24	28.24	*BB	17.7104
2	3.90		44172.00	3854.20	9.10	9.10	*BB	11.4607
3	4.35		252128.50	15219.80	51.93	51.93	*BB	16.5658
-	5.00	carbadox	0.00	0.00	0.00	0.00		
4	5.95		7142.00	281.19	1.47	1.47	*BB	25.3988
5	7.65		32869.50	1128.99	6.77	6.77	*BB	29.1140
6	8.47		3322.00	163.86	0.68	0.68	*BB	20.2740
7	9.57		8735.00	236.45	1.80	1.80	*BB	36.9416
			485471.50	28625.84	100.00	100.00		

Missing Component Report

Component	Expected Retention (Calibration File)
carbadox	5.000

Software Version	: 6.1.1.0.0:K20	Date	: 11/13/00 9:30:12 AM
Sample Name	: 22005-b	Data Acquisition Time	: 11/10/00 10:20:50 AM
Instrument Name	: HPLC-2	Channel	: A
Rack/Vial	: 0/0	Operator	:
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 21		

Result File : \\rik004s\TCdata\ Residue\HPLC-1\carbadox\091100-021.rst
Sequence File : \\rik004s\TCdata\ Residue\HPLC-1\carbadox\carbadox 091100.seq



carbadox

Peak #	Time [min]	Component Name	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	3.92		977.50	81.29	1.44	1.44	*BB	12.0248
2	4.51		11982.00	684.47	17.59	17.59	*BB	17.5055
3	5.06	carbadox	55150.00	3278.54	80.97	80.97	*BB	16.8215
			68109.50	4044.30	100.00	100.00		

Missing Component Report

Component	Expected Retention (Calibration File)

All components were found

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: **CARBADOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
321103	negative	negative
321104	8,50	8,62
321149	1,46	1,62
321150	1,59	1,56
321190	8,84	8,92
321217	negative	negative

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

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: 1.6 ml/min
- Injection volume: 20 (µL)
- Retention time of carbadox: 3.73 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

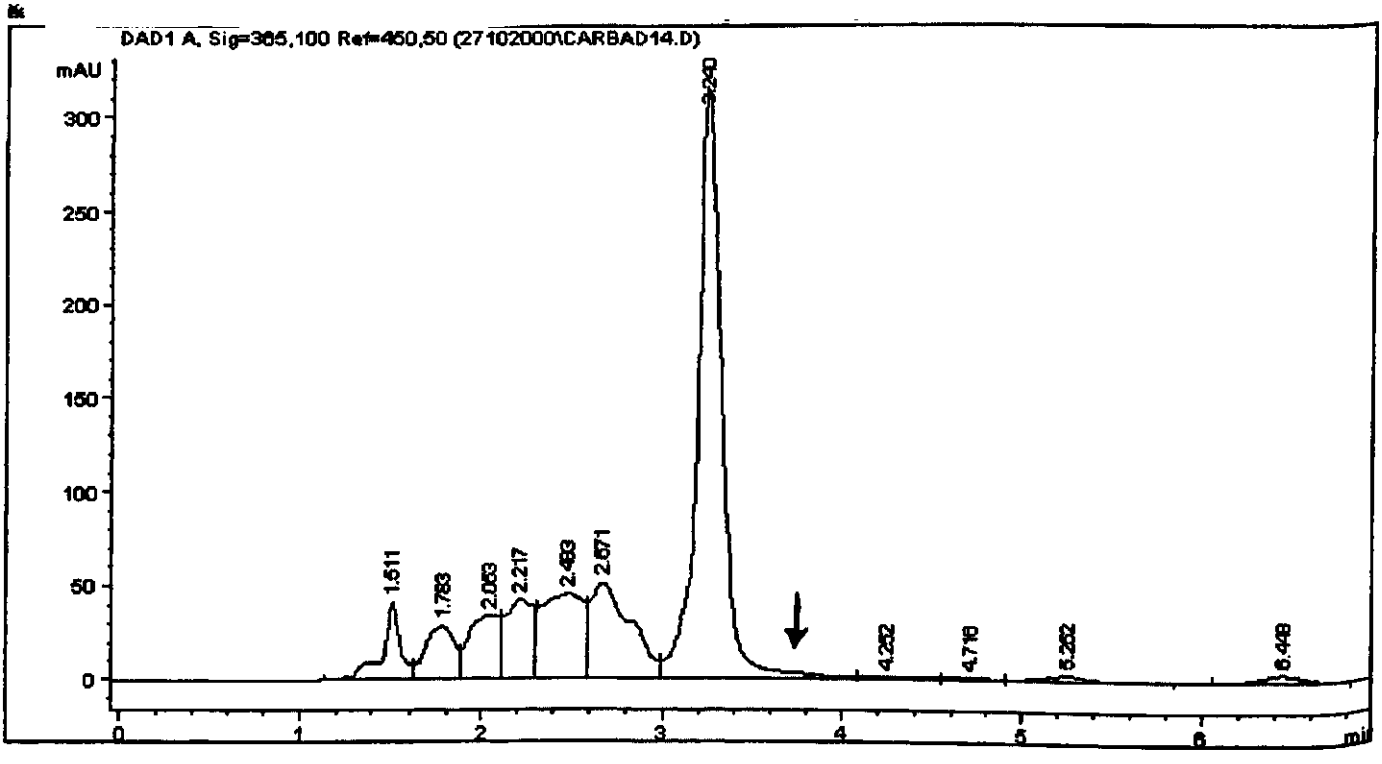
Recovery results:

- Percentage recovery: 100.6 %
- Single/duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 101.0% and 100.3%
- Spiking level: 10 mg/kg

32

m - 10.0019g

Injection Date	: 10/27/00 11:14:17 PM	Seq. Line :	9
Sample Name	: 321183	Vial :	9
Acq. Operator	: ..	Inj :	1
		Inj Volume :	20 µl
Acq. Method	: C:\HPCHEM\1\	X.M	
Last changed	: 10/27/00 11:12:17 PM by (modified after loading)		
Analysis Method	: C:\HPCHEM\1\		
Last changed	: 10/30/00 12:25:59 PM by (modified after loading)		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	10/30/00 12:25:57 PM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=365,100 Ref=450,50

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

Totals : 0.00000

Results obtained with enhanced integrator!
1 Warnings or Errors :

File C:\HPCHEM\1\DATA\27102000\CARBAD19.D

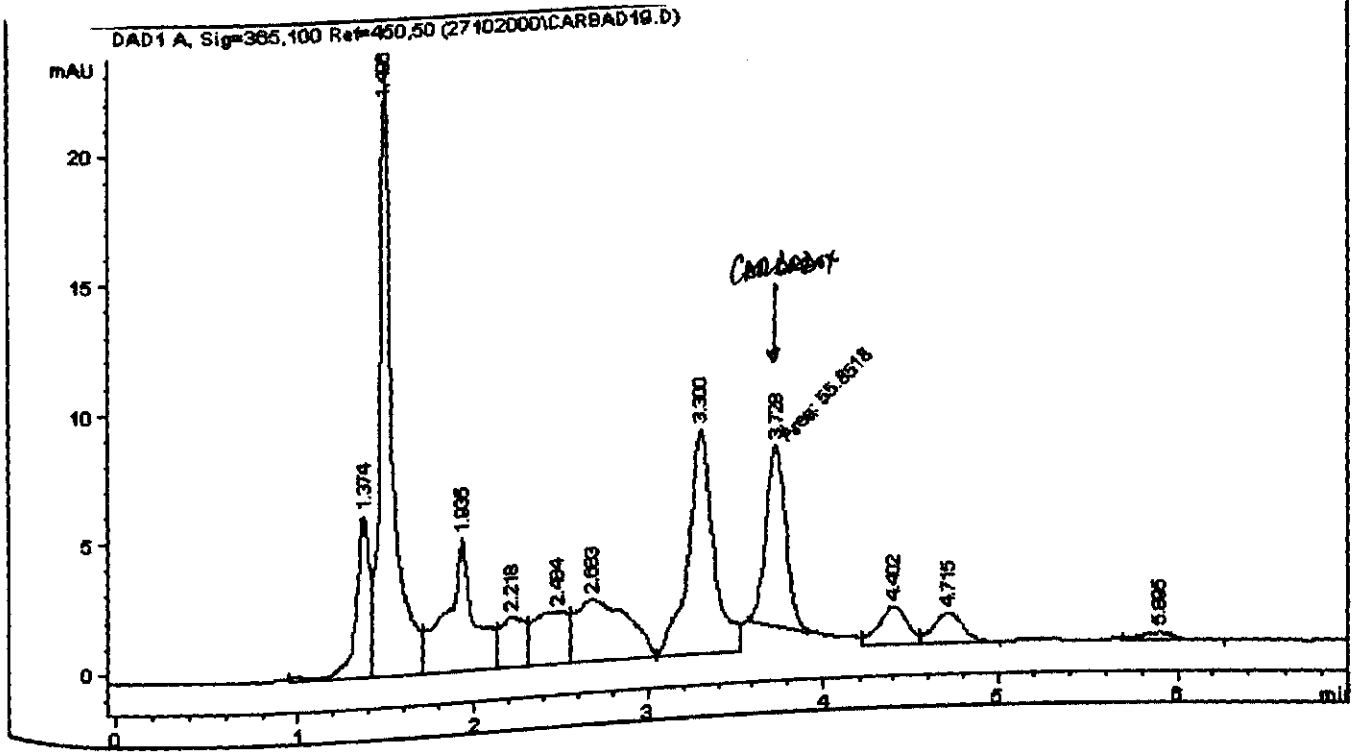
m = 10.0035g

Injection Date : 10/20/00 12:00:54 AM
Sample Name : 321184/
Acq. Operator :

Seq. Line : 14
Vial : 13
Inj : 1
Inj Volume : 20 µl

Acq. Method : C:\HPCHEM\1\METHOD
Last changed : 10/20/00 12:06:54 AM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\
Last changed : 10/30/00 12:25:59 PM by
(modified after loading)

HPLC.



External Standard Report

Injected By : Signal
Calib. Data Modified : 10/30/00 12:25:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,100 Ref=450,50

RTime min	Type	Area mAU*s	Amt/Area	Amount ug/ml	Grp	Name
3.728	MM	55.85188	3.04379e-2	1.70001		Carbadox
Total :				1.70001		

Results obtained with enhanced integrator!

