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

Co-ordinator: Dr. J. de Jong

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

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**Section 3.1.2 Sample homogeneity**

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

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

<table>
<thead>
<tr>
<th>Product</th>
<th>Declared content (mg/kg)</th>
<th>Measured content (mg/kg)</th>
<th>Homogeneity results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piglet feed</td>
<td>2</td>
<td>1,5</td>
<td>3,9</td>
</tr>
<tr>
<td>Piglet feed</td>
<td>7,5</td>
<td>4,9</td>
<td>3,9</td>
</tr>
</tbody>
</table>

The correction of CV's (between samples) does not influence the conclusion drawn about the homogeneity.
This report describes the results of a collaborative study of an HPLC method for the growth promoter olaquindox in three piglet feeds. The collaborative study forms part of the EU-project “Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (CANFAS-SMT4-CT98-2216).

The principle of the method is as follows: The sample is extracted by a mixture of water - methanol. The content of olaquindox is determined by reversed-phase high-performance liquid chromatography (HPLC) with UV-detection at 380 nm.

The samples that were tested in the collaborative study were 2 piglet feeds with declared olaquindox contents of 2 and 7.5 mg/kg and 1 blank piglet feed. The feed samples were sent to the participants as blind duplicates. The participants were asked to do duplicate determinations per sample.

Results were reported by 19 laboratories. Statistical evaluation was performed according to ISO 5725. The results show that acceptable results are obtained for repeatability (rsd, < 10 %) and reproducibility (Horrat ratios < 2). However, many laboratories reported difficulties with the practicability of the method due to the low ratio between the volume of extraction solvent (50 ml) and the weight of feed (25 g). For this reason it is recommended to modify the CANFAS-method in such a way that the ratio between the extraction volume and the sample weight is increased to 5 and to organise a second round of collaborative studies for final validation of the method.

The results of the collaborative study were evaluated in a meeting attended by the participants. It can be concluded that the repeatability and reproducibility of the method is acceptable. The results obtained for the blank feed are also acceptable. The panel agreed that, due to the problems with the practicability of the method, the method cannot be recommended for adoption as an official method. A second collaborative study will be organised with a modified method.
1 INTRODUCTION

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

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

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

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

The samples that were prepared for the collaborative study were two piglet feeds with declared olaquindox contents of 2 and 7,5 mg/kg respectively and one blank feed. The feed samples were sent to the participants as blind duplicates. Before these samples were shipped, the between- and within-sample homogeneity of the feed samples containing olaquindox was checked with satisfactory results (see par. 3.1.2).

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

This report describes the results of the collaborative study.
The following laboratories/persons participated in the collaborative study.

- Administration des Services Technique de l'Agriculture Division des Laboratoires, Ettenbruck, Luxemburg; R. Meyers
- Bundesamt und Forschungszentrum für Landwirtschaft (BFL), Wien, Austria; B. Stoisser, M. Wieshaider
- INETI/DTIA, Lisbon, Portugal; I. Felgueiras, C. Saldanha
- 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 Zooprofilattico 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-Avidia.
- Laboratory of the Government Chemist, Teddington, United Kingdom; G. Merson, J. Cowles, S. Jayakumar
- LUFA – Augustenburg, Karslruhe, Germany; K. Michels, S. Witzemann.
- LUFA-ITL Kiel, Kiel, Germany; F.H. Johannsen, Kollwitz.
- Masterlab, Putten, The Netherlands; K. van Schalm, A. Schaaf.
- National Veterinary Institute, Uppsala, Sweden; E. Nordkvist, A. Stepinska
- Rijksontledingslaboratorium, Tervuren, Belgium; K. Haustraete, A. Fontaine, M. Bral, R. van San
- RIKILT, Wageningen, The Netherlands; H. Kleijnen, H. van der Kamp
- State Laboratory Dublin, Ireland; P. Shearan, A. Cunningham, A. Murphy
- Universität Hamburg, Institut für Angewandte Botanik, Hamburg, Germany; H. Putzka, D. Böhm.
- Universität Hohenheim, Landesanstalt für Landwirtschaftliche Chemie, Stuttgart, Germany; B. Eckstein, K. Schwadendorf, 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

<table>
<thead>
<tr>
<th>Type of feed</th>
<th>Declared content</th>
<th>Units</th>
<th>Subcontractor</th>
<th>Date of production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piglet feed</td>
<td>2</td>
<td>mg/kg</td>
<td>IPC - Dier, Barneveld (NL)</td>
<td>05/09/2000</td>
</tr>
<tr>
<td>Piglet feed</td>
<td>7,5</td>
<td>mg/kg</td>
<td>IPC - Dier, Barneveld (NL)</td>
<td>05/09/2000</td>
</tr>
</tbody>
</table>

The feed sample with 2 mg/kg olaquindox also contained 10 mg/kg carbadox, the feed sample with 7,5 mg/kg olaquindox also contained 2,5 mg/kg carbadox. The complete composition of the feeds is given in Appendix 2 (in Dutch). The main composition of the two feeds is given in Table 2.

Table 2: Main composition of the two feeds

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Piglet feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein (%)</td>
<td>18,1</td>
</tr>
<tr>
<td>Crude fat (%)</td>
<td>4,3</td>
</tr>
<tr>
<td>Starch (%)</td>
<td>39,4</td>
</tr>
<tr>
<td>Crude fibre (%)</td>
<td>4,4</td>
</tr>
<tr>
<td>Crude ash (%)</td>
<td>4,7</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>12,4</td>
</tr>
</tbody>
</table>

The composition of the feeds was the same as the composition of the products which were produced by IPC-Dier in September 1999 for stability testing (see Report on homogeneity and stability studies of samples for the collaborative studies for olaquindox, K. Michels, LUFA Augustenborg, Germany, 05/05/2000).
The feed products have been prepared in a quantity of 500 kg each. To achieve a maximum degree of homogeneity halfway through the production 54 kg of feed are withdrawn from the stream for subsampling activities and put into three sacks of 18 kg. After discarding the top layer (ca. 2 kg) about 30 - 50 subsamples of approx. 250 grams have been taken (manual distribution with a shovel) from each of these sacks. The subsamples were stored in double paper sacks.

All subsamples have been stored at room temperature (ca. 20 °C). Next to the above mentioned samples that contained olaquindox, a blind blank feed was sent to the participants as well as a blank feed labelled “blank feed for olaquindox recovery purposes” (see Appendix 1). The blind blank feed was a bull feed containing 5 mg/kg virginiamycin (see the corresponding CANFAS report). This feed was analysed at LUFA Augustenberg prior to the collaborative studies and was found to contain a small interfering peak at the retention time of olaquindox which corresponds to ca. 0,5 mg/kg. The blank feed for olaquindox recovery purpose was a standard piglet feed produced by IPC-Dier. This feed was also analysed prior to the collaborative study and contained no detectable amounts of olaquindox or interfering substances.

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

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

<table>
<thead>
<tr>
<th>Results</th>
<th>Declared content (mg/kg)</th>
<th>Measured content (mg/kg)</th>
<th>Homogeneity results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product</td>
<td></td>
<td></td>
<td>Between sample CV (%)</td>
</tr>
<tr>
<td>Piglet feed</td>
<td>2</td>
<td>1,5</td>
<td>2,8</td>
</tr>
<tr>
<td>Piglet feed</td>
<td>7,5</td>
<td>4,9</td>
<td>2,5</td>
</tr>
</tbody>
</table>

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

The samples were sent as blind duplicates. The codes are given in Appendix 4. The samples were sent to the participants by courier service on 2 October 2000 together with a letter with instructions (Appendix 1). During transport no special precautions were taken with regards to the temperature of the samples.

3.2 Reference standard

The reference standard was supplied by Dr. A. Plöger, Danish Plant Directorate, Lyngby (DK). According to the specifications (see Appendix 5), the purity of the reference standard (Lot Nr. 890416) is 99.46%. The participants were instructed to set the purity of the reference standard to 100% (see Appendix 1).

The expiration date of the reference standard was April 2001. The identity and content was checked by RIKILT. The identity could be confirmed by UV, $^1$H-NMR as well as mass spectrometry. The purity was determined by $^1$H-NMR and UV spectroscopy and was shown to be approx. 100% (see Appendix 5).
4 METHODS

4.1 Method of analysis

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

4.1.1 HPLC-conditions

Various types of HPLC-columns were used (the column which was recommended in the method is a C18, 250 mm x 4 mm, 5 μm packing or equivalent).

The mobile phase described in the method is a mixture of water and methanol 900:100 (v/v). Three laboratories used a different mobile phase.

The HPLC conditions (Column and mobile phase) used by the participants are shown in Table 4.

4.2 Method for statistical evaluation

Statistical evaluation was performed according to ISO 5725 Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method (First edition, 1994-12-15).

The scrutiny of results for consistency and outliers was checked by

Graphical consistency techniques: Mandel's h plot for between-laboratory variability, Mandel's k plot for within-laboratory variability

Numerical outlier techniques: Cochran's test of the within-laboratory variability, Grubbs' test (single and double) for between-laboratory variability

Whenever necessary and appropriate, laboratories which showed consistently high within-cell variation and/or extreme cell means across many levels and/or Cochran or Grubbs' outliers were contacted to try to ascertain the cause of the discrepant behaviour.

The Horwitz equation and the HORRAT ratios form the basis for the evaluation of the reproducibility of the method. The HORRAT ratios are given in Table 5. The HORRAT ratios should be lower than 2 (see W. Horwitz and R. Albert, J.A.O.A.C. 74 (1991) 718-744).
<table>
<thead>
<tr>
<th>Partner</th>
<th>Column</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>As described in the method</td>
<td>As described in the method</td>
</tr>
<tr>
<td>15</td>
<td>Hypersil ODS 5 μm 200 x 4,6 mm + guard column</td>
<td>As described in the method</td>
</tr>
<tr>
<td>16</td>
<td>Sperisorb ODS 2, 10 μm 250 x 4,6 mm</td>
<td>As described in the method</td>
</tr>
<tr>
<td>17</td>
<td>Sperisorb S10 ODS-1 10 μ</td>
<td>As described in the method</td>
</tr>
<tr>
<td>18</td>
<td>150 x 4,6 mm; 5 μm; Sperisorb ODS2 C18</td>
<td>As described in the method</td>
</tr>
<tr>
<td>20</td>
<td>Alltimo Alltech C18, 250x4,6 mm, 5 μm</td>
<td>As described in the method</td>
</tr>
<tr>
<td>21</td>
<td>Supelcosil LC-18 25 cm x 4,6 mm (5 μm) + supelguard LC-18</td>
<td>Acetonitrile: ammoniumacetate buffer (0,01M; pH 4,6) Gradient elution</td>
</tr>
<tr>
<td>23</td>
<td>Not reported</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>250 x 4,6 mm C18 5 μm</td>
<td>As described in the method</td>
</tr>
<tr>
<td>25</td>
<td>As described in the method</td>
<td>Water/methanol = 800:200 (v/v)</td>
</tr>
<tr>
<td>26</td>
<td>Sperisorb ODS 2 250 mm x 4,6 mm 5 μm</td>
<td>As described in the method</td>
</tr>
<tr>
<td>27</td>
<td>As described in the method</td>
<td>As described in the method</td>
</tr>
<tr>
<td>29</td>
<td>Nova Pack, 4,6 x 250 mm; C18; 4 μ</td>
<td>As described in the method</td>
</tr>
<tr>
<td>31</td>
<td>As described in the method</td>
<td>As described in the method</td>
</tr>
<tr>
<td>32</td>
<td>Waters symmetry, C18, 5μm, 4,6x250 mm</td>
<td>As described in the method</td>
</tr>
<tr>
<td>33</td>
<td>As described in the method</td>
<td>0,01 M ammoniumacetate pH 5: acetonitrile = 95:5 (v/v)</td>
</tr>
<tr>
<td>34</td>
<td>As described in the method,</td>
<td>As described in the method</td>
</tr>
<tr>
<td>37</td>
<td>Lichrosper RP18-5 endcapped</td>
<td>As described in the method</td>
</tr>
<tr>
<td>38</td>
<td>Hypersil ODS C-18, 250 x 4,6 mm, 5 μm</td>
<td>As described in the method</td>
</tr>
<tr>
<td>40</td>
<td>C18 sperical 5 μm 3,9 x 15 cm waters</td>
<td>As described in the method</td>
</tr>
</tbody>
</table>
5 RESULTS

For each participant the reported results for the samples, the completed questionnaire and representative chromatograms are annexed in Appendix 6.