*** End of Report ***

Printed at 10/30/00 12:33:42 PM

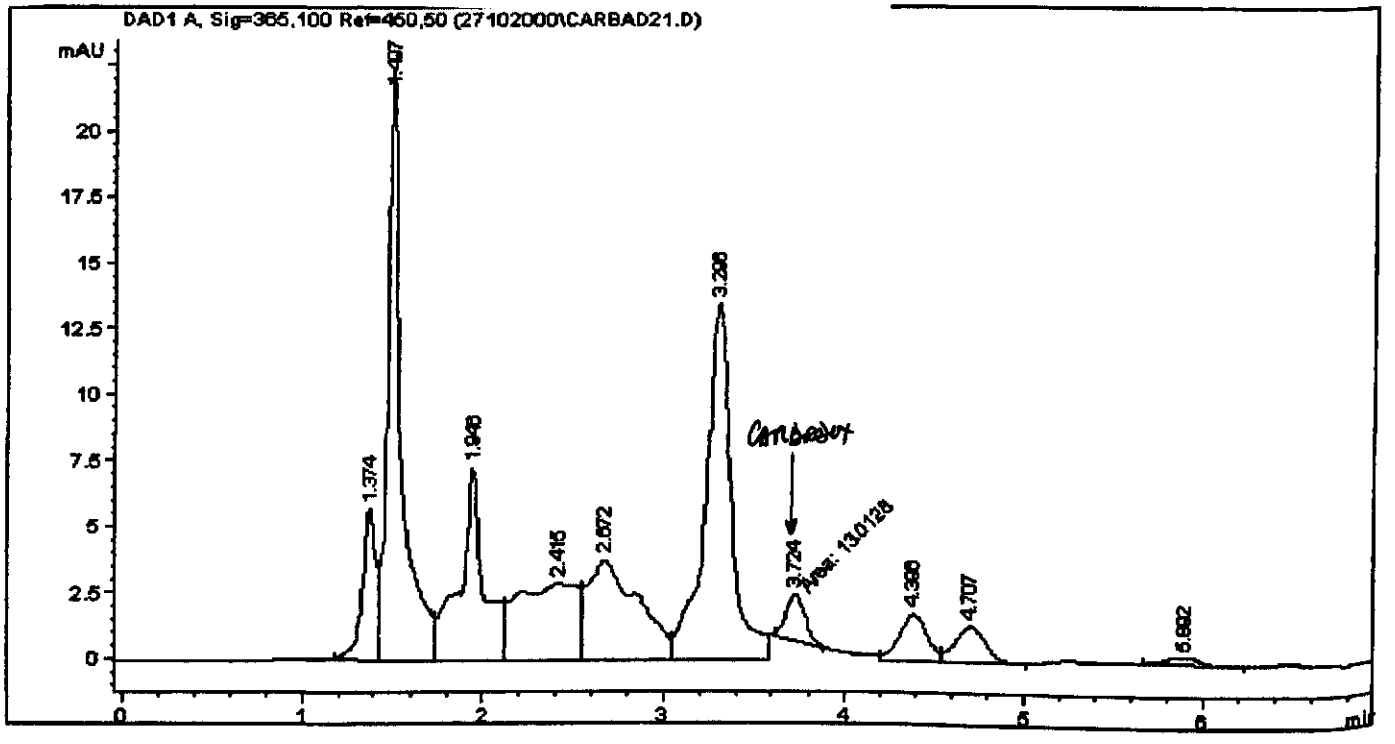
a File C:\HPCHEM\1\DATA\27102000\CARBAD21.D Sample Name: 321149

(32)

m - 10.0022g

Injection Date : 10/28/00 12:30:51 AM Seq. Line : 16
Sample Name : 321149 Vial : 15
Acq. Operator : Inj : 1
 Inj Volume : 20 µl
Acq. Method : C:\HPCHEM\1\METHODS\CARBADOX.M
Last changed : 10/28/00 12:28:51 AM by
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\CARBADOX.M
Last changed : 10/30/00 12:25:59 PM by
(modified after loading)

HPLC.



External Standard Report

Sorted By : Signal
Calib. Data Modified : 10/30/00 12:25:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,100 Ref=450,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ug/ml]	Grp	Name
3.724	MM	13.01282	2.24483e-2	2.92111e-1		Carbadox

Totals : 2.92111e-1

Results obtained with enhanced integrator!

*** End of Report ***

rument 1 10/30/00 12:35:32 PM

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: CARBADOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
331130	1,2	1,3
331140	5,6	5,8
331174	5,1	4,7
331206	< 1	< 1
331224	1,3	1,5
331226	< 1	< 1

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

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

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase: Buffer/acetonitrile 80/20
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: ....0.5..... ml/min
- Injection volume: ...20....µl
- Retention time of carbadox: ..3,1.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: ..85.. %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ..84 % and ..86 %
- Spiking level: .....2.. mg/kg

M. 33 1174

(33)

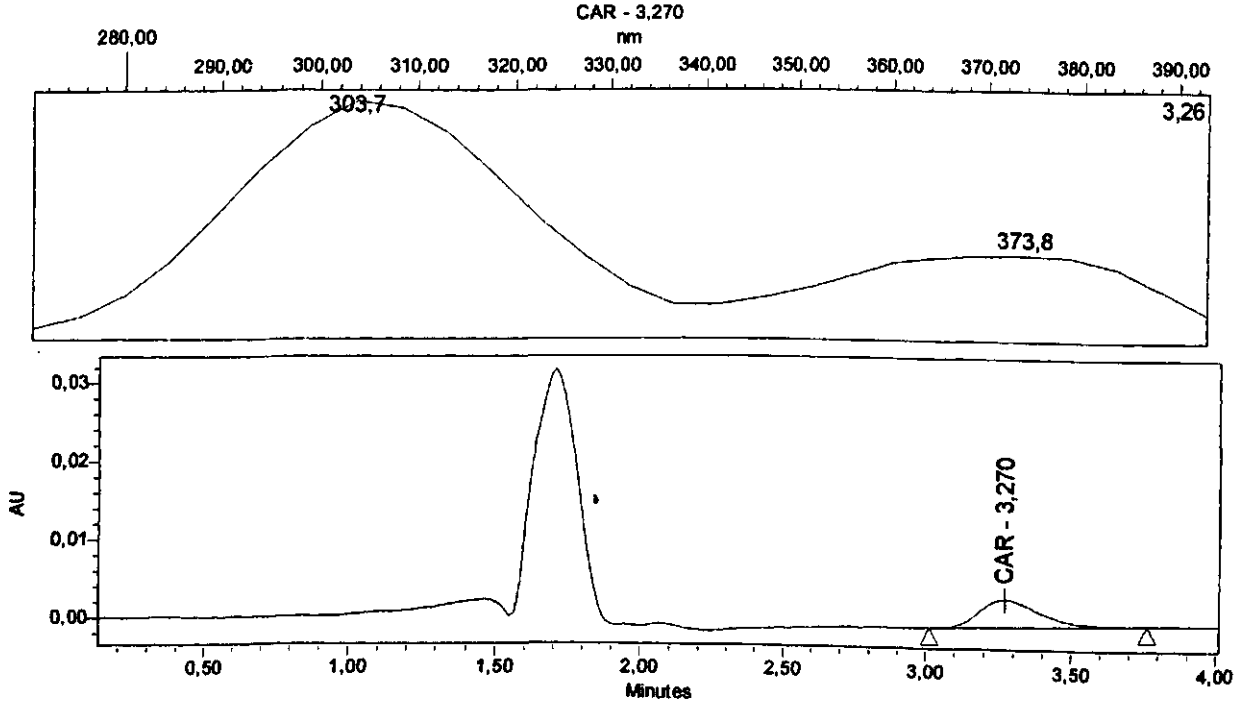
Sample Set Name CAR24

User Name RVSA

Current Date 25/10/2000

Current Time 9:04:22

Spectrum Index Plot



SampleName 7833/00 Vial 5 Injection 1 Date Acquired 24/10/2000 12:16:37

Peak Results

	Name	RT	Area	Height	Amount	Units
1	CAR	3,270	51420	3570	4,732	mg/kg

Blind Blank

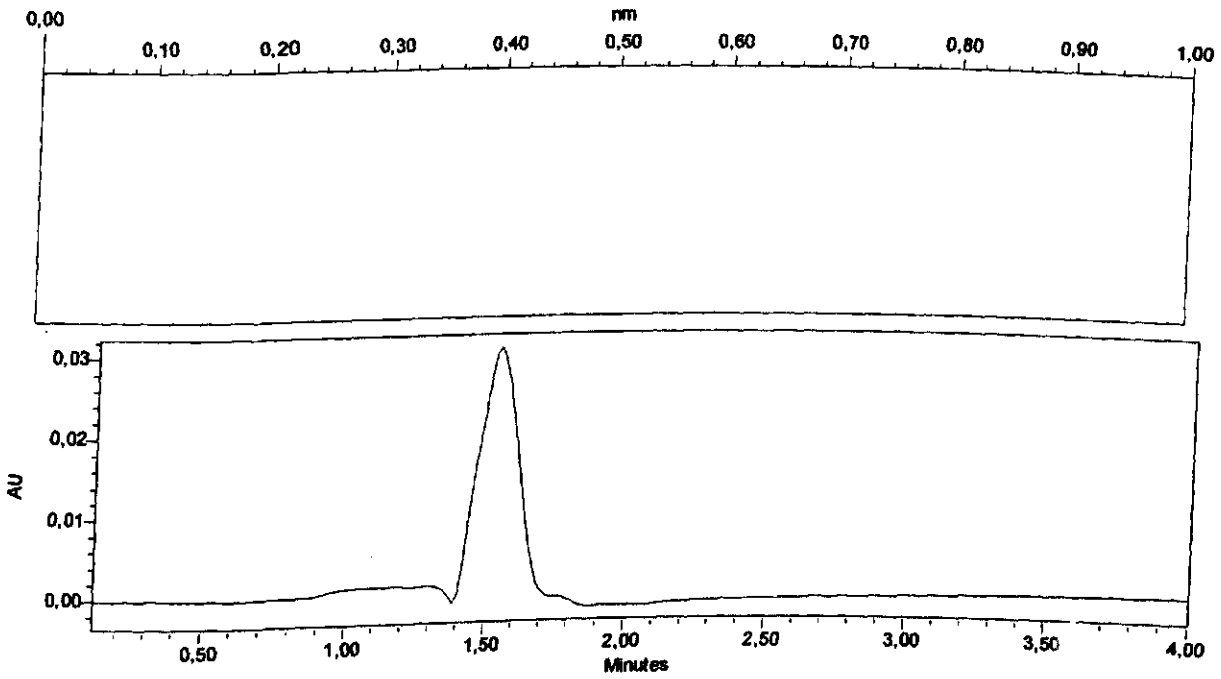
Sample Set Name CAR24

User Name RVSA

Current Date 25/10/2000

Current Time 9:04:19

Spectrum Index Plot



SampleName 7834/00 Vial 6 Injection 1 Date Acquired 24/10/2000 12:21:44

Peak Results

	Name	RT	Area	Height	Amount	Units
1	CAR	3,165				

Nr. 331224

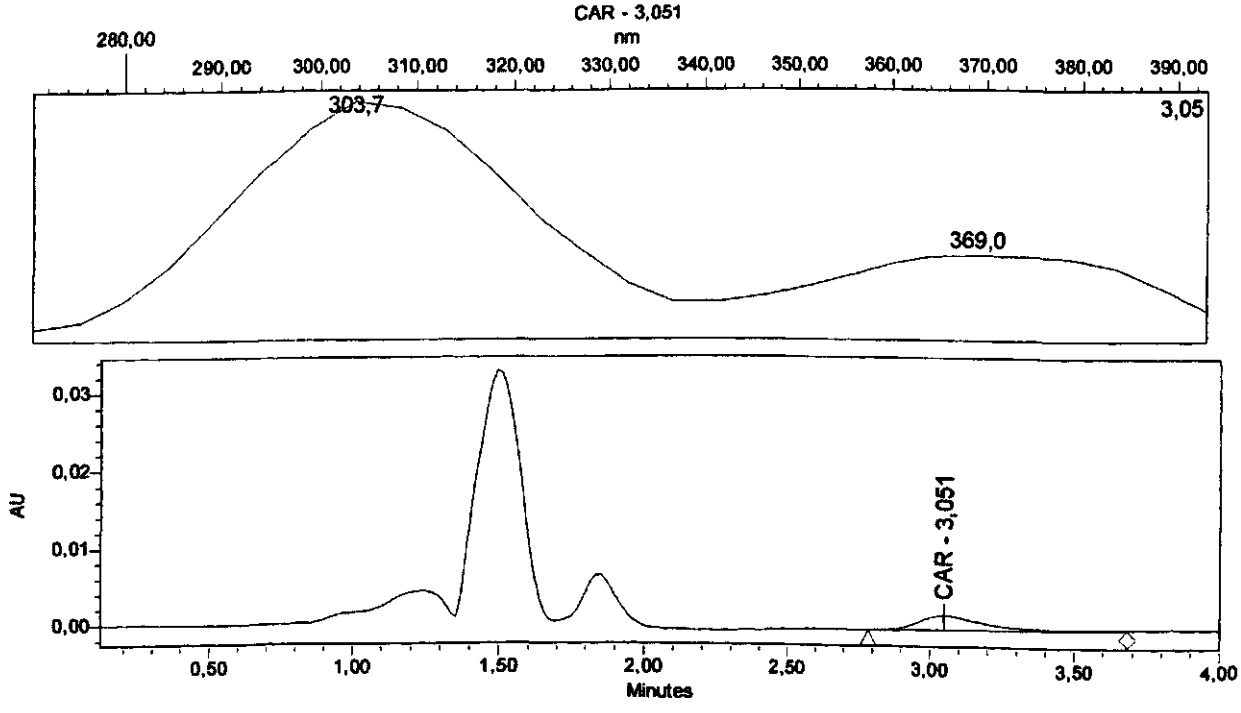
Sample Set Name CAR24

User Name RVSA

Current Date 25/10/2000

Current Time 9:04:18

Spectrum Index Plot



SampleName 7836/00 Vial 8 Injection 1 Date Acquired 24/10/2000 12:31:56

Peak Results

	Name	RT	Area	Height	Amount	Units
1	CAR	3,051	29477	1834	1,460	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: **CARBADOX**

Unit / Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
341125	0	0
341147	6,2	6,3
341167	0	0
341180	6,2	6,2
341216	1,2	1,1
341221	1,1	1,1

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

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 carbadox: ..3.8.. min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

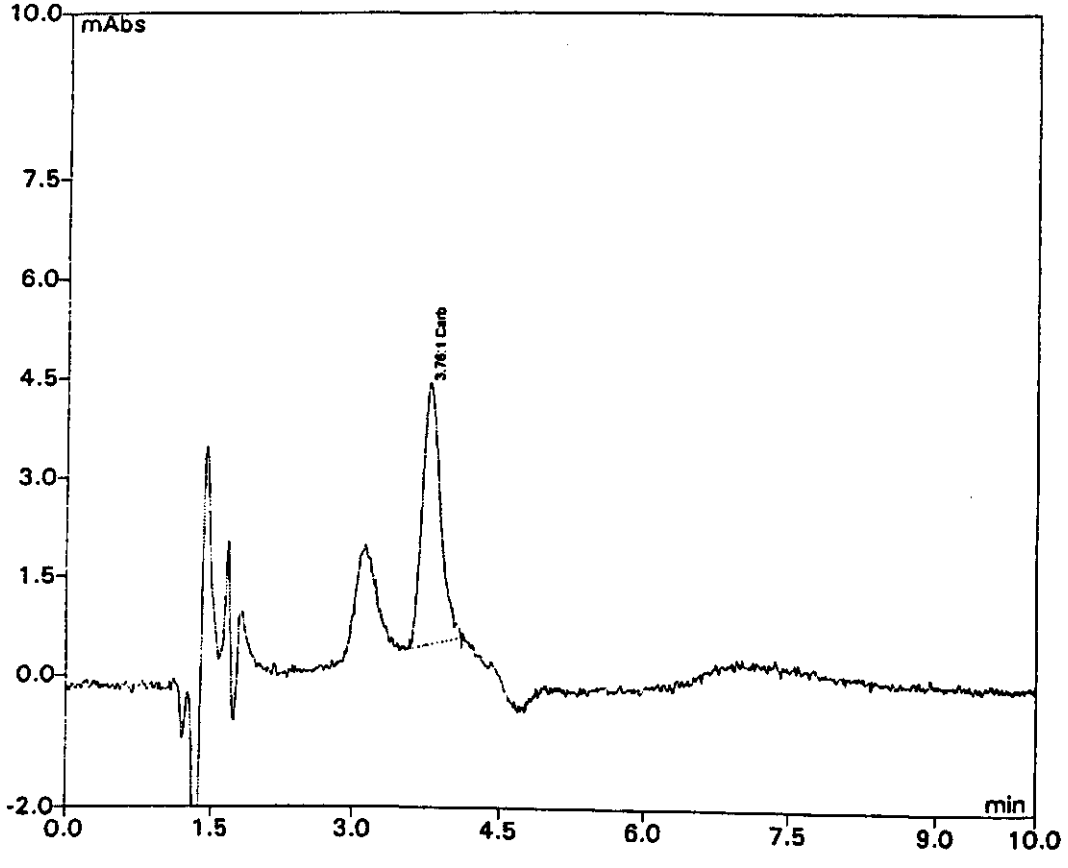
- Percentage recovery: ..87.. %
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: ..86.. % and ..88.. %
- Spiking level: ..5.. mg/kg

Channel 3

KromaSystem 2000 Version 1.83 RESULT REPORT: INTEGRATION

SYS1 - CBXEG039.SMP (modified): C
No. 17: C1905" 10g/50ml Acquired : 24.10.00 13:14:28
Channel 3: DAD 3 chrom. 365/ 5 Processed: 25.10.00 08:13
No Text

Program File ..... CBXEG ..... Carbadox EG-Methode
Worksheet ..... CBXEG2 .....
Peak Table ..... CBXEG .....
Parameter Table .. CBXEG .....
Report File ....
Document File ....



No.	PNo	Ret.Time min	Type	Name	Area mAbs*min	Amount	Rel.Ar %
1	1	3.76	MOD	Carbadox	8.3877e-001	6.2399e+000	100.00
					8.3877e-001	6.2399e+000	100.00

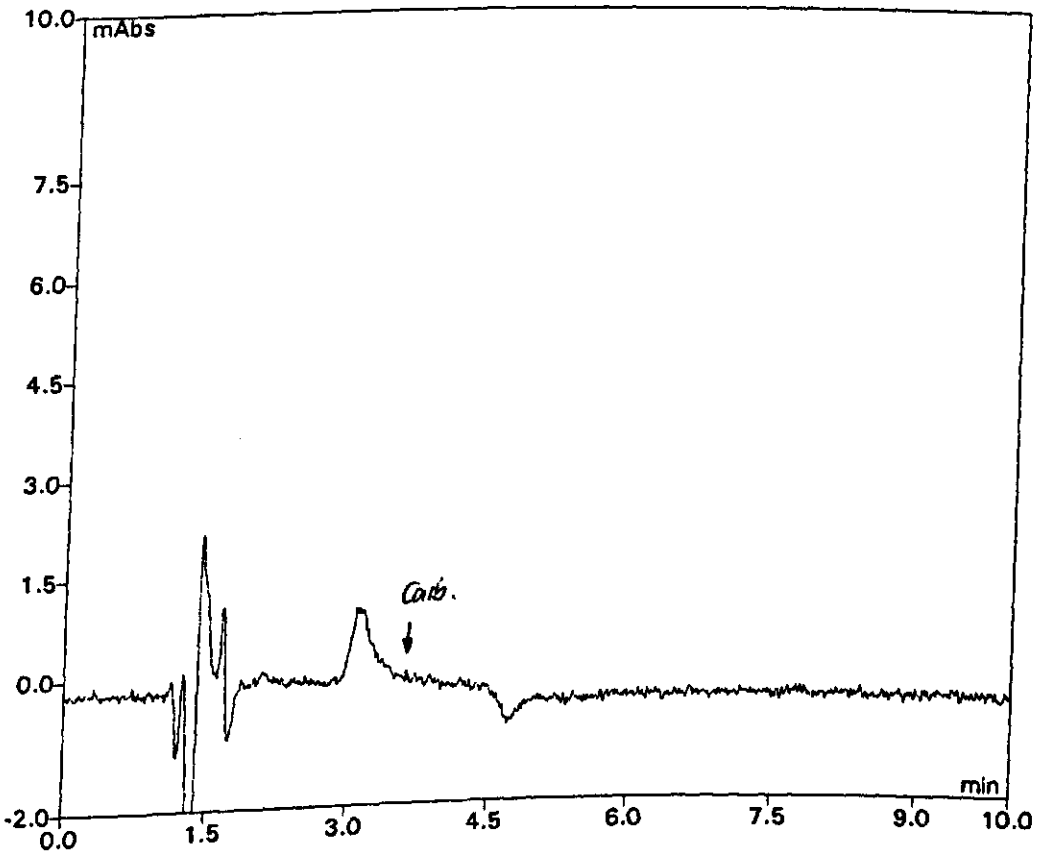
Channel 3

KromaSystem 2000 Version 1.83 RESULT REPORT: INTEGRATION

SYS1 - CBXEG039.SMP (modified):
No. 05: BL " 10g/50ml
Channel 3: DAD 3 chrom. 365/ 5
No Text