5.1 Statistical evaluation

The results reported by the participants are given in Table 6. Due to problems in obtaining enough solvent after the extraction step two laboratories used a modified method with a higher ratio between extraction volume and sample weight. The results of these laboratories are not included in Table 6 and will be described in par. 5.5.

The results reported by lab 25 clearly show that this lab has interchanged (the results of) the samples (see also par. 5.2): two samples are reported as "nd", two samples at 1,70 mg/kg and two samples at 4,86 - 4,88 mg/kg but only in one case the code corresponds to the right sample. This lab was contacted but was not able to trace back the origin of the interchange. For this reason the results of lab 25 were not taken into account in the statistical evaluation. Statistical analysis shows that the results of the other laboratories do not contain Cochran or Grubbs' outliers or stragglers. The values for the statistical parameters (mean, relative standard deviations for repeatability and reproducibility) are given in Table 6. According to the Project Plan, the rsd-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-values are in line with values predicted by the Horwitz equation. Consequently it can be concluded that the reproducibility of the method is satisfactory.

Table 5: Horrat ratios of the olaquindox collaborative study

<table>
<thead>
<tr>
<th>Mean after discarding lab 25 (mg/kg)</th>
<th>Predicted rsdR</th>
<th>Established rsdR</th>
<th>Horrat1</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,721</td>
<td>14,745</td>
<td>21,12</td>
<td>1,43</td>
<td>Reproducibility OK</td>
</tr>
<tr>
<td>5,284</td>
<td>12,454</td>
<td>16,65</td>
<td>1,34</td>
<td>Reproducibility OK</td>
</tr>
</tbody>
</table>

1 = Horrat is the ratio between the established rsdR and the predicted rsdR

The Mandel h and k plots are shown in Figure 1. The Mandel h plot shows that 3 laboratories (nr. 15, 29 and 37) reported low values for both samples. Laboratories 29 and 37 reported the lowest recoveries, viz. 69 and 49 % while the recovery reported by lab 15 (78 %) is a normal value. Lab 37 is considered as a Grubbs outlier with regard to the recovery (see par. 5.3). Nevertheless it is unjustified to discard the results of lab 37 from statistical evaluation because of the problems encountered by many laboratories (among them lab 37, see par. 5.4) with the extraction step in the method, which is regarded as the main causative factor for the low recovery.
Table 6: Results reported by the participants.

Table 6. Olaquindox in two piglet feeds

<table>
<thead>
<tr>
<th>Sample</th>
<th>OLA 2 mg/kg</th>
<th>OLA 2 mg/kg</th>
<th>OLA 2 mg/kg</th>
<th>OLA 7.5 mg/kg</th>
<th>OLA 7.5 mg/kg</th>
<th>OLA 7.5 mg/kg</th>
<th>OLA 7.5 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1.97</td>
<td>2.01</td>
<td>1.54</td>
<td>1.51</td>
<td>4.84</td>
<td>4.78</td>
<td>5.84</td>
</tr>
<tr>
<td>15</td>
<td>1.25</td>
<td>1.02</td>
<td>1.12</td>
<td>1.34</td>
<td>4.60</td>
<td>3.52</td>
<td>4.14</td>
</tr>
<tr>
<td>16</td>
<td>1.56</td>
<td>1.70</td>
<td>1.66</td>
<td>1.65</td>
<td>5.66</td>
<td>4.98</td>
<td>5.65</td>
</tr>
<tr>
<td>17</td>
<td>2.07</td>
<td>1.99</td>
<td>1.99</td>
<td>1.92</td>
<td>5.60</td>
<td>5.66</td>
<td>5.58</td>
</tr>
<tr>
<td>18</td>
<td>1.56</td>
<td>1.52</td>
<td>1.67</td>
<td>1.65</td>
<td>5.78</td>
<td>5.92</td>
<td>6.18</td>
</tr>
<tr>
<td>20</td>
<td>1.72</td>
<td>1.60</td>
<td>1.52</td>
<td>1.59</td>
<td>4.85</td>
<td>4.78</td>
<td>4.65</td>
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<tr>
<td>21</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>5.40</td>
<td>5.30</td>
<td>5.30</td>
</tr>
<tr>
<td>23</td>
<td>1.67</td>
<td>1.76</td>
<td>1.91</td>
<td>1.86</td>
<td>4.23</td>
<td>5.77</td>
<td>5.40</td>
</tr>
<tr>
<td>24</td>
<td>2.00</td>
<td>2.10</td>
<td>1.80</td>
<td>1.70</td>
<td>5.70</td>
<td>6.20</td>
<td>6.00</td>
</tr>
<tr>
<td>25</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>4.88</td>
<td>4.88</td>
<td>1.70</td>
</tr>
<tr>
<td>26</td>
<td>1.60</td>
<td>1.50</td>
<td>0.70</td>
<td>1.20</td>
<td>5.10</td>
<td>4.80</td>
<td>6.00</td>
</tr>
<tr>
<td>27</td>
<td>2.05</td>
<td>1.98</td>
<td>1.81</td>
<td>1.89</td>
<td>5.22</td>
<td>5.34</td>
<td>5.30</td>
</tr>
<tr>
<td>29</td>
<td>1.20</td>
<td>1.30</td>
<td>1.30</td>
<td>1.30</td>
<td>3.80</td>
<td>3.70</td>
<td>3.80</td>
</tr>
<tr>
<td>31</td>
<td>1.88</td>
<td>1.90</td>
<td>2.08</td>
<td>1.80</td>
<td>6.28</td>
<td>6.13</td>
<td>6.05</td>
</tr>
<tr>
<td>32</td>
<td>2.06</td>
<td>2.24</td>
<td>2.07</td>
<td>2.03</td>
<td>5.91</td>
<td>5.92</td>
<td>5.63</td>
</tr>
<tr>
<td>33</td>
<td>2.30</td>
<td>2.40</td>
<td>2.50</td>
<td>2.50</td>
<td>7.40</td>
<td>6.80</td>
<td>7.20</td>
</tr>
<tr>
<td>34</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>4.70</td>
<td>4.80</td>
<td>4.80</td>
</tr>
<tr>
<td>37</td>
<td>1.38</td>
<td>1.22</td>
<td>1.11</td>
<td>1.02</td>
<td>3.70</td>
<td>3.63</td>
<td>3.50</td>
</tr>
<tr>
<td>38</td>
<td>1.72</td>
<td>1.76</td>
<td>2.01</td>
<td>1.94</td>
<td>6.00</td>
<td>5.52</td>
<td>5.53</td>
</tr>
<tr>
<td>40</td>
<td>1.50</td>
<td>1.80</td>
<td>2.21</td>
<td>1.62</td>
<td>4.75</td>
<td>4.56</td>
<td>5.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>number of labs</th>
<th>m (mg/kg)</th>
<th>rsd (%)</th>
<th>rsdR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>19</td>
<td>1,721</td>
<td>9,47</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>5,284</td>
<td>6,22</td>
</tr>
</tbody>
</table>

Remark: Italic printed results are not taken into account in the statistical evaluation!
Figure 1: Mandel h and k plots of results reported by the participants.
### 5.2 Blank samples

Table 7: Reported results of the blank samples

<table>
<thead>
<tr>
<th>Partner</th>
<th>Blank sample 1</th>
<th>Blank sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Result 1</td>
<td>Result 2</td>
</tr>
<tr>
<td>12</td>
<td>Nsd</td>
<td>Nsd</td>
</tr>
<tr>
<td>15</td>
<td>Blank</td>
<td>Blank</td>
</tr>
<tr>
<td>16</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Not detected &lt;0,5</td>
<td>Not detected &lt;0,5</td>
</tr>
<tr>
<td>20</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>21</td>
<td>0,0 ND</td>
<td>0,0 ND</td>
</tr>
<tr>
<td>23</td>
<td>&lt;0,11</td>
<td>&lt;0,21</td>
</tr>
<tr>
<td>24</td>
<td>Blank</td>
<td>Blank</td>
</tr>
<tr>
<td>25</td>
<td>1,70</td>
<td>4,88</td>
</tr>
<tr>
<td>26</td>
<td>0,1</td>
<td>0,1</td>
</tr>
<tr>
<td>27</td>
<td>Not detect.</td>
<td>Not detect.</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>33</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>37</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>Not analysed</td>
</tr>
</tbody>
</table>

Only laboratories 25 and 26 reported positive results for the blank samples. For lab 25 this was caused by the fact that this lab had interchanged the samples (see par. 5.1).

For lab 26 the reported values (0,1 mg/kg) are at the limit of detection defined for the method. Consequently it can be concluded that no interfering substances are detected in the blank samples.
5.3 Recoveries

Table 8: Recoveries

<table>
<thead>
<tr>
<th>Partner</th>
<th>Recovery 1 in %</th>
<th>Recovery 2 in %</th>
<th>Average recovery in (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>78</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>15</td>
<td>78</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>16</td>
<td>77</td>
<td>76</td>
<td>77</td>
</tr>
<tr>
<td>17</td>
<td>85</td>
<td>83</td>
<td>84</td>
</tr>
<tr>
<td>18</td>
<td>80</td>
<td>76</td>
<td>78</td>
</tr>
<tr>
<td>20</td>
<td>101</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>21</td>
<td>83</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>71</td>
<td>86</td>
<td>79</td>
</tr>
<tr>
<td>25</td>
<td>84</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>26</td>
<td>75</td>
<td>71</td>
<td>73</td>
</tr>
<tr>
<td>27</td>
<td>76</td>
<td></td>
<td>76</td>
</tr>
<tr>
<td>29</td>
<td>68</td>
<td>70</td>
<td>69</td>
</tr>
<tr>
<td>31</td>
<td>78</td>
<td>86</td>
<td>82</td>
</tr>
<tr>
<td>32</td>
<td>82</td>
<td>87</td>
<td>85</td>
</tr>
<tr>
<td>33</td>
<td>93</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>34</td>
<td>82</td>
<td>86</td>
<td>84</td>
</tr>
<tr>
<td>37</td>
<td>48</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>38</td>
<td>81</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>40</td>
<td>83</td>
<td></td>
<td>83</td>
</tr>
</tbody>
</table>

Recoveries range from 48 - 101 %. This range is broader than the range (60 - 90 %) which was measured in the between-lab validation of the method (see Second Annual Report CANDAS, J. de Jong, 12-08-2000).

Most probably, the problems encountered by many laboratories with the extraction step in the method are the main causative factor for the low recovery.

The mean recovery value reported by lab 37 (49 %) is a Grubbs outlier.
5.4 Remarks

Table 9: Remarks made by the partners

<table>
<thead>
<tr>
<th>Partner</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>In some cases (we try the method as well with real samples of feedingstuffs), the volume of liquid for the extraction is not enough to get a proper volume of extractant solution, even with centrifugation. We think the weight/volume could be 1/3, not $\frac{1}{2}$. Perhaps, this is one of the reasons because of the recovery percentages are not so good as with carbadox method (where the relation is 1/5).</td>
</tr>
<tr>
<td>15</td>
<td>We have encountered some troubles performing the centrifugation step of 50 ml solvent extract in the extraction procedure. However the results were obtained utilising the method without modification. In another occasion, we have tried to increase extraction volume to 100 ml (20 ml methanol and 80 ml water) obtaining an increase of recovery from 78% to 88% at 3.0 mg/kg spiking level and the following results on the samples:</td>
</tr>
<tr>
<td></td>
<td>Sample code</td>
</tr>
<tr>
<td></td>
<td>155711</td>
</tr>
<tr>
<td></td>
<td>155735</td>
</tr>
<tr>
<td></td>
<td>155741</td>
</tr>
<tr>
<td></td>
<td>155761</td>
</tr>
<tr>
<td></td>
<td>155776</td>
</tr>
<tr>
<td></td>
<td>155784</td>
</tr>
<tr>
<td></td>
<td>spike 3.0 mg/kg</td>
</tr>
<tr>
<td>16</td>
<td>1. 5.1.2 Recovery test: &quot;Proceeding with the extraction step (5.2)&quot;. Remark: Transfer of 1.5 ml stock standard solution (3.5.1) results in our opinion in the addition of 38.5 ml water and not in 40 ml water (see 5.2). This volume error amounts to about 3 %.</td>
</tr>
<tr>
<td></td>
<td>2. 5.2 Extraction: Remark: weight in of 25 g of sample in relation with a volume of 50 ml of liquid strongly recommends centrifugation. Our parameters: 10 minutes with 7200 rpm. The supernatant liquids were subsequently filtered by using membrane filters (Macherey&amp;Nagel, Chromafil Type A-45/25, 0.45 μm).</td>
</tr>
</tbody>
</table>
Partner | Remarks
--- | ---
17 | Ad 3.5.1 The olaquindox-standard is soluble within 1 minute by ultrasonic treatment. An ultrasonic treatment of 10 minutes warms up the fluid. 
Ad. 5.2.1 Attention: the total volume by the recovery test is 51,5 ml!!
Ad. 5.2 a) It is hardly possible too moisten 25 g sample with 10 ml methanol.
b) It is hardly possible to shake or stir the sample (25g/50 ml liquor)
c) It is better to centrifuge the sample than to filter through an folded filter (suck up of the liquid).
All samples were analysed by the existing EU-Methode 98/64

<table>
<thead>
<tr>
<th>mg/kg</th>
<th>recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+3 mg olaquAg: 88%</td>
<td></td>
</tr>
<tr>
<td>175703</td>
<td>-</td>
</tr>
<tr>
<td>175718</td>
<td>6,09</td>
</tr>
<tr>
<td>175730</td>
<td>2,10</td>
</tr>
<tr>
<td>175775</td>
<td>-</td>
</tr>
<tr>
<td>+ 6 mg olaquAg: 88%</td>
<td></td>
</tr>
<tr>
<td>175817</td>
<td>2,14</td>
</tr>
<tr>
<td>175828</td>
<td>6,21</td>
</tr>
</tbody>
</table>

18 | HPLC equipment: 15 and 16/11/00: pump; autosampler->HP 1050 (40 ul); DAD-> HP 1100
23/11/00: pump Spectra Physics; autosampler Marathon (50 ul); single wave length Milton Roy; Chromjet Recorder

Differences with CANFAS/ola/02/10/2000 method:
- 3.5.1 Stock standard solution -> 50 mg/1000 ml; weigh to the nearest 1 mg
- 3.5.2 Standard solutions -> point at 2,5 μg/ml -> 5 ml of (3.5.1) in a 100 ml graduated flask.
- 5.2 Instead of filtration through a folded filter, centrifugation was carried out as mentioned at 7.1.
- Receival of sample package on 5/10/00, storage of samples until analysis at < 8 °C, in a refrigerated room
- 15 and 16/11/2000: direct analysis of the 6 feeds with DAD; test of recovery; identity confirmation
- 23/11/2000: Analysis, with single wavelength detector, of the 2 blank feeds and the lowest content sample to estimate LOD and LOQ

Results:
- Reported results are the average of height and area results.
- Calibration based on height and area (10 points; forced through origin) -> see example

20 | No remarks

21 | We found difficulties during the extraction because of the large amount of the feed compared to the volume of the solvent.
In two samples (n. 770-791) the solvent was almost completely absorbed by the feed and this made the extraction of the samples very difficult. We were forced to centrifuge these extracts in order to obtain supernatant.
<table>
<thead>
<tr>
<th>Partner</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Not reported</td>
</tr>
<tr>
<td>24</td>
<td>The ratio between the sample amount and the extraction solvent volume resulted to be a very critical step of the method which could affect the reproducibility.</td>
</tr>
<tr>
<td>25</td>
<td>We had some difficulties in the extraction method (5.2). It was very difficult to have enough filtrate in the filtration step so centrifugation has been applied (4000 rpm for 15 min.) Then we followed the standard procedure: Transfer the sample extract in a 50 ml volumetric flask Filter the solution through a folded filter Filter an aliquot through a membrane filter (0.45 μm) for analysis by HPLC</td>
</tr>
<tr>
<td>26</td>
<td>No remarks</td>
</tr>
<tr>
<td>27</td>
<td>As to the samples with the code numbers 275720 and 275773 strong swelling avoided a shakeable suspension to be formed. Along with 275720 the lot of fluid was enlarged to 70 ml, along with 275773 the volume of extraction fluid was doubled; in this case the injection volume was doubled as well as that of the calibration standards so that the limit of detection (with 0.1 mg/kg) could be ensured.</td>
</tr>
<tr>
<td>29</td>
<td>No remarks</td>
</tr>
<tr>
<td>31</td>
<td>During HPLC-analyses the area/height ratio changed, the peaks got broader. Quantification was only possible on area. Sample preparation: The samples were centrifuged after extraction, then filtrated on GFA-filter, followed by filtration on acrodisc 0.45 μm</td>
</tr>
<tr>
<td>32</td>
<td>No remarks</td>
</tr>
<tr>
<td>33</td>
<td>No remarks</td>
</tr>
<tr>
<td>34</td>
<td>No remarks</td>
</tr>
<tr>
<td>37</td>
<td>We found it impossible to proceed with the method particularly with the use of conical flask + filtration of sample. In this situation no filtrate was collected. The entire study was done using 150 ml glass centrifuge tubes (instead of conical flasks) with a centrifugation step (15 min at 3000) (instead of filtration). It was also necessary to further separate the filtrate and centrifuge this prior to HPLC.</td>
</tr>
<tr>
<td>38</td>
<td>Please note that our detection system has been DAD; not a single wavelength UV-detection (as it has been indicated at particularly instruction) because we have not it.</td>
</tr>
<tr>
<td>40</td>
<td>No remarks</td>
</tr>
</tbody>
</table>

The remarks clearly indicate that the practicability of the method is unsatisfactory. Ten out of nineteen laboratories reported difficulties in obtaining enough solvent after the extraction step due to the low ratio between the volume of solvent (50 ml) and the weight of feed (25 g). Moreover, two laboratories decided to use a modified method with a higher ratio between extraction volume...
and sample weight (see par. 5.5) and one laboratory informed the co-ordinator that they abstained from participation due to the same problem.

The results of laboratories 15 and 17 show that when the ratio between the extraction volume and the sample weight is increased, the recoveries increase and the values for the blind positive samples are higher than with the CANFAS-method.

For these reasons it is proposed to modify the CANFAS-method in such a way that the ratio between the extraction volume and the sample weight is increased to 5 and to organise a second round of collaborative studies for final validation of the method.

5.5 Special requests

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

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

5.5.1 HPLC conditions

Table 10: HPLC conditions

<table>
<thead>
<tr>
<th>Partner</th>
<th>Column</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masterlab, Putten, The Netherlands</td>
<td>As described in the method.</td>
<td>As described in the method</td>
</tr>
<tr>
<td>National Veterinary Institute, Uppsala, Sweden</td>
<td>Hypersil C18 ODS BDS 250 x 4,6 mm; 5 μm</td>
<td>As described in the method</td>
</tr>
</tbody>
</table>

5.5.2 Recoveries

Table 11: Recoveries

<table>
<thead>
<tr>
<th>Partner</th>
<th>Recovery 1 in %</th>
<th>Recovery 2 in %</th>
<th>Recovery average in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masterlab, Putten, The Netherlands</td>
<td>98</td>
<td>82</td>
<td>90</td>
</tr>
<tr>
<td>National Veterinary Institute, Uppsala, Sweden</td>
<td>95</td>
<td>96</td>
<td>96</td>
</tr>
</tbody>
</table>
5.5.3 Remarks

Table 12: Remarks made by the partners

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

5.5.4 Results of the samples

Table 13: Results reported by the partners

<table>
<thead>
<tr>
<th>Partner</th>
<th>Masterlab, Putten, The Netherlands</th>
<th>National Veterinary Institute, Uppsala, Sweden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample content (mg/kg)</td>
<td>Result 1 (mg/kg)</td>
<td>Result 2 (mg/kg)</td>
</tr>
<tr>
<td>0</td>
<td>&lt;0,1</td>
<td>&lt;0,1</td>
</tr>
<tr>
<td>0</td>
<td>&lt;0,1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>1,5</td>
<td>1,4</td>
</tr>
<tr>
<td>2</td>
<td>1,6</td>
<td>1,5</td>
</tr>
<tr>
<td>2</td>
<td>1,6</td>
<td>2,27</td>
</tr>
<tr>
<td>2</td>
<td>1,6</td>
<td>2,34</td>
</tr>
<tr>
<td>7,5</td>
<td>4,6</td>
<td>4,5</td>
</tr>
<tr>
<td>7,5</td>
<td>6,2</td>
<td>6,81</td>
</tr>
<tr>
<td>7,5</td>
<td>6,2</td>
<td>6,79</td>
</tr>
<tr>
<td>7,5</td>
<td>6,2</td>
<td>6,81</td>
</tr>
</tbody>
</table>

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

The results of the collaborative study were evaluated in a meeting in Tervuren (Belgium) on June 19-20, 2001.

The panel has accepted the results of the statistical evaluation, as described in par. 5.1, Table 6. Consequently it can be concluded that the repeatability and reproducibility of the method is acceptable. The results obtained for the blank feed are also acceptable. Large differences are observed in the recovery (range 49 – 101 %), most probably due to the problems in the extraction step, caused by the unfavourable ratio between extraction volume and sample weight.

The panel agreed that, due to the problems with the practicability of the method (see par. 5.4), the method cannot be recommended for adoption as an official method. A second collaborative study will be organised with a modified method. The ratio between the extraction volume and the feed weight will be increased from 2 : 1 to 5 : 1.

The results obtained for the blind blank feed indicate that different columns lead to differences in interfering peaks (large peak eluting prior to olaquindox).

The following columns will be recommended in the method:
- Hypersil ODS 5 μm, 200 x 4,6 mm;
- Spherisorb ODS-2 5 μm, 250x4,6 mm;
- LUNA C18(2) 250 x 4,6 mm.

The following remarks, related to the method description have been accepted:
- Lab 16, remark 1 (see par. 5.4 of this report).
ACKNOWLEDGEMENTS

Financial support from the European Commission, DG Research, Standards, Measurements and Testing Programme (SMT) is gratefully acknowledged.
Dr. A. Plöger, Danish Plant Directorate, Lyngy, Denmark, is thanked for supplying the olaquindox reference standard.
Dr. H. van de Voet, Biometris, Wageningen University and Research Centre is thanked for statistical advice.
APPENDIX 1

Letter with instructions, sent with the samples (with four annexes)
cc Driessen/J. de Jong

Dear colleague,

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

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

The samples must be analysed in duplicate.

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

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

Annex 2 contains the reporting form. This form will also be send to you by E-mail as an Excel 5.0 file. We strongly prefer to get the results back in electronic form by E-mail (please send the results to the following E-mail address: j.m.driessen@rikilt.wag-ur.nl). Of course you can also fill in the form and send it by fax or normal mail. The deadline for reporting the results is 8 December 2000.

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

Annex 4 is a questionnaire. We kindly ask you to give us information about the experimental conditions, recoveries, etc. On this form you can also give your remarks about the method.

The reference standard of olaquindox which has to be used (980416) was already sent to you with my letter of 31 May 2000. In the calculations this reference standard can be regarded as 100 % pure.
We wish you and your colleagues the best with the collaborative studies. If you have any questions, do not hesitate to contact us.