Acquired : 24.10.00 11:04:10
Processed: 25.10.00 08:11

Program File ..... CBXEG ..... Carbadox EG-Methode
Worksheet ..... CBXEG2 .....
Peak Table ..... CBXEG .....
Parameter Table .. CBXEG .....
Report File ....
Document File ....



No.	PNo	Ret.Time min	Type	Name	Area mAbs*min	Amount	Rel.Ar %
					0.0000e+000	0.0000e+000	0.00

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 35

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: CARBADOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
351113	1,7	1,6
351177	1,7	1,7
351188	< 0,1	< 0,1
351193	7,9	7,8
351200	7,4	7,4
351213	< 0,1	< 0,1

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 30-11-2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ■ Other: Lichrospher RP-18 5mm (125 x 40 mm)
- Mobile phase:
  - ■ As described in the method
  - ☐ Other: .....
- Flow-rate: 1,0 ml/min
- Injection volume: 20 µl
- Retention time of carbadox: 4,1 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: 100 %
- Single / duplicate determinations: ☐ single ■ duplicate
- If duplicate, please give both percentages: 101,3 % and 98,7 %
- Spiking level: 15 mg/kg

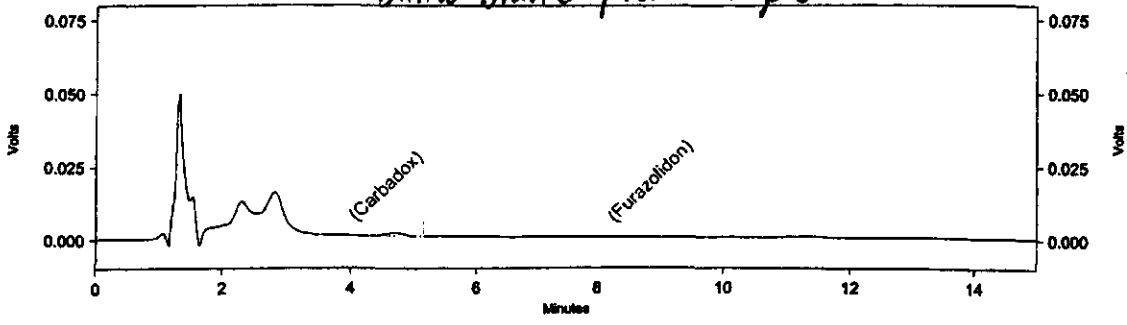
Carbadox/Furazolidon

: \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Carbadox\Method\Carbadox.met

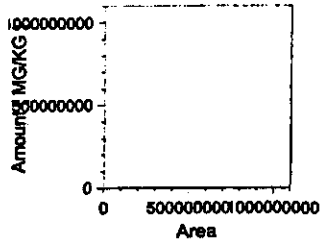
File : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Carbadox\Data\carbadox_301100_012

Sequence : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Carbadox\Sequence\carabdox.seq

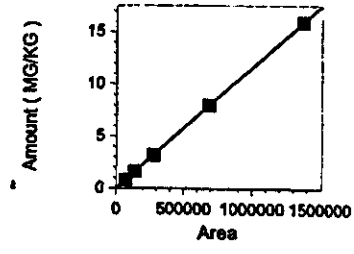
Blind blank feed sample



Peak: Furazolidon – ESTD – UV-Detector



Peak: Carbadox – ESTD – UV-Detector



UV-Detector Results

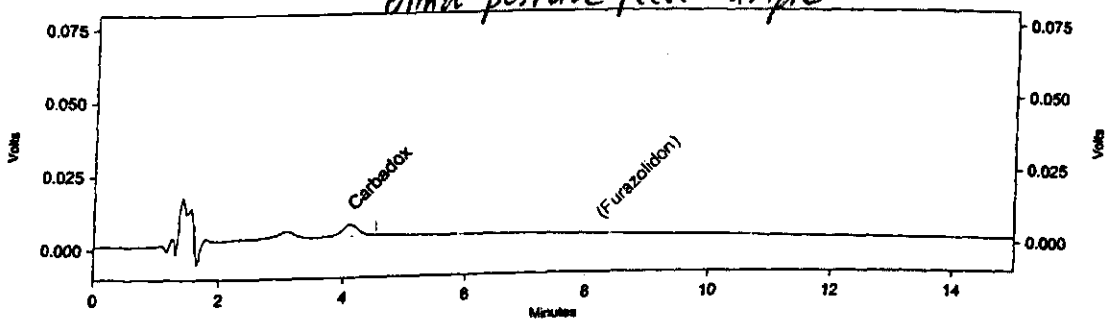
	Pk #	Retention Time	Area	Height	ESTD concentration	Units
[redacted]rbadox					0.00000 BDL	MG/KG
Furazolidon					0.00000 BDL	MG/KG

Carbadox/Furazolidon

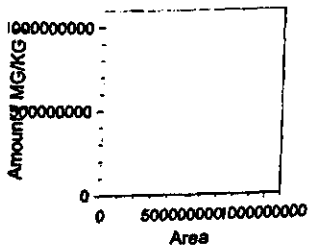
CANFAS code 351193

File : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Carbadox\Method\Carbadox.met
Sequence : \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Carbadox\Data\carbadox_301100_014
: \\Fs_mlab\VOL1\DATA\Elite_Admin\Projects\Carbadox\Sequence\carsbdox.seq

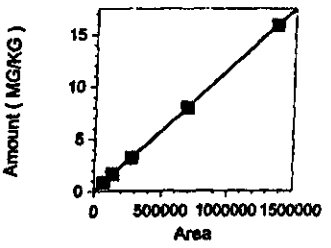
Blind positive feed sample



Peak: Furazolidon – ESTD – UV-Detector



Peak: Carbadox – ESTD – UV-Detector



UV-Detector Results

	Pk #	Retention Time	Area	Height	ESTD concentration	Units
Carbadox	1	4.10	141050	7799	7.88765	MG/KG
Furazolidon					0.00000 BDL	MG/KG

APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 37

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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
371101	8,36	8,76
371169	2,51	2,57
371179	ND	ND
371189	ND	ND
371191	2,40	2,56
371223	9,27	8,67

(S) 39

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

- **Annex 4 - Questionnaire**

Date(s) of analysis: 5th Dec 2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☐ Other: Hichrosphere RP18 - 5 Endcapped
- Mobile phase:
  - ☐ As described in the method
  - ☐ Other: .....
- Flow-rate: 1ml/min ml/min
- Injection volume: 50 µl
- Retention time of carbadox: 5.4 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

- Percentage recovery: 109 %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 109 % and 109 %
- Spiking level: 5 mg/kg

(37)

Olaquindox in Feed
Mode: Reprocessed Data
Original Results: C:\TSP\SYSTEM1\Data\carb51200ps.RES
Reprocessed Results: C:\TSP\SYSTEM1\Data\carb61200ps.RMS

Analysis Report

Name: 7
Description: 7
Type: Sample
Injection Volume: 50.0 µL

Vial: 819

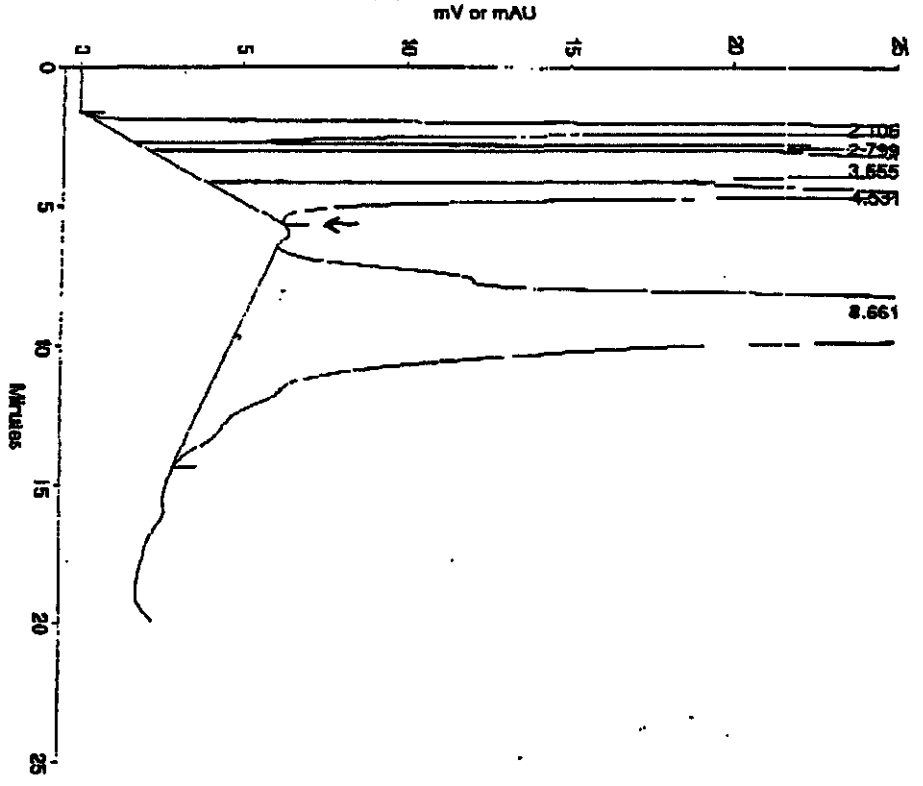
Injection: 1 of 1
Injected On: 06-12-00 02:37:41