Kind regards,

dr. Jacob de Jong
CANDAS co-ordinator

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

cc mrs. I. de Froidmont-Görtz, European Commission, DG Research, CI/3, Brussels
Determiniation of low level contents of Olaquindox in Feedingstuffs

1. Purpose and scope
   The method is for the determination of olaquindox in feedingstuffs. The limit of
determination (=quantification) is 1.5 mg/kg. The limit of detection (=qualification) is
0.1 mg/kg

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

3. Reagents
   3.1. Methanol
   3.2. Methanol, HPLC grade
   3.3 Water, HPLC grade
   3.4. Mobile phase for HPLC
      Water (3.3)-methanol (3.2) mixture, 900+100 (V + V)
   3.5. Standard substance: pure olaquindox 2-[N-2'-(hydroxyethyl)carbamoyl]-3-
methylquinoxaline-N¹,N¹-dioxide, E 851
   3.5.1. Olaquindox stock standard solution, 50 µg/ml
      Weigh to the nearest 0.1 mg 10 mg of olaquindox (3.5) in a 200 ml graduated flask
and add ca. 190 ml water. Then place the flask for 10 min in a ultrasonic bath (4.1).
After ultrasonic treatment, bring the solution to room temperature, make up to the
mark with water and mix. Wrap the flask with aluminium foil and store in a refrigerator.
At this temperature of ≤ 4°C the solution is stable for 1 month.

   3.5.2. Calibration solutions
      Into a series of 50 ml graduated flasks transfer 0.5, 1.0, 2.5, 5.0 and 10.0 ml of the
standard stock solution (3.5.1). Make up to the mark with water (3.3) and mix. These
solutions correspond to 0.5, 1.0, 2.5, 5.0 and 10.0 µg of olaquindox per ml
respectively.
These solutions must be prepared fresh each day.

4. Apparatus
4.1. Ultrasonic bath
4.2. Mechanical shaker
4.3. Membrane filter, 0.45 μm
4.4. HPLC equipment with variable wavelength ultraviolet detector
4.4.1. Liquid chromatographic column, 250 mmx4mm, C 18, 5 μm packing, or equivalent

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

5.1. General

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

5.1.2. Recovery test
A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of olaquindox, similar to that present in the sample. To fortify at a level of 3 mg/kg, transfer 1.5 ml of the stock standard solution (3.5.1) to a 250 ml conical flask, add 25 g of the blank feed, mix thoroughly and leave for 10 min mixing again several times before proceeding with the extraction step (5.2). Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, prepare two independent laboratory sample aliquots (A and B) of the feed to be examined. Spike one of them (A), before extraction with a quantity of olaquindox, similar to that already present in the sample. Both samples are analysed. Calculate the analyte content in sample A and B and calculate the recovery by subtraction.

5.2. Extraction
Weigh to the nearest 0.01 g, approximately 25 g of the sample. Transfer to a 250 ml
conical flask, add 10 ml of methanol (3.1) and place the flask for 5 min in the ultrasonic bath (4.1). Add 40 ml water and leave in the ultrasonic bath for further 15 min. Remove the flask from the ultrasonic bath, shake it for 30 min on the shaker (4.2) and filter through a folded filter or a glass fibre filter (GFA, Whatman) (see remark 7.1). It is highly recommended to filter the clear samples by using a membrane filter (4.3) additionally. Proceed to the HPLC determination (5.3).

5.3. HPLC determination

5.3.1. Parameters:
The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.
Analytical column (4.4.1)
Mobile Phase (3.4): water (3.3) - methanol (3.1.) mixture, 900 + 100 (V+ V)
Flow rate: 1.5 - 2 ml/min
Detection wavelength: 380 nm
Injection volume: 20 μl -100 μl
Check the stability of the chromatographic system, injecting several times the calibration solution (3.5.3) containing 2.5 μg/ml, until constant peak heights and retention times are achieved.

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

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

6. Calculation of the results
From the height (area) of the olaquindox peaks of the sample solution determine the concentration of the sample solution in μg/ml by reference to the calibration graph (5.3.2).
The olaquindox content w (mg/kg) of the sample is given by the following formular:

\[ w = \frac{c \times 50}{m} \]
in which:

\[
c = \text{o}laquindox \text{ concentration of the sample extract (5.2) in } \mu \text{g/ml}
\]

\[
m = \text{mass of the test portion in g}
\]

7 Remarks

7.1 Instead of filtration a centrifugation step could be carried out.
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: 
Date of analysis: 
Analyte: OLAQUINDOX

<table>
<thead>
<tr>
<th>Unit Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>315707</td>
<td></td>
<td></td>
</tr>
<tr>
<td>315709</td>
<td></td>
<td></td>
</tr>
<tr>
<td>315710</td>
<td></td>
<td></td>
</tr>
<tr>
<td>315794</td>
<td></td>
<td></td>
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<td>315801</td>
<td></td>
<td></td>
</tr>
<tr>
<td>315811</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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.
Annex 4 - Questionnaire

Laboratory: .................................................................................................................................
Contact person: ............................................................................................................................... 

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

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

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: ...... %
- Single / duplicate determinations: □ single □ duplicate
- If duplicate, please give both percentages: ...... % and ...... %
- Spiking level: ............ mg/kg
Please complete this questionnaire and return it together with representative chromatograms to:
Ing. J.J.M. Driessen
RIKILT
P.O. Box 230
6700 AE Wageningen
The Netherlands
Fax +31-317-417717

Thank you for your cooperation!
APPENDIX 2

Composition of the feed samples
## BESTMIX - Afdruk mengopdracht

- **05/09/00 - IPC DIER BARNEVELD**

**2 250.00 Biggen opfok korrel**

- *Rikilt*
- biggenvoer van 12 tot 25/30 kg
  - 10 ppm Carbadox + 2 ppm olaquindox

### Grondstof

<table>
<thead>
<tr>
<th>Silo</th>
<th>Gewicht</th>
<th>Tol.</th>
<th>Cumul Gew.</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>kg</td>
<td>kg</td>
<td>+/-Afw.</td>
<td>kg</td>
<td>kg</td>
</tr>
</tbody>
</table>

#### Weegschaal DW 1

| Zonbl. schr. 290re | (2) | 2.00 | 10.00 | 0.30 | 10.00 | V. |
| Tapioca 65%zetmeel | (4) | 7.50 | 37.50 | 1.13 | 47.50 | V. |
| Soja 45/46(arg/braz)| (9) | 13.00| 65.00 | 1.95 | 112.50| V. |

#### Weegschaal DW 2

| Tarwe | (voer) | 10.00 | 50.00 | 1.50 | 50.00 | V. |
| Gerst | (11) | 37.10 | 185.50 | 5.57 | 235.50 | V. |
| Mais | (12) | 12.00 | 60.00 | 1.80 | 295.50 | V. |

#### Bijstort SP4

| Lynzaad | (0) | 5.00 | 25.00 | 0.75 | 25.00 | V. |
| Vismee | (0) | 4.40 | 22.00 | 0.66 | 47.00 | V. |

#### Bijstort SP6

| Powerfood Twil melkv | (0) | 4.00 | 20.00 | 0.60 | 20.00 | V. |

#### Bijstort SP7

| Fumaarzuur | (0) | 0.25 | 1.25 | 0.01 | 1.25 | V. |
| L-lysine HCl | (0) | 0.17 | 0.85 | 0.01 | 2.10 | V. |
| DL-Methio-nine | (0) | 0.03 | 0.15 | 0.00 | 2.25 | V. |
| Krijt/kalksteen | (0) | 0.45 | 2.25 | 0.02 | 4.50 | V. |
| Monocal Belgie | (0) | 0.50 | 2.50 | 0.03 | 7.00 | V. |
| Zout | (0) | 0.10 | 0.50 | 0.01 | 7.50 | V. |
| Prem biggen Rikilt | (0) | 1.00 | 5.00 | 0.05 | 12.50 | V. |

#### Vloeistoffen

| Melasse riet >450s | (3) | 2.50 | 12.50 | 0.38 | 12.50 | V. |

**Totaal: 500.00**

## RETOURPRODUKT

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## INSTELLINGEN

- **T.R.**: aardappelen 50%
- **V.Z.**: grof 60%...
- **Z.P.**: 25... mm
- **H.M.**: hoog/laag toerend kningloop: ja/nee
- **L.M.**: voortmengen 3.00 sec
- **M.D.**: 73... 1/h
- **Meel temp**: 75,5°C karmeldemp 78°C
- **Matrijs diam.**: 2.5 x 35. mm
- **K.P.**: 28. Amp
- **Kruimelen**: ja/nee
- **Holmen**: 46.8%
- **Vocht**: 

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# BESTMIX - Afdruk mengopdracht

## 2 250.00 Biggen opfok korrel Rikill

Biggenvoer van 12 tot 25/30 kg
2,5 kg/kg carbox + 7,5 mg/kg olaquindox

### Grondstof

<table>
<thead>
<tr>
<th>Silo</th>
<th>Gewicht</th>
<th>Tol.</th>
<th>Cumul Gew.</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kg +/-Afw.</td>
<td>kg</td>
<td></td>
<td>5^-</td>
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#### Weegschaal DW 1

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<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>113</td>
<td>Zonbl.schr.2390re</td>
<td>(2)</td>
<td>2.00</td>
<td>10.00</td>
<td>0.30</td>
</tr>
<tr>
<td>460</td>
<td>Tapioca65%zetmeel</td>
<td>(4)</td>
<td>7.50</td>
<td>37.50</td>
<td>1.13</td>
</tr>
<tr>
<td>77</td>
<td>Soja 45/46(arg/braz)</td>
<td>(9)</td>
<td>13.00</td>
<td>65.00</td>
<td>1.95</td>
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</table>

#### Weegschaal DW 2

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</thead>
<tbody>
<tr>
<td>145</td>
<td>Tarwe (voer)</td>
<td>(9)</td>
<td>10.00</td>
<td>50.00</td>
<td>1.50</td>
</tr>
<tr>
<td>14</td>
<td>Gerst</td>
<td>(11)</td>
<td>37.10</td>
<td>185.50</td>
<td>5.57</td>
</tr>
<tr>
<td>40</td>
<td>Maïs</td>
<td>(12)</td>
<td>12.00</td>
<td>60.00</td>
<td>1.80</td>
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#### Bijstort SP4

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</thead>
<tbody>
<tr>
<td>34</td>
<td>Lynzaad</td>
<td>(0)</td>
<td>5.00</td>
<td>25.00</td>
<td>0.75</td>
</tr>
<tr>
<td>105</td>
<td>Vismee</td>
<td>(0)</td>
<td>4.40</td>
<td>22.00</td>
<td>0.66</td>
</tr>
</tbody>
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#### Bijstort SP6

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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>476</td>
<td>Powerfood Twil melkv</td>
<td>(0)</td>
<td>4.00</td>
<td>20.00</td>
<td>0.60</td>
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</tbody>
</table>

#### Bijstort SP7

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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>Fumaarzuur</td>
<td>(0)</td>
<td>0.25</td>
<td>1.25</td>
<td>0.01</td>
</tr>
<tr>
<td>78</td>
<td>L-lysine HCl</td>
<td>(0)</td>
<td>0.17</td>
<td>0.85</td>
<td>0.01</td>
</tr>
<tr>
<td>79</td>
<td>DL-Methio-nine</td>
<td>(0)</td>
<td>0.03</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td>117</td>
<td>Krijt/kalksteen</td>
<td>(0)</td>
<td>0.45</td>
<td>2.25</td>
<td>0.02</td>
</tr>
<tr>
<td>228</td>
<td>Monocal Belg</td>
<td>(0)</td>
<td>0.50</td>
<td>2.50</td>
<td>0.03</td>
</tr>
<tr>
<td>485</td>
<td>Zout</td>
<td>(0)</td>
<td>0.10</td>
<td>0.50</td>
<td>0.01</td>
</tr>
<tr>
<td>508</td>
<td>Prem biggen Rikil</td>
<td>(0)</td>
<td>1.00</td>
<td>5.00</td>
<td>0.05</td>
</tr>
</tbody>
</table>

#### Vloeistoffen

<p>| | | | | | |</p>
<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>474</td>
<td>Melasse riet &gt;450s</td>
<td>(3)</td>
<td>2.50</td>
<td>12.50</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Totaal: 500.00

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## RETOURPRODUKT

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## INSTITELLINGEN

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T.R.</td>
<td>and 50%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V.Z.</td>
<td>grof 80%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z.F.</td>
<td>2/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.M.</td>
<td>30min laag toeren</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.M.</td>
<td>voormengen 9 sec</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.D.</td>
<td>73 1/h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

## Meel temp: 25.5°C konden 77°C

## Matrijs diam.: 25 x 35 mm

## K.P.: 28 Amp

## Laagdikte Ko: 35 cm

## Zeef Ko: 0.75 mm

## Kruimelen: 96,8 %
APPENDIX 3

Homogeneity of samples
Homogeneity test collaborative study

Additive: Olaquindox
Product: Feed sample: 2 ppm

Date of determination: September 22th, 2000

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content mg/kg</th>
<th>Duplicate average mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>341024 A</td>
<td>1,6</td>
<td>1,6</td>
</tr>
<tr>
<td>341024 B</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>341030 A</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
<td>341030 B</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>341028 A</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
<td>341028 B</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>341029 A</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
<td>341029 B</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>341021 A</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
<td>341021 B</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>341023 A</td>
<td>1,4</td>
<td>1,5</td>
</tr>
<tr>
<td>341023 B</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>341027 A</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
<td>341027 B</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>341026 A</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
<td>341026 B</td>
<td>1,4</td>
<td></td>
</tr>
<tr>
<td>341022 A</td>
<td>1,4</td>
<td>1,4</td>
</tr>
<tr>
<td>341022 B</td>
<td>1,4</td>
<td></td>
</tr>
<tr>
<td>341025 A</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
<td>341025 B</td>
<td>1,5</td>
<td></td>
</tr>
</tbody>
</table>

Homogeneity
Criterion: CV{\text{between}} = < 15%

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>1,5</td>
<td></td>
</tr>
<tr>
<td>SD (between samples)</td>
<td>0,04</td>
<td></td>
</tr>
<tr>
<td>CV (between samples)</td>
<td>2,8</td>
<td></td>
</tr>
<tr>
<td>Grubb's test, single lower</td>
<td>2,065</td>
<td>no outlier</td>
</tr>
<tr>
<td>Grubb's test, single upper</td>
<td>1,579</td>
<td>no outlier</td>
</tr>
<tr>
<td>Grubb's test, double lower</td>
<td>0,3279</td>
<td>no outliers</td>
</tr>
<tr>
<td>Grubb's test, double upper</td>
<td>0,6557</td>
<td>no outliers</td>
</tr>
</tbody>
</table>

Repeatability

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>SD (within samples)</td>
<td>(sd_{r})</td>
<td>0,04</td>
</tr>
<tr>
<td>CV (within samples)</td>
<td>(CV (%))</td>
<td>2,6</td>
</tr>
</tbody>
</table>

Result Grubb's test
no outlier
no outliers
Homogeneity test collaborative study

Additive: Olaquindox
Product: Feed sample: 7.5 ppm

Date of determination: September 22\textsuperscript{th}, 2000

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content (mg/kg)</th>
<th>Duplicate average (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>345016 A</td>
<td>4,8</td>
<td>5,1</td>
</tr>
<tr>
<td>345016 B</td>
<td>5,3</td>
<td></td>
</tr>
<tr>
<td>345014 A</td>
<td>5,0</td>
<td>5,0</td>
</tr>
<tr>
<td>345014 B</td>
<td>4,9</td>
<td></td>
</tr>
<tr>
<td>345013 A</td>
<td>5,0</td>
<td>5,1</td>
</tr>
<tr>
<td>345013 B</td>
<td>5,2</td>
<td></td>
</tr>
<tr>
<td>345012 A</td>
<td>5,0</td>
<td>4,9</td>
</tr>
<tr>
<td>345012 B</td>
<td>4,8</td>
<td></td>
</tr>
<tr>
<td>345020 A</td>
<td>5,0</td>
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**Homogeneity**

Criterion: $\text{CV}_{\text{between}} \leq 15\%$

- **Average**: 4,9
- **SD (between samples)**: 0,12
- **CV (between samples)**: 2,5
- **Grubb's test, single lower**: 1,225, no outlier
- **Grubb's test, single upper**: 1,633, no outlier
- **Grubb's test, double lower**: 0,5833, no outliers
- **Grubb's test, double upper**: 0,4236, no outliers

**Repeatability**

- **SD (within samples)**: (sd\textsubscript{r}) 0,15
- **CV (within samples)**: (CV (%)) 3,1
APPENDIX 4

Sample codes
Sample codes supplied to the participants in the olaquindox collaborative study

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APPENDIX 5

Olaquindox reference standard profile, identity and purity
CERTIFICATO DI ANALISI N. 01/99
CERTIFICATE OF ANALYSIS No.01/99

<table>
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<tr>
<td>Batch no:</td>
<td>080416</td>
</tr>
<tr>
<td>Mfg date:</td>
<td>Aprile 98/Apr 98</td>
</tr>
<tr>
<td>Exp date:</td>
<td>Aprile 01/Apr 01</td>
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**RISULTATI/RESULTS:**

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<th>Specifiche/Specification</th>
<th>Riferimento/Reference</th>
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<td>Aspetto/Appearance</td>
<td>polvere cristallina/crystalline powder</td>
<td>Corrisponde/Corresponds</td>
</tr>
<tr>
<td>01 COL</td>
<td>Colore/Colour</td>
<td>giallo/yellow</td>
<td>Corrisponde/Corresponds</td>
</tr>
<tr>
<td>01 ODO</td>
<td>Odore/Odour</td>
<td>inodore/odourless</td>
<td>Corrisponde/Corresponds</td>
</tr>
<tr>
<td>UV016</td>
<td>Titolo/Assay</td>
<td>min 98 max 101.5%</td>
<td>99.46%</td>
</tr>
<tr>
<td>01PFPE</td>
<td>Perdita di peso por</td>
<td>max 0.5%</td>
<td>0.07%</td>
</tr>
<tr>
<td></td>
<td>essicamento/Loss on drying</td>
<td>max 0.5%</td>
<td>max 0.5%</td>
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<tr>
<td></td>
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<td>max 0.25%</td>
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<tr>
<td></td>
<td>Metilsteres</td>
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<td>max 0.2%</td>
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Il sopranominato Prodotto è stato analizzato secondo i metodi analitici DOX-AL ITALIA SpA ed è stato approvato per la vendita dal CONTROLLO QUALITÀ/The above mentioned product has been analyzed according to Dox-al Italia Spa analytical methods and has been approved for sale by QUALITY CONTROL.

Analista/Analyst: Lucia Corbetta

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

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

<table>
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<tr>
<th>Supplier</th>
<th>Pfizer</th>
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<tbody>
<tr>
<td>Lotnr</td>
<td>3E121-84QCS</td>
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<tr>
<td>Drying loss (%)</td>
<td>0.02</td>
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<tr>
<td>Purity (%)</td>
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</table>

**Olaquindox**

<table>
<thead>
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<th>DOX-AL Italia</th>
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<tr>
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<td>311363</td>
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<tr>
<td>Lotnr</td>
<td>980416</td>
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<tr>
<td>Drying loss (%)</td>
<td>&lt;0.5</td>
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<td>Purity (%)</td>
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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 \(\mu\)g/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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorbance maxima (nm)</th>
<th>Δ (nm)</th>
<th>Absorbance (AU)</th>
<th>Δ (%)</th>
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</thead>
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<tr>
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<td>383.5</td>
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<td>0.1856</td>
<td>-4.9</td>
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</table>

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.

1H-NMR

1H-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).

1H-NMR experiments: 1H-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.

1H-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.

1H-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 percent 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.
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.
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

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<th>e-mail:</th>
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<td>Contact person:</td>
<td>fax:</td>
</tr>
<tr>
<td>Date of analysis:</td>
<td>telephone:</td>
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**Analyte:** OLAQUINDOX

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Annex 4 - Questionnaire

Date(s) of analysis: 22 November 2000

Chromatographic conditions:
- Column:
  - ☑ As described in the method.
  - ☐ Other: .................................................................
- Mobile phase:
  - ☑ As described in the method.
  - ☐ Other: .................................................................
- Flow-rate: 1.2 ml/min
- Injection volume: 50 μl
- Retention time of olaquindox: ~ 9 min.

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample
Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 78%
- Single / duplicate determinations: ☑ single ☐ duplicate
- If duplicate, please give both percentages: ......% and ......%
- Spiking level: 3 mg/kg
Injection Date: 22/11/00 13:10:36
Sample Name: 
Acq. Operator: 

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

Area Percent Report

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Dilution: 1.0000

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

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Totals: 43.85185 1.75619

*** End of Report ***

Instrument 1 22/11/00 19:51:48
Injection Date: 22/11/00 14:01:28
Sample Name: 
Acq. Operator: 

Vial: 1
Inj Volume: 50 µl

Acq. Method: C:\HPCHEM\OLAQCQL.M
Last changed: 22/11/00 13:08:59 by

Analysis Method: C:\HPCHEM\OLAQCQL.M
Last changed: 22/11/00 19:51:44 by (modified after loading)

CANFAS/Olaquindox

Area Percent Report

Sorted By: Signal
Multiplier: 1.0000
Dilution: 1.0000

Signal 1: DAD1 A, Sig=380.4 Ref=500.20
Results obtained with enhanced integrator!

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<th>Area %</th>
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Totals: 139.76018 5.39218

*** End of Report ***
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:  

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<th>Result 2 (mg/kg)</th>
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<td></td>
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<tr>
<td>Sample code</td>
<td></td>
<td></td>
</tr>
<tr>
<td>155711</td>
<td>blank</td>
<td>blank</td>
</tr>
<tr>
<td>155735</td>
<td>1,25</td>
<td>1,02</td>
</tr>
<tr>
<td>155741</td>
<td>1,12</td>
<td>1,34</td>
</tr>
<tr>
<td>155761</td>
<td>blank</td>
<td>blank</td>
</tr>
<tr>
<td>155776</td>
<td>4,60</td>
<td>3,52</td>
</tr>
<tr>
<td>155784</td>
<td>4,14</td>
<td>4,76</td>
</tr>
</tbody>
</table>
CANFAS COLLABORATIVE STUDIES OCTOBER 2000 • OLAQUINDOX

Annex 4 - Questionnaire

Data(s) of analysis: O 3

Chromatographic conditions:

- Column:
  - □ As described in the method
  - ⃝ Other: Hypersil ODS 5 μm 200 x 4.6 mm + guard column

- Mobile phase:
  - □ As described in the method
  - □ Other: ..........................................................

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

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

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

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

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

RetTime Type Area Amt/Area Amount Grp Name
[min] [mAU*s] [ng/ul] 
5.110 - - - 0.0000 olaquindox

Totals: 0.00000

Results obtained with enhanced integrator!
1 Warnings or Errors:
Warning: Calibrated compound(s) not found

Page 1 of 2
Injection Date: 11/3/00 6:08:37 PM
Sample Name: 155741
Acq. Operator: adl
Seq. Line: 11
Vial: 11
Inj Volume: 20 μl

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

---

External Standard Report
---

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

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

RetTime Type Area Amt/Area Amount Grp Name
[min] [mAUs] [ng/ul]  [ng/ul]

5.107 PB 20.12256 3.3420e-2 6.72497e-1 olaquindox

Totals:
6.72497e-1 × 2 = 1.34 mg/kg

Results obtained with enhanced integrator!
---

*** End of Report ***
---

Instrument 1 11/6/00 2:28:09 PM

Page 1 of 1
ata File C:\HPCHEM\DATA\ADL\olaq0324.D

Sample Name: 1

Injection Date: 11/3/00 7:33:32 PM
Sample Name: 155776
Acq. Operator: adl

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

---

External Standard Report

---

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

Signal 1: DAD1 A, Sig=380.4 Ref=450.50

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

Totals: 1.76595 x 2 = 3.532 mg/kg

Results obtained with enhanced integrator!