Acquisition Log
Column Pressure (PSI): 2669
Noise (microAU): 3e+01
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): 1e+02

Pump Flow Stability: 7.4

Signal 1: UV2000 A 365 nm
Calculation Type: External Standard (Height)



Typical chromatograms for Carbadox

Blind Blank Feed (371179)

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

System: Reprocess
Analyst: ps
Acquisition Method: C:\TSP\SYSTEM1\Methods\carbadox.AQM
Calculation Method: C:\TSP\Methods\carb.CAM
Report Method: C:\TSP\Methods\olaq.RPM

PC1000 Ver 3.5 1
07-12-00 13:33:34
07-12-00 15:25:56
06 12 00 12:57:28

Olaquindox in Feed
Mode: Reprocessed Data
Original Results: C:\TSP\SYSTEM1\Data\carb51200ps.RES
Reprocessed Results: C:\TSP\SYSTEM1\Data\carb51200ps.RMS

Reported On: 07-12-00 17:02:02

Analysis Report

Name: 5
Description: 5
Type: Sample
Injection Volume: 50.0 µL

Vial: B15

Injection: 1 of 1

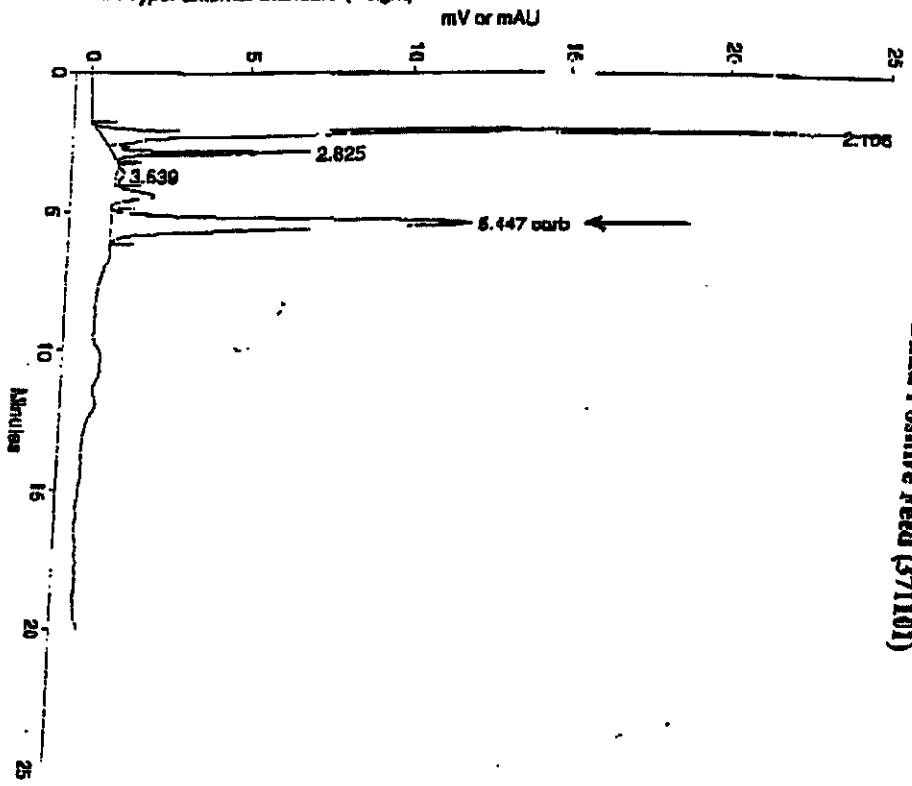
Injected On: 06-12-00 01:18:19

Acquisition Log
Column Pressure (PSI): 2636
Noise (microAU): 2e+01
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): 1e+02

Pump Flow Stability: 14.0

Signal 1: UV2000 A 365 nm
Calculation Type: External Standard (Height)



Typical chromatograms for Carbadox

Blind Positive Feed (37101)

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

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

Analyst: ps

PC1000 Ver 3.5.1
07-12-00 13:33:34
07-12-00 15:25:56
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: **CARBADOX**

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
381110	7,17	7,24
381116	7,10	7,55
381127	0	0
381156	1,40	1,53
381160	2,01	1,95
381202	0	0

CANFAS COLLABORATIVE STUDIES - CARBADOX

Annex 4 – *Questionnaire*

Date(s) of analysis: **11/30/00**

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:* **7** *min*

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank samples

Please indicate the carbadox peak with an arrow

Recovery results:

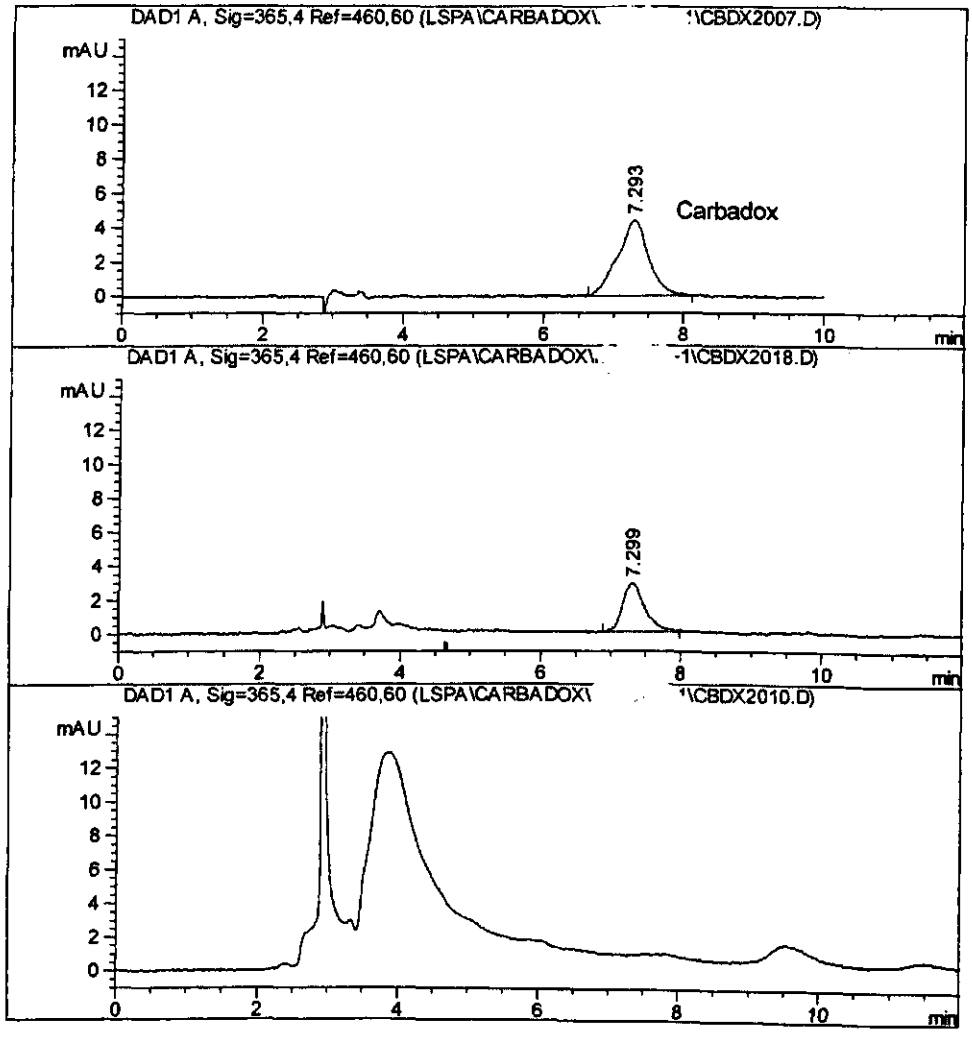
- Percentage recovery: ... %
- Single / duplicate determinations: ☐ single **X** duplicate
- If duplicate, please give both percentages: **99** % and **97** %
- Speaking level: **5** mg/kg

CANFAS COLLABORATIVE STUDIES - CARBADOX

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 (1,27 ppm), sample (381116) and blank



APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 41

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: CARBADOX

Unit Sample code	Result 1 (mg/kg)	Result 2 (mg/kg)
411112	0	0
411131	6,44	6,55
411141	0	0
411171	6,00	6,00
411211	1,55	1,58
411215	1,58	1,61

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Date(s) of analysis: 15.11, 20.11, 21.11.2000

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Lichrosorb RP18 (5 µm) (250mm x 4)
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1 ml/min
- Injection volume: 20 µl
- Retention time of carbadox: 6.1 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

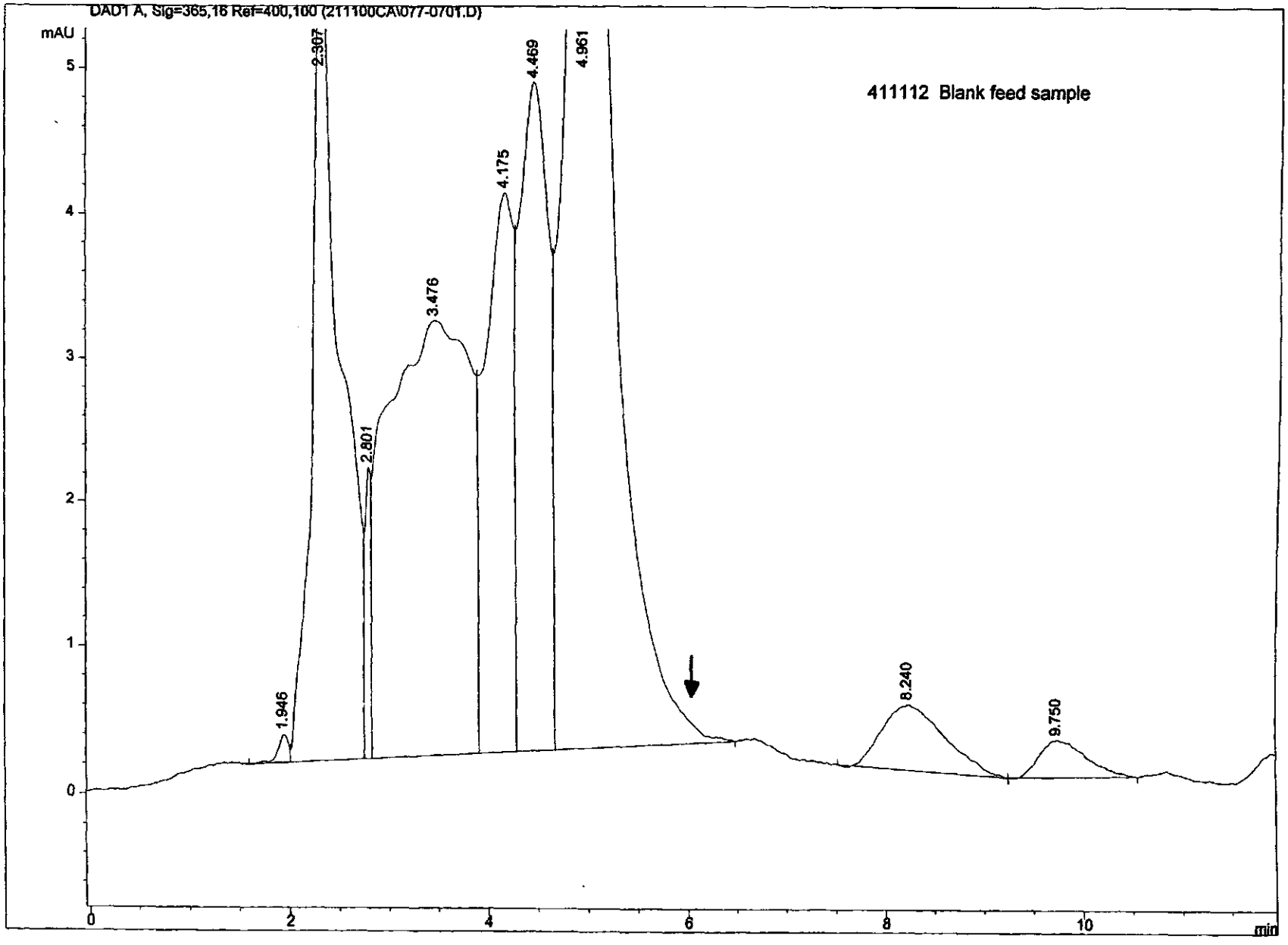
Please indicate the carbadox peak with an arrow

Recovery results:

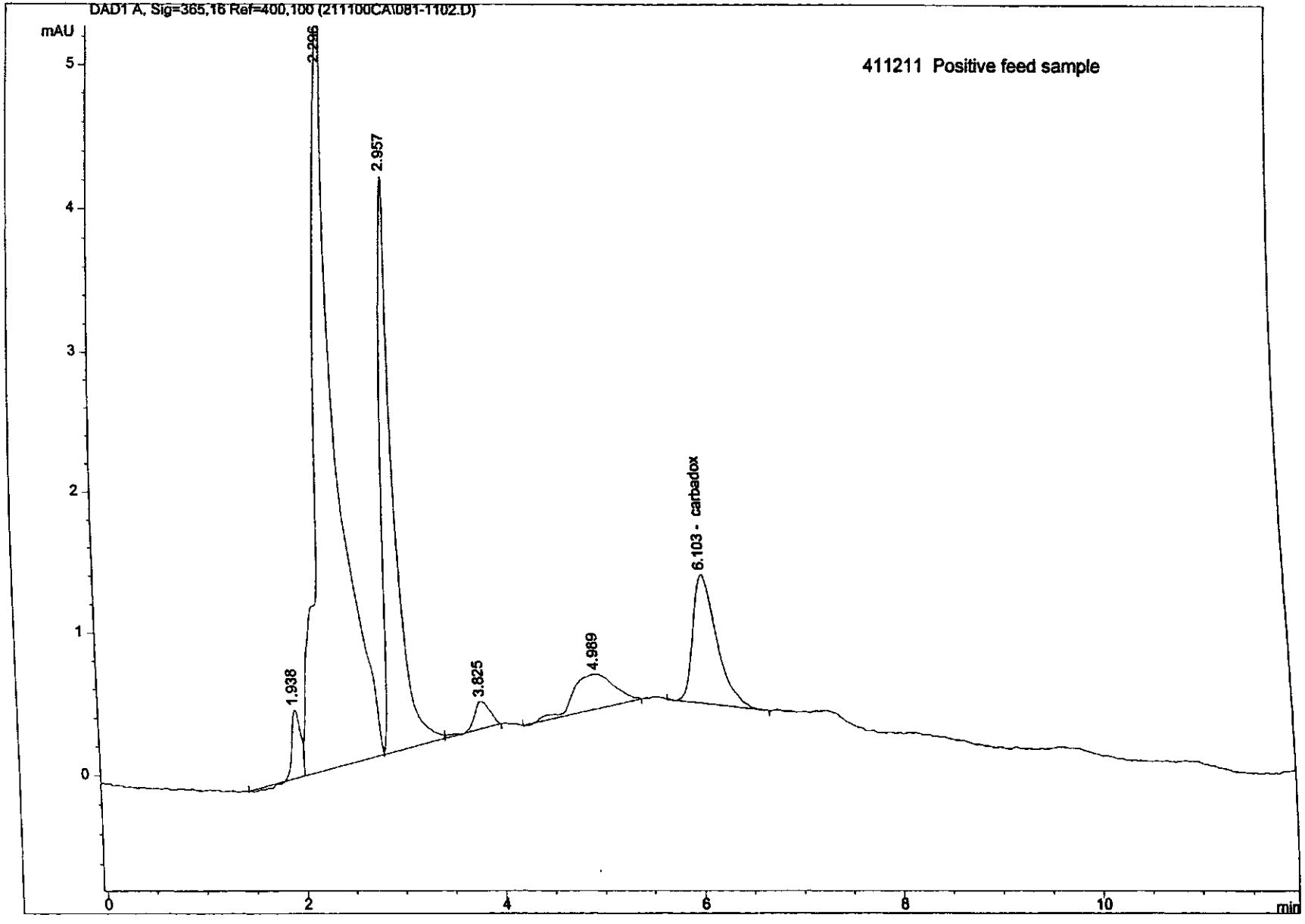
- Percentage recovery: 100.7%
- Single / duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 99.7 % and 101.7 %
- Spiking level: 5 mg/kg

(41)

Current Chromatogram(s)

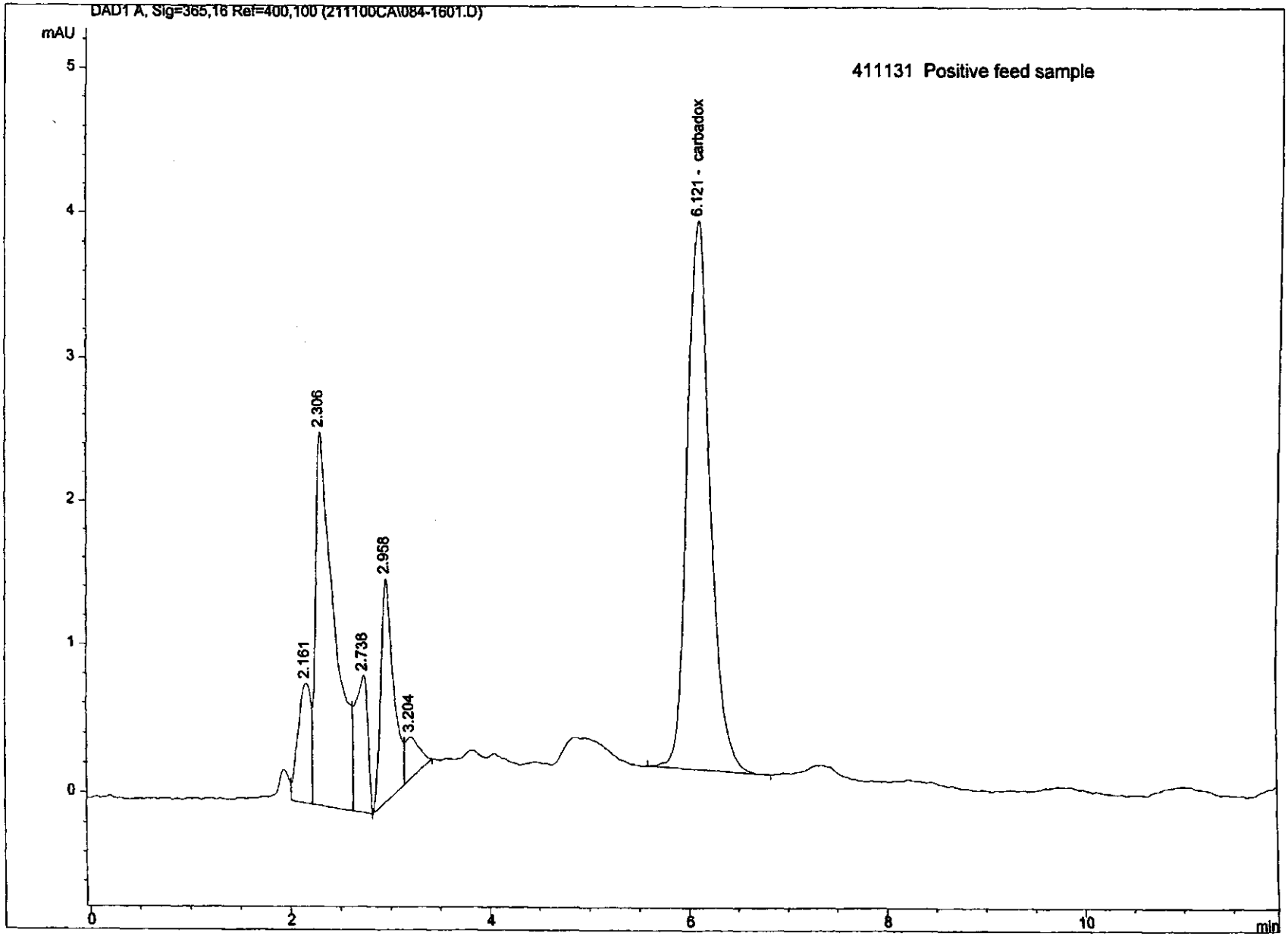


Current Chromatogram(s)