---

*** End of Report ***

---

instrument 1 11/6/00 2:28:39 PM
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 16
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:

<table>
<thead>
<tr>
<th>Analyte: OLAQUINDOX</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample code</td>
<td>Result 1 (mg/kg)</td>
<td>Result 2 (mg/kg)</td>
</tr>
<tr>
<td>165708</td>
<td>5.66</td>
<td>4.98</td>
</tr>
<tr>
<td>165719</td>
<td>1.56</td>
<td>1.7</td>
</tr>
<tr>
<td>165751</td>
<td>not found</td>
<td>not found</td>
</tr>
<tr>
<td>165774</td>
<td>1.66</td>
<td>1.65</td>
</tr>
<tr>
<td>165802</td>
<td>not found</td>
<td>not found</td>
</tr>
<tr>
<td>165816</td>
<td>5.65</td>
<td>5.54</td>
</tr>
</tbody>
</table>
Date(s) of analysis: 2000-10-23, 2000-11-14, 2000-11-15 (each assay one day)

Chromatographic conditions:
- Column:
  - □ As described in the method
  - ✗ Other: Spherisorb ODS 2, 10 μm, 250 x 4.6 mm
- Mobile phase:
  - □ As described in the method
  - ✗ Other:
- Flow-rate: 1.7 ml/min
- Injection volume: 20 μl
- Retention time of olaquindox: 9.5 min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 76.5 %
- Single / duplicate determinations: □ single ✗ duplicate
- If duplicate, please give both percentages: 76.9 and 76.0 %
- Spiking level: 3.0 mg/kg
INJ NO. OF STD : 1 / 1 REP , 1st level

D-2500

METHOD: TAG: 495 CH: 1
FILE: 3 CALC-METHOD: EXT-STD TABLE: 9 CONC: HEIGHT

<table>
<thead>
<tr>
<th>NO.</th>
<th>RT</th>
<th>AREA</th>
<th>HEIGHT</th>
<th>UG/ML</th>
<th>NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9.40</td>
<td>4351</td>
<td>172</td>
<td>400</td>
<td>OLA</td>
</tr>
</tbody>
</table>

D-2500

METHOD: TAG: 496 CH: 1
FILE: 3 CALC-METHOD: EXT-STD TABLE: 9 CONC: HEIGHT

Sample code: 165754
CH. 1 CS 5.00 ATT 2 OFFS 10 11/15/00 10:23

1.63
2.76
3.97

S.P. 400

9.38 → Olaquindox

INJ NO. OF STD : 1 / 1 REP , 1st level
D-2500
11/15/00 10:23
METHOD:
TAG: 483 CH: 1
FILE: 3 CALC-METHOD: EXT-STD TABLE: 9 CONC: HEIGHT

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

CH. 1 CS 5.00 ATT 2 OFFS 10 11/15/00 10:36

1.40

4.65

S.P. 400

9.46 → Olaquindox

D-2500
11/15/00 10:36

Sample code 165708
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 17
Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

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

Date of analysis:

Analyte: OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>175703</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>175718</td>
<td>5.60</td>
<td>5.66</td>
</tr>
<tr>
<td>175730</td>
<td>2.07</td>
<td>1.99</td>
</tr>
<tr>
<td>175775</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>175817</td>
<td>1.99</td>
<td>1.92</td>
</tr>
<tr>
<td>175828</td>
<td>5.58</td>
<td>5.63</td>
</tr>
</tbody>
</table>
CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 11.10.2000

Chromatographic conditions:
- Column:
  - □ As described in the method
  - □ Other: Spherisorb S10 ODS-1 10μ
- Mobile phase:
  - □ As described in the method
  - □ Other: ..........................................................
- Flow-rate: ...... ml/min
- Injection volume: ...... μl
- Retention time of olaquindox: ...... min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

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

The recovery was not respected in calculation
the results.
**Sample Name**: Standard 2,5µg/ml

**Analyzed**: 11.10.00 13:39  
**Reported**: 13.11.00 10:46

**Data Path**: C:\Win32App\HSM\OLAQU\DATA\0199\  
**Application**: Olaquindox

**Injection from this vial**: 1 of 1  
**Vial Number**: 1  
**Volume**: 20,0 µl

**Sample Description**:

**Chrom Type**: Fixed WL Chromatogram, 380 nm

![Chromatogram Image]

**Acquisition Method**: Olaquindox  
**Column Type**: RP 18  
**Pump A Type**: L-7100  
**Solvent A**: MeOH/H2O  
**Solvent C**: MeOH/H2O  
**Peak Quantitation**: AREA  
**Calculation Method**: EXT-STD

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Conc 1</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olaqu</td>
<td>4.19</td>
<td>558</td>
<td>0.000</td>
<td>BB</td>
</tr>
<tr>
<td></td>
<td>4.71</td>
<td>141</td>
<td>0.000</td>
<td>BB</td>
</tr>
<tr>
<td></td>
<td>5.66</td>
<td>124</td>
<td>0.000</td>
<td>BB</td>
</tr>
<tr>
<td></td>
<td>7.02</td>
<td>39485</td>
<td>2.607</td>
<td>MC</td>
</tr>
<tr>
<td></td>
<td>7.87</td>
<td>0</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

**Developed by**: 

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

Page Indicator 1 / 2
**D-7000 HSM: Olaquindox**  
**Sample Name:** 175775

Analyzed: 11.10.00 17:40  
Data Path: C:\Win32App\HSM\OLAQU\DATA\0200\  
Application: Olaquindox  
Injection from this vial: 1 of 1

Sample Description:

Chrom Type: Fixed WL Chromatogram, 380 nm

---

**Acquisition Method:** Olaquindox  
**Column Type:** RP 18  
Pump A Type: L-7100  
Solvent A: MeOH/H2O  
Solvent C: MeOH/H2O  
Peak Quantitation: AREA  
Calculation Method: EXT-STD

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

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Conc 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.65</td>
<td>6506</td>
<td>0.000</td>
</tr>
</tbody>
</table>
|     |     | 6506 | 0.000  | BC

Peak rejection level: 0
D-7000 HSM: Olaquindox

Sample Name: 175828

Analyzed: 11.10.00 15:39
Data Path: C:\Win32App\HSM\OLAQU\DATA\0199\Application: Olaquindox
Injection from this vial: 1 of 1

Sample Description:

Chrom Type: Fixed WL Chromatogram, 380 nm

Acquisition Method: Olaquindox
Column Type: RP 18
Pump A Type: L-7100
Solvent A: MeOH/H2O
Solvent C: MeOH/H2O
Peak Quantitation: AREA
Calculation Method: EXT-STD

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

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Conc 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olaqu</td>
<td>6,99</td>
<td>42790</td>
<td>5,634</td>
</tr>
<tr>
<td></td>
<td>8,42</td>
<td>253</td>
<td>0,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BC</th>
<th>MC</th>
<th>BB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

43043 5,634

Peak rejection level: 0

Page Indicator 1 / 1
Sample Name: 175817

Analyzed: 11.10.00 17:50
Data Path: C:\Win32App\HSM\OLAQU\DATA\0200\ Application: Olaquindox
Injection from this vial: 1 of 1
Series: 0200
Vial Number: 10
Volume: 20,0 ul

Sample Description:

Chrom Type: Fixed WL Chromatogram, 380 nm

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Conc 1</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cone 1</td>
<td>3,83</td>
<td>2176</td>
<td>0,000</td>
<td>BB</td>
</tr>
<tr>
<td>Cone 1</td>
<td>4,33</td>
<td>663</td>
<td>0,000</td>
<td>BB</td>
</tr>
<tr>
<td>Cone 1</td>
<td>5,12</td>
<td>0</td>
<td>0,000</td>
<td></td>
</tr>
<tr>
<td>Cone 1</td>
<td>7,28</td>
<td>14085</td>
<td>1,990</td>
<td>MC</td>
</tr>
<tr>
<td>Cone 1</td>
<td></td>
<td></td>
<td>16924</td>
<td></td>
</tr>
</tbody>
</table>

Developed by: I

Solvent B: MeOH/H2O
Solvent D: MeOH/H2O
Sample Amount: 0,500
Scale Factor 1: 1,000
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 18
### Task 4 COLLABORATIVE STUDY

**Analyte:** OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>185693</td>
<td>5,78</td>
<td>5,92</td>
</tr>
<tr>
<td>185695</td>
<td>6,18</td>
<td>6,12</td>
</tr>
<tr>
<td>185731</td>
<td>1,56</td>
<td>1,52</td>
</tr>
<tr>
<td>185733</td>
<td>1,67</td>
<td>1,65</td>
</tr>
<tr>
<td>185758</td>
<td>Not Detected ; LOD&lt;0,5</td>
<td>Not Detected ; LOD&lt;0,5</td>
</tr>
<tr>
<td>185823</td>
<td>Not Detected ; LOD&lt;0,5</td>
<td>Not Detected ; LOD&lt;0,5</td>
</tr>
</tbody>
</table>
CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

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

Chromatographic conditions:
- Column:
  - ☐ As described in the method
  - ☑ Other: 150 x 4.6 mm; 5 μm; Spherisorb ODS2 5 μm
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other: .................................................................
- Flow rate: 1.00 ml/min
- Injection volume: 10 μl
- Retention time of olaquindox: 8.4 min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 17.3%
- Single / duplicate determinations: ☑ single ☐ duplicate
- If duplicate, please give both percentages: 14.6% and 16.2%
- Spiking level: 3.0 mg/kg
Current Chromatogram(s)

Sample feed 185733 (#1.5 mg/kg)

Area: 77.785

01/12/00 12:37:44 mcr
Current Chromatogram(s)

Blank feed

Olaquindox standard 1 µg/ml

Olaquindox peak

Are: 36.3923

01/12/00 12:33:59 mcr

Page 1 of
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:**  
**Date of analysis:**  
**Analyte:** OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>205713</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>205729</td>
<td>1.72</td>
<td>1.6</td>
</tr>
<tr>
<td>205739</td>
<td>1.52</td>
<td>1.59</td>
</tr>
<tr>
<td>205796</td>
<td>4.85</td>
<td>4.78</td>
</tr>
<tr>
<td>205809</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>205822</td>
<td>4.65</td>
<td>4.78</td>
</tr>
</tbody>
</table>
CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

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

Chromatographic conditions:
- Column:
  - ☑ As described in the method: AGRILTECH C18, 150 x 4.6 mm, 5 μm
  - ☐ Other:
- Mobile phase:
  - ☑ As described in the method
  - ☐ Other:
- Flow rate: 0.5 ml/min
- Injection volume: 10 μl
- Retention time of olaquindox: 3.4 min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow.

Recovery results:
- Percentage recovery: 100%
- Single/duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 100% and 100%
- Spiking level: 0.5 mg/kg
External Standard Report

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

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

RetTime Type Area Amt/Area Amount Grp Name
[min] [mAU*s] [ug/ml] | 178.33518 1.4758e-2 2.63371 Olaquindox
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6.414 MM</td>
<td></td>
</tr>
</tbody>
</table>

Totals: 2.63371

Results obtained with enhanced integrator.