Current Chromatogram(s)

(41)



Appendix 7

Results of special requests:

SPE clean-up

Istituto Superiore di Sanita, Lab. Med. Veterinaria, Roma, Italy

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

Annex 4 - Questionnaire

Laboratory: ISS

Contact person: Dr. G. Brambilla

Date(s) of analysis: 23-11-2000

Chromatographic conditions:

- Column:
  - ☒ As described in the method
  - ☐ Other: .....
- Mobile phase:
  - ☐ As described in the method
  - ☒ Other: Mix 80 ml of acetate buffer solution with 20 ml of acetonitr
- Flow-rate: ....1,3..... ml/min
- Injection volume: ..20.....µl
- Retention time of carbadox: 4,34 min

Chromatograms: Please include representative chromatograms of:

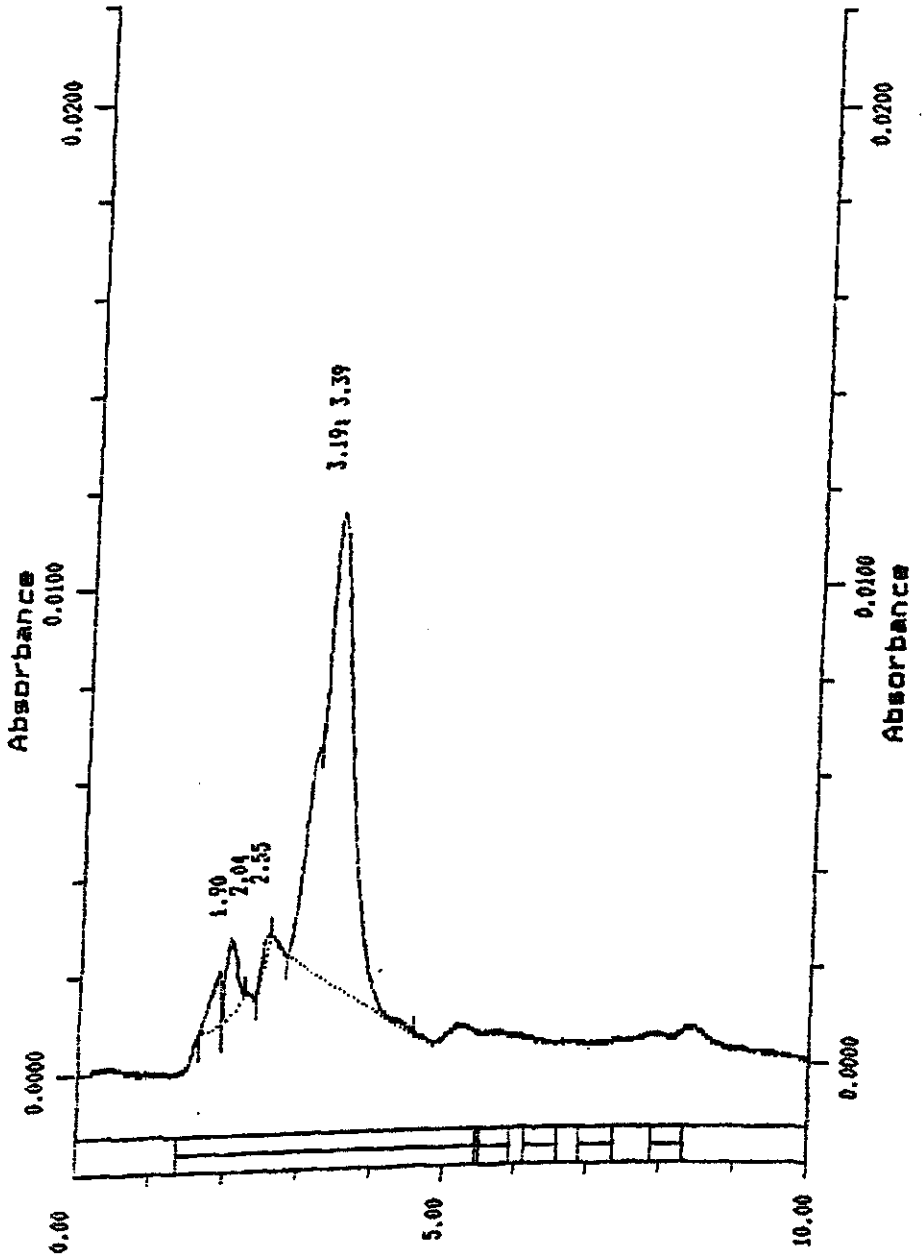
- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

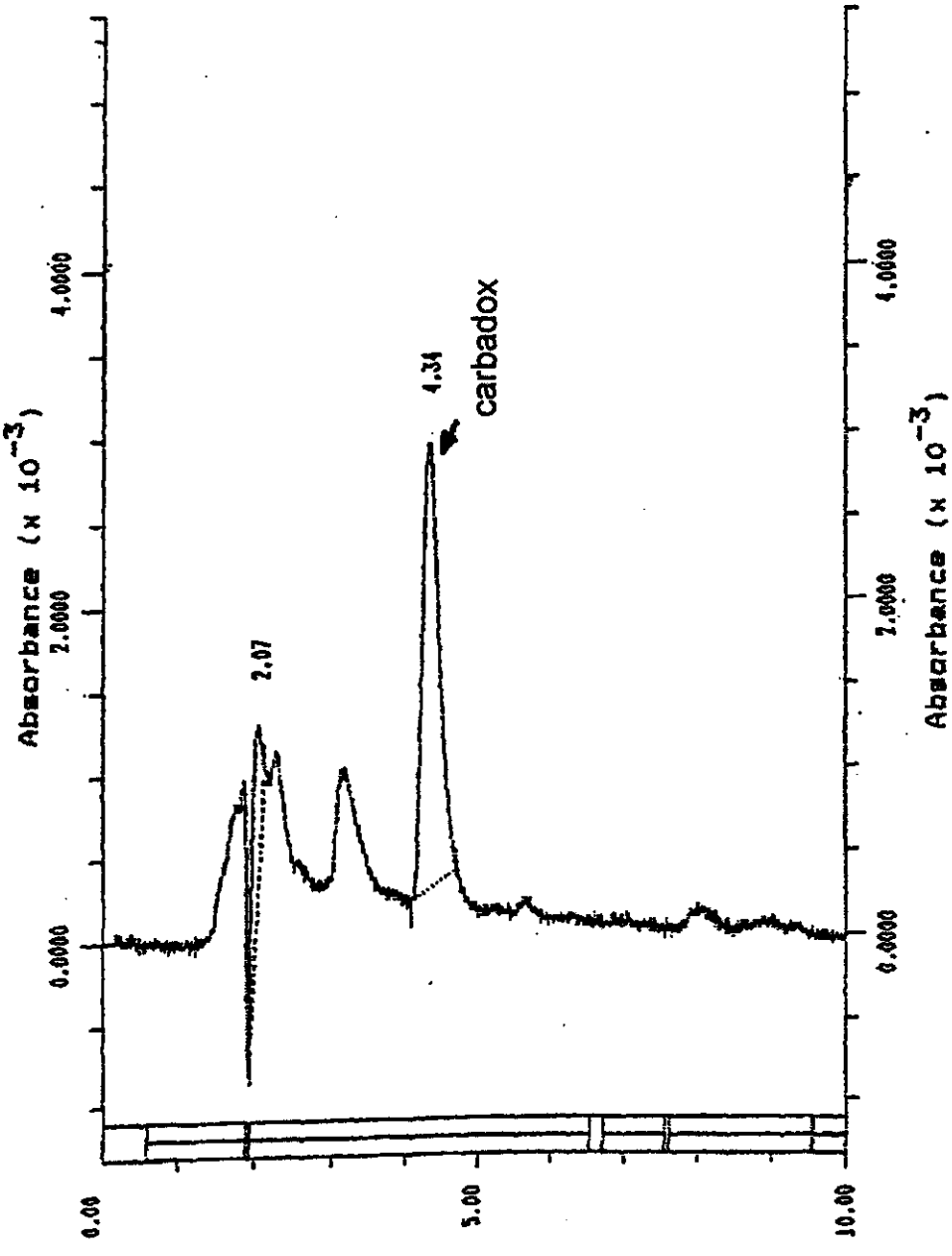
- Percentage recovery: 96,5. %
- Single / duplicate determinations: ☒ single ☐ duplicate
- If duplicate, please give both percentages: ..... % and ..... %
- Spiking level: ...5..... mg/kg

Chromatogram for carbadox study



Feed Blank

Chromatogram for carbadox study



Feed 10 mg/kg

Appendix 7

Results of special requests:

SPE clean-up

LNIV, Lisbon, Portugal

CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - CARBADOX

SPECIAL REQUEST

Annex 4 - Questionnaire

Laboratory: Laboratório Nacional de Investigação Veterinária (LNIV) - Portugal

Contact person: Dr. José Manuel Nunes Costa

Date(s) of analysis: 2001.01.17

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☒ Other: Waters Symmetry, C18, 5 um, 4.6mmX250mm (Part Nº WAT 054215)
- Mobile phase:
  - ☒ As described in the method
  - ☐ Other: .....
- Flow-rate: 1.6 ml/min
- Injection volume: 20 (µL)
- Retention time of carbadox: 3.50 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the carbadox peak with an arrow

Recovery results:

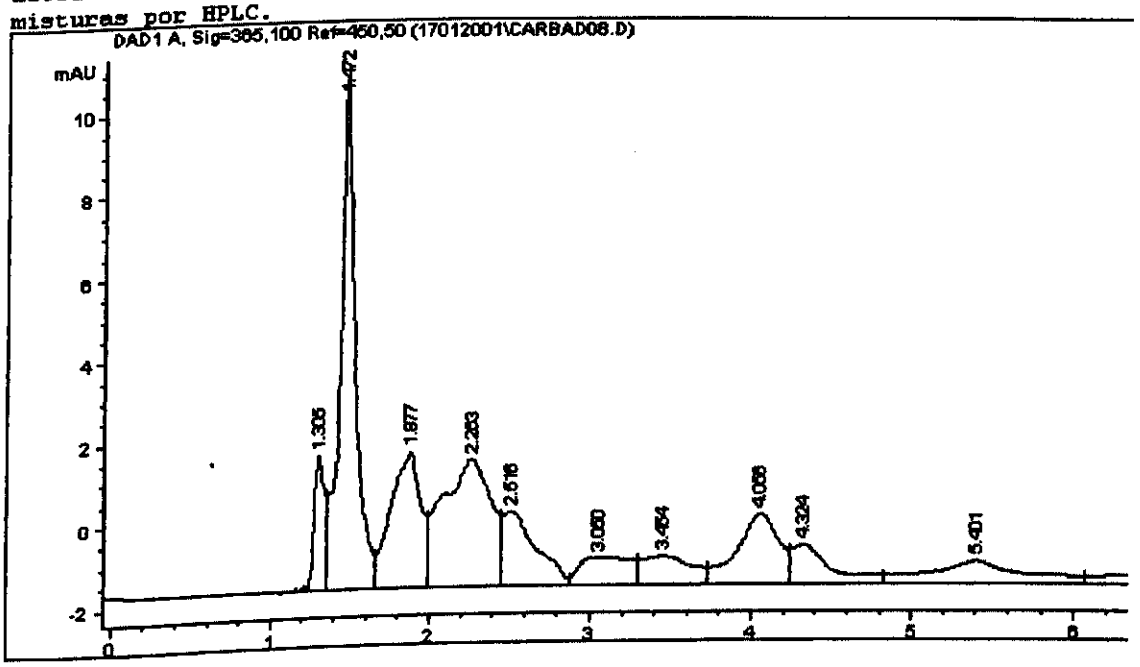
- Percentage recovery: 96.6 %
- Single/duplicate determinations: ☐ single ☒ duplicate
- If duplicate, please give both percentages: 98.2% and 95.0%
- Spiking level: 5 mg/kg

Data File C:\HPCHEM\1\DATA\17012001\CARBAD08.D Sample Name: B

Amostra de racao branca BLANK SAMPLE

Injection Date	: 1/17/01 5:32:35 PM	Seq. Line :	8
Sample Name	: B	Vial :	8
Acq. Operator	: Jose Casqueira	Inj :	1
		Inj Volume :	20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\CARBADOX.M		
Last changed	: 1/17/01 5:33:43 PM by Jose Casqueira (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\CARBADOX.M		
Last changed	: 1/18/01 9:24:40 AM by Jose Casqueira (modified after loading)		

Metodo de doseamento de Carbadox em alimentos compostos para animais e pre-misturas por HPLC.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	1/18/01 9:23:52 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=365,100 Ref=450,50

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
3.454	VV	7.21079e-1	1.99268e-1	1.43688e-1		Carbadox
Totals :				1.43688e-1		

Results obtained with enhanced integrator!

19-01-2001

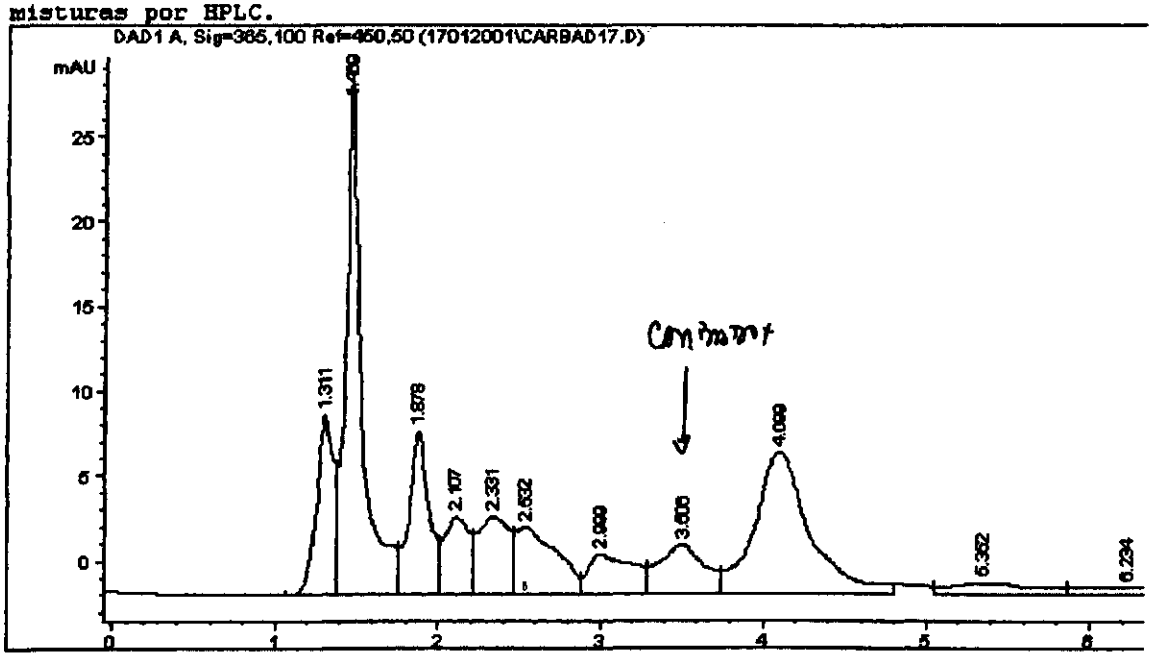
file://A:\Carbadoxspe2.gif

Data File C:\HPCHEM\1\DATA\17012001\CARBAD17.D Sample

2,5 mg/kg

Injection Date : 1/17/01 7:10:41 PM Seq. Line : 17
Sample Name : 321150/ Vial : 17
Acq. Operator : Jose Casqueira Inj : 1
Inj Volume : 20 µl
Acq. Method : C:\HPCHEM\1\METHODS\CARBADOX.M
Last changed : 1/17/01 7:08:44 PM by Jose Casqueira
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\CARBADOX.M
Last changed : 1/18/01 9:24:40 AM by Jose Casqueira
(modified after loading)

Metodo de doseamento de Carbadox em alimentos compostos para animais e pre-misturas por HPLC.



Carbadox

External Standard Report

Sorted By : Signal
Calib. Data Modified : 1/18/01 9:23:52 AM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=365,100 Ref=450,50

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
3.506	VV	2.93446	2.61214e-1	7.66521e-1		Carbadox

Totals : 7.66521e-1

Results obtained with enhanced integrator!

*** End of Report ***

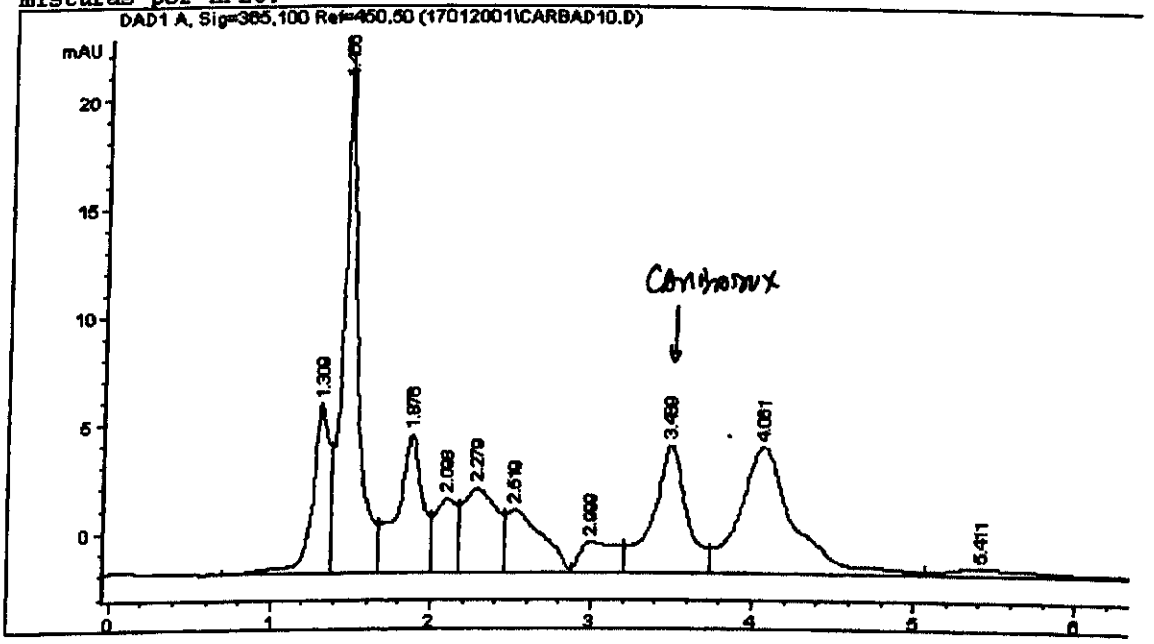
Data File C:\HPCHEM\1\DATA\17012001\CARBAD10.D Sample

m - 10.0033g

10 mg/kg

Injection Date	: 1/17/01 5:54:28 PM	Seq. Line :	10
Sample Name	: 321104	Vial :	10
Acq. Operator	: Jose Casqueira	Inj :	1
		Inj Volume :	20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\CARBADOX.M		
Last changed	: 1/17/01 5:52:30 PM by Jose Casqueira (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\CARBADOX.M		
Last changed	: 1/18/01 9:24:40 AM by Jose Casqueira (modified after loading)		

Metodo de doseamento de Carbadox em alimentos compostos para animais e pre-misturas por HPLC.



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	1/18/01 9:23:52 AM
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: DAD1 A, Sig=365,100 Ref=450,50

RetTime [min]	Type	Height [mAU]	Amt/Height	Amount [ug/ml]	Grp	Name
3.489	VV	5.88144	2.71326e-1	1.59579		Carbadox
Totals :				1.59579		

Results obtained with enhanced integrator!