*** End of Report ***

4.84 µg/g

Page 1 of 1
**External Standard Report**

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

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

<table>
<thead>
<tr>
<th>Ret.Time [min]</th>
<th>Type</th>
<th>Area [mAU's]</th>
<th>Amt/Area</th>
<th>Amount [ug/ml]</th>
<th>Grp Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.618</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Olaquindox</td>
</tr>
</tbody>
</table>

**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
Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2: Task 4 COLLABORATIVE STUDY)

Subtitle: OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>215715</td>
<td>2,0</td>
<td>2,0</td>
</tr>
<tr>
<td>215770</td>
<td>0,0 N.D.</td>
<td>0,0 N.D.</td>
</tr>
<tr>
<td>215791</td>
<td>0,0 N.D.</td>
<td>0,0 N.D.</td>
</tr>
<tr>
<td>215797</td>
<td>5,4</td>
<td>5,3</td>
</tr>
<tr>
<td>215813</td>
<td>2,0</td>
<td>2,0</td>
</tr>
<tr>
<td>215827</td>
<td>5,3</td>
<td>5,2</td>
</tr>
</tbody>
</table>
CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

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

Chromatographic conditions:
- Column:
  - ☐ As described in the method
  - ☑ Other: SuperCosil LC-18 25cm x 4.6 mm (5μm)
  + SuperCharm LC-18
- Mobile phase:
  - ☐ As described in the method
  - ☑ Other: GRADIENT ELUTION (See Table enclosed)
- Flow-rate: 4.2 ml/min
- Injection volume: 20 μl
- Retention time of olaquindox: 7.6 min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample
Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 83.5 %
- Single / duplicate determinations: ☐ single ☑ duplicate
- If duplicate, please give both percentages: 82.7 % and 84.6 %
- Spiking level: 3.0 mg/kg
**Sample Name:** 791-B

**Action Date:** 23/10/2000 21.30.34

**Sample Name:** 791-B

**Acq. Operator:**

**Different Inj Volume from Sequence!**

**Actual Inj Volume:** 20 μl

**Acq. Method:** C:\HPCHEM\1\IZ

**Last changed:** 28/08/2000 16.09.10 bv

**Analysis Method:** C:\HPCHEM\1\IZ

**Last changed:** 04/12/2000 15.32.39

*(modified after loading)*

**External Standard Report**

<table>
<thead>
<tr>
<th>Sorted By</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calib. Data Modified</td>
<td>04/12/2000 15.32.39</td>
</tr>
<tr>
<td>Multiplier</td>
<td>1.0000</td>
</tr>
<tr>
<td>Dilution</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

**Signal 1:** DAD1 A, Sig=365.8 Ref=off (OLACANF1091-B.D)

**Retention Time**

<table>
<thead>
<tr>
<th>Type</th>
<th>Area</th>
<th>Amount</th>
<th>Group</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>mAU's</td>
<td>[ng inj.]</td>
<td></td>
<td>OLAQ-olaquindox</td>
</tr>
<tr>
<td>7.658</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00000</td>
</tr>
</tbody>
</table>

**Totals:**

Results obtained with enhanced integrator!

1 Warnings or Errors:

Warning: Calibrated compound(s) not found

---

Page 1 of 2
External Standard Report

Reported By:  
Calib. Data Modified: 24/10/2000 13.49.03
Multiplier: 1.0000
Dilution: 1.0000

Signal 1: DAD1 A, Sig=365.8 Ref=off

<table>
<thead>
<tr>
<th>RetTime</th>
<th>Type</th>
<th>Area</th>
<th>Amt/Area</th>
<th>[ng inj.]</th>
<th>Amount</th>
<th>Grp</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.655</td>
<td>MM</td>
<td>126.16067</td>
<td>4.26466e-1</td>
<td>53.80323</td>
<td>53.80323</td>
<td>OLAQ-olaquindox</td>
<td></td>
</tr>
</tbody>
</table>

Totals: 53.80323

Results obtained with enhanced integrator!

Visible 24/10/2000 14.40.13
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 23
Canfas

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

subtitle: Task 4 Collaborative Study

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

Date of analysis:  

Analyte: Olaquindox

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>235694</td>
<td>4.23</td>
<td>5.77</td>
</tr>
<tr>
<td>235699</td>
<td>5.40</td>
<td>5.37</td>
</tr>
<tr>
<td>235712</td>
<td>1.67</td>
<td>1.76</td>
</tr>
<tr>
<td>235767</td>
<td>&lt; 0.11</td>
<td>&lt; 0.21</td>
</tr>
<tr>
<td>235787</td>
<td>1.91</td>
<td>1.86</td>
</tr>
<tr>
<td>235793</td>
<td>&lt; 0.11</td>
<td>&lt; 0.21</td>
</tr>
</tbody>
</table>
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 24
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: e-mail:
Contact person: fax:
Date of analysis: telephone:

Analyte: OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>245723</td>
<td>2,0</td>
<td>2,1</td>
</tr>
<tr>
<td>245746</td>
<td>5,7</td>
<td>6,2</td>
</tr>
<tr>
<td>245780</td>
<td>blank</td>
<td>blank</td>
</tr>
<tr>
<td>245781</td>
<td>blank</td>
<td>blank</td>
</tr>
<tr>
<td>245825</td>
<td>1,8</td>
<td>1,7</td>
</tr>
<tr>
<td>245829</td>
<td>6,0</td>
<td>5,9</td>
</tr>
</tbody>
</table>
CANSAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

Date(s) of analysis: 26 October 2000

Chromatographic conditions:
- Column:
  - ☐ As described in the method
  - ☐ Other: 250 mm x 4.6 mm C18 5 µm
- Mobile phase:
  - ☐ As described in the method
  - ☐ Other: ...........................
- Flow-rate: 1.5 ml/min
- Injection volume: 50 µl
- Retention time of olaquindox: 8.2 min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 7.9 % (average)
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: 7.1 % and 8.6 %
- Spiking level: 3 mg/kg
Injection Date: 26/10/00 14.12.05
Sample Name: Location: Vial 1
Acq. Operator: Method: C:\HECHEM1\METHODS\IZS_ME-1\OLAQUID.M
Last changed: 26/10/00 13.00.32 by
(modified after loading)

Olaquindox canfas

Normal 0 /a/quindox

External Standard Report

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

Signal 1: DAD1 A, Sig=380,4 Ref=550,100 (CANFAS\OLAQUI18.D)

Retention Time Sig Type Area Amt/Area Amount Grp Name
[min] [mAUs] [ng/ul]
8.189 1 BB 124.84376 2.7446e-2 2.83955 olaquindox

Totals: 2.83955

Results obtained with enhanced integrator!

*** End of Report ***
### External Standard Report

**Sort By:** Retention Time  
**Calib. Data Modified:** 26/10/00 12.09.03  
**Multiplier:** 1.0000  
**Dilution:** 1.00000

**Signal 1:** DAD1 A, Sig=380.4 Ref=550,100

<table>
<thead>
<tr>
<th>RetTime</th>
<th>Sig Type</th>
<th>Area [mV*sec]</th>
<th>Amt/Area</th>
<th>Amount [ng/ul]</th>
<th>Grp Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.338</td>
<td></td>
<td>1</td>
<td></td>
<td>0.00000</td>
<td>olaquindox</td>
</tr>
</tbody>
</table>

**Results obtained with enhanced integrator!**

**Warnings or Errors:**

**Warning:** Calibrated compound(s) not found
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 25
**CANFAS**

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

Subtitle: Task 4 COLLABORATIVE STUDY  
Lab-name:  
Contact person:  
e-mail:  
fax:  
telephone:  
Date of analysis:  

<table>
<thead>
<tr>
<th>Analyte: OLAQUINDOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample code</td>
</tr>
<tr>
<td>255696</td>
</tr>
<tr>
<td>255728</td>
</tr>
<tr>
<td>255742</td>
</tr>
<tr>
<td>255782</td>
</tr>
<tr>
<td>255814</td>
</tr>
<tr>
<td>255819</td>
</tr>
</tbody>
</table>
Date(s) of analysis: 2 - 12 - 2000

Chromatographic conditions:

- Column:
  - ☑ As described in the method
  - ☐ Other:

- Mobile phase:
  - ☐ As described in the method
  - ☑ Other: Water - Methanol 800:200 (v/v)

- Flow rate: ... ml/min
- Injection volume: ... μl
- Retention time of olaquindox: 5.33 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ☑ 4.5 %
- Single / duplicate determinations: ☑ single ☐ duplicate
- If duplicate, please give both percentages: ...... % and ...... %
- Spiking level: ...... mg/kg
Chromatogram for olaquindox study

Absorbance

Feed n° 255696
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 26
**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

**Contact person:**

**Date of analysis:** 7/12/00

**Analyte:**

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>265743</td>
<td>1,6</td>
<td>1,5</td>
</tr>
<tr>
<td>265749</td>
<td>5,1</td>
<td>4,8</td>
</tr>
<tr>
<td>265755</td>
<td>0,1</td>
<td>0,1</td>
</tr>
<tr>
<td>265763</td>
<td>0,7</td>
<td>1,2</td>
</tr>
<tr>
<td>265764</td>
<td>0,1</td>
<td>0,1</td>
</tr>
<tr>
<td>265768</td>
<td>6,0</td>
<td>5,3</td>
</tr>
</tbody>
</table>
Date(s) of analysis: 7.12.00

Chromatographic conditions:

• Column:
  - As described in the method
  - Other: SHERWIN ODS 2 5μm 250mm x 4.6mm

• Mobile phase:
  - As described in the method
  - Other:

• Flow-rate: 1.5 ml/min
• Injection volume: 20 μl
• Retention time of olaquindox: 9.8 min

Chromatograms: Please include representative chromatograms of:

• Blind positive feed samples
• Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

• Percentage recovery: 73 %
• Single/duplicate determinations: single duplicate
• If duplicate, please give both percentages: 75 % and 71 %
• Spiking level: 3 mg/kg
File name: OLAQUIN0812021.CH1

Info:
50A1

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

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>RT</th>
<th>Area [μV.Sec]</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>olaquindox</td>
<td>9.917</td>
<td>303752.400</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Total Area of Peak = 303752.400 [μV.Sec]
File name: OLAQUINO812025.CH1

Info:
51A1

Vial # = 25 Rack # = I
Injection Date: 8-Dec-2000 21:25:02
User:
Group: OLAQUIN
Control Method:

Peak Detection Not Available
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms

of partner 27
Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

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

Date of analysis:

<table>
<thead>
<tr>
<th>Sample code</th>
<th>OLAQUINDOX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unit</td>
</tr>
<tr>
<td>275700</td>
<td></td>
</tr>
<tr>
<td>275720</td>
<td></td>
</tr>
<tr>
<td>275745</td>
<td></td>
</tr>
<tr>
<td>275773</td>
<td></td>
</tr>
<tr>
<td>275805</td>
<td></td>
</tr>
<tr>
<td>275830</td>
<td></td>
</tr>
</tbody>
</table>
Date(s) of analysis: 41st week 2000

Chromatographic conditions:
- Column:
  - ✓ As described in the method
  - □ Other: ..........................................................
- Mobile phase:
  - ✓ As described in the method
  - □ Other: ..........................................................
- Flow-rate: 0.8 - 1.1 ml/min, depending on the back pressure of the column
- Injection volume: 50.0 µl, for code number 275773: 100 µl
- Retention time of olaquindox: 10.4 min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample
Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 75.7%
- Single / duplicate determinations: ✓ single □ duplicate
- If duplicate, please give both percentages: ...... % and ...... %
- Spiking level: ......3 mg/kg
<table>
<thead>
<tr>
<th>#</th>
<th>Retention (min)</th>
<th>Type</th>
<th>Area (eV*sec)</th>
<th>Height (eV)</th>
<th>Int Type</th>
<th>Start Time (min)</th>
<th>End Time (min)</th>
<th>Baseline Start (min)</th>
<th>Baseline End (min)</th>
<th>Slope</th>
<th>Offset</th>
<th>% Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.317</td>
<td>Unknown</td>
<td>255710</td>
<td>7983</td>
<td>MM</td>
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<td>9.683</td>
<td>11.600</td>
<td>-0.335826</td>
<td>6.186183</td>
<td>100.00</td>
</tr>
</tbody>
</table>

---

**Diagram:**

- **Code nr.:** 275700
- **Date:** 11.0. OKT. 2000
- **Compound:** Olaquindox

---

**Sample Name:** OLA la Vial
**Injection:** 6
**Channel:** SATIN
**Type:** Unknown
**Page:** 1 of 1
Concerning Olaquindox

Code nr. 275720 (= blank)

RT for olaquindox
Sample Name: OLA 3 a Vial: 6 Inj: 1 Ch: SATIN Type: Unknown

code: 275745

Olaquindox
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 29
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:

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>295704</td>
<td>3.8</td>
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<tr>
<td>295721</td>
<td>3.8</td>
<td>3.7</td>
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<tr>
<td>295732</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>295756</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>295762</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>295786</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>
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: .......................... ml/min
- Injection volume: .............. μl
- Retention time of olaquindox: .......................... min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

  Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ........ %
- Single / duplicate determinations: □ single □ duplicate
- If duplicate, please give both percentages: ........................ % and ........................ %
- Spiking level: ........ mg/kg
Sample Name: olaquindox 7211
Sample Type: Unknown
Vial: 32
Injection #: 1
Injection Volume: 100.00 µl
Run Time: 15.0 Minutes
Sample Set Name: OLAQUINDOX

Acquired By: System
Date Acquired: 16-11-2000 15:34:13
Acq. Method Set: Olaquindox
Date Processed: 18-11-2000 18:03:28
Processing Method: 'olaquindox 18 11 00
Proc. Chnl. Descr.: PDA 380.0 nm

Cromatogram

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.973</td>
<td>305140</td>
<td>8855</td>
<td>1.947</td>
<td>µg/ml</td>
</tr>
</tbody>
</table>

\[(3.8 \text{ mg l}^{-1})\]
Sample Name: olaquindox 732 I/II
Sample Type: Unknown
Vial: 34
Injection #: 1
Injection Volume: 100,00 ul
Run Time: 15.0 Minutes
Sample Set Name: olaquindox 2

Acquired By: System
Date Acquired: 16-11-2000 18:01:25
Acq. Method Set: Olaquindox
Date Processed: 16-11-2000 18:03:28
Processing Method: olaquindox 18 11 00
Proc. Chnl. Descr.: PDA 390.0 nm

Cromatogram

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.137</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Report Method: Olaquindox
Printed 11:20:42 05-12-2000
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 31
#### CANFAS

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

**Subtitle:** Task 4 COLLABORATIVE STUDY

**Lab-name:**

**Contact person:**

**e-mail:**

**fax:**

**telephone:**

**Date of analysis:**

**Analyte:** OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Unit (mg/kg)</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>315707</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>315709</td>
<td></td>
<td>6.28</td>
<td>6.13</td>
</tr>
<tr>
<td>315710</td>
<td></td>
<td>1.88</td>
<td>1.90</td>
</tr>
<tr>
<td>315794</td>
<td></td>
<td>6.05</td>
<td>5.86</td>
</tr>
<tr>
<td>315801</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>315811</td>
<td></td>
<td>2.08</td>
<td>1.80</td>
</tr>
</tbody>
</table>
Date(s) of analysis: 6 - 11, 2000

Chromatographic conditions:

- Column:
  - ☑ As described in the method
  - □ Other: .................................................................

- Mobile phase:
  - ☑ As described in the method
  - □ Other: .................................................................

- Flow-rate: 1.5 ml/min
- Injection volume: 10 µl
- Retention time of olaquindox: 7.2 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ...... %
- Single / duplicate determinations: □ single ☑ duplicate
- If duplicate, please give both percentages: 40. % and 42. %
- Spiking level: 3. .... mg/kg
No peaks available to report.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>7,353</td>
<td>176368</td>
<td>8792</td>
<td>176368</td>
<td>100,00</td>
<td>1,000000</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>176368</td>
<td>8792</td>
<td>176368</td>
<td>100,00</td>
<td>1,000000</td>
</tr>
</tbody>
</table>
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)

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>325705</td>
<td>5,91</td>
<td>5,92</td>
</tr>
<tr>
<td>325716</td>
<td>2,06</td>
<td>2,24</td>
</tr>
<tr>
<td>325744</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>325747</td>
<td>5,63</td>
<td>5,71</td>
</tr>
<tr>
<td>325798</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>325807</td>
<td>2,07</td>
<td>2,03</td>
</tr>
</tbody>
</table>
Annex 4 - Questionnaire

Chromatographic conditions:

- Column:
  - ☐ As described in the method
  - ☑ Other: Waters Symmetry, C18, 5 μm, 4.6mmX250mm (Part Nº WAT 054215)
- Mobile phase:
  - ☐ As described in the method
  - ☑ Other: ..............................................................
- Flow-rate: 1.4 ml/min
- Injection volume: 20 (μL)
- Retention time of olaquindox: 7.05 min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

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

**Date of analysis:**

**Analyze:**

### OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Unit</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>335702</td>
<td></td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>335706</td>
<td></td>
<td>2,3</td>
<td>2,4</td>
</tr>
<tr>
<td>335753</td>
<td></td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>335759</td>
<td></td>
<td>7,4</td>
<td>6,9</td>
</tr>
<tr>
<td>335772</td>
<td></td>
<td>7,2</td>
<td>7,2</td>
</tr>
<tr>
<td>335804</td>
<td></td>
<td>2,5</td>
<td>2,5</td>
</tr>
</tbody>
</table>
CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

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

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

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: ................................ %
- Single/duplicate determinations: □ single □ duplicate
- If duplicate, please give both percentages: ................................ % and ................................ %
- Spiking level: .................................. mg/kg
Sample Set Name OLA18
User Name RVSA

Current Date 18/10/00
Current Time 02:29:43

Auto-Scaled Chromatogram

Peak Results

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLA</td>
<td>2.683</td>
<td>126858</td>
<td>15667</td>
<td>7.239</td>
<td>mg/kg</td>
</tr>
</tbody>
</table>
Sample Set Name OLA18
User Name RVSA
Current Date 18/10/00
Current Time 02:29:38

Auto-Scaled Chromatogram

Peak Results

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLA</td>
<td>2.744</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sample Set Name OLA18
User Name RVSA
Current Date 18/10/00
Current Time 02:29:34

Auto-Scaled Chromatogram

Peak Results

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLA</td>
<td>2,685</td>
<td>88628</td>
<td>10323</td>
<td>2,495</td>
<td>mg/kg</td>
</tr>
</tbody>
</table>
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:**

**Lab-name:**

**Contact person:**

**Date of analysis:**

**Analyte:**

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>345698</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>345717</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>345750</td>
<td>4.7</td>
<td>4.8</td>
</tr>
<tr>
<td>345752</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>345778</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>345826</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
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: ................................ ml/min
- Injection volume: .................. μl
- Retention time of olaquindox: .......... min

Chromatograms: Please include representative chromatograms of:

- Blind positive feed samples
- Blind blank feed sample

Please indicate the olaquindox peak with an arrow

Recovery results:

- Percentage recovery: ........ %
- Single / duplicate determinations: 0 single 0 duplicate
- If duplicate, please give both percentages: ........ % and ........ %
- Spiking level: ........ mg/kg
Channel 2
KromaSystem 2000 Version 1.83 RESULT REPORT: INTEGRATION

SVS2 - OLAQ025.SHP: Olaquindox-
No. 07: C1908" 25g/50ml
Channel 2: DETECT 332
No Text

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

<table>
<thead>
<tr>
<th>No. PNo Ret. Time Type min</th>
<th>Name</th>
<th>Area mV*min</th>
<th>Amount</th>
<th>Rel. Ar %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00000e+000 0.00000e+000 0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample Report Page 1 of 1 Printed: 15.11.00 11:06
Channel 2

<table>
<thead>
<tr>
<th>No.</th>
<th>PNo</th>
<th>Ret. Time</th>
<th>Type</th>
<th>Name</th>
<th>Area (mV*min)</th>
<th>Amount (µg/g)</th>
<th>Rel. A.r.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>7.83</td>
<td>MOD</td>
<td>olaquindox</td>
<td>5.7859e+000</td>
<td>4.7822e+000</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Sample Report

Page 1 of 1

Printed: 15.11.00 11:07
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 37
CIA quindox in Feed
Mode: Reprocessed Data
Original Results: CATSPS/ SYSTEM\Data\ola011200AM.RES
Reprocessed Results: CATSPS/ SYSTEM\Data\ola011200AM.RMS

Analysis Report

Name: R
Description: Sample
Injection Volume: 50.0 μL

Injection:
Injection: 1 of 1
Injection On: 02-12-00 02:34:50

Acquisition Log
Column Pressure (PSI): 2580
Column Temperature (C): N/A
Noise (microAU): 1e+02
Drift (microAU/min): 0.00002
Run-Time Messages: None

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

mV or microAU

Component
CIA quindox

RT(min)
10.429

Area
260545
7062

Height

ug/ml
1.7086

Peak Type
Modified

Component: CIA quindox

Report Method: CATSPS/Methods/olaa.RPM

System: Reprocess
Analyzer: AM

PC1000 Ver 3.5.1
29-11-00 18:31:34
U4-1K-UU 4U1:40-49

04-12-00 18:10:48
09-12-00 12:17:59
Analysis Report

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

Injection: 1 of 1
Injected On: 02-12-00 03:40:08

Acquisition Log
Column Pressure (PSI): 2575
Column Temperature (C): N/A
Drift (microAU/min): -d*+fl?
Pump Flow Stability: 2.2
Run-Time Messages: None

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

Component
RT(min) | Area  | Height | ugm/ml | Peak Type
---------|-------|--------|--------|----------
Tota ls

Typical chromatograms for Olaquindox

Analyst: AM

System: Repromax
Acquisition Method: C:\TSPSYSTE M1\Method\olaquindox.ACM
Calculation Method: C:\\TSPMethod\olaq.CAM
Report Method: C:\TSP\Method\olaq.RPM

PCI1000 Ver 3.3.1
29-11-00 18:31:34
06-12-00 18:10:48
08-12-00 12:25/29
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:**

**Date of analysis:**

**Analyte:**

**OLAQUINDOX**

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>385726</td>
<td>6,00</td>
<td>5,52</td>
</tr>
<tr>
<td>385737</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>385783</td>
<td>1,72</td>
<td>1,76</td>
</tr>
<tr>
<td>385789</td>
<td>2,01</td>
<td>1,94</td>
</tr>
<tr>
<td>385803</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>385815</td>
<td>5,53</td>
<td>5,38</td>
</tr>
</tbody>
</table>
CANFAS COLLABORATIVE STUDIES - OLAQUINDOX

Annex 4 – Questionnaire

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

Chromatographic conditions:

• Column:
  □ As described in the method
  X Other: Hypersil ODS C-18, 250 x 4,6 mm, 5 μm

• Mobile phase:
  X As described in the method
  □ Other:

• Flow-rate: 1 ml/min
• Injection volume: 20 μl
• Retention time of Carbadox: 6 min

Chromatograms: Please include representative chromatograms of:

• Blind positive feed samples
• Blind blank samples
Please indicate the olaquindox peak with an arrow

Recovery results:

• Percentage recovery: 81%
• Single / duplicate determinations: X single □ duplicate
• If duplicate, please give both percentages: ...% and ...%
• Speaking level: 2 mg/kg
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 particular instruction) because we have not it.

Chromatograms for standard (3 ppm), sample (385726) and blank
APPENDIX 6

Table with results, questionnaire (page 1) and chromatograms of partner 40
Development and Validation of HPLC-methods for the official control of Coccidiostats and Antibiotics used as Feed Additives (SMT4-CT98-2216)

Subtitle: Task 4 COLLABORATIVE STUDY
Lab-name:  
Contact person:  
e-mail:  
fax:  
telephone:  
Date of analysis:  
Analyte: OLAQUINDOX

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Result 1 (mg/kg)</th>
<th>Result 2 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>405697</td>
<td>blanco</td>
<td>blanco</td>
</tr>
<tr>
<td>405701</td>
<td>blanco</td>
<td>blanco</td>
</tr>
<tr>
<td>405734</td>
<td>1,50</td>
<td>1,80</td>
</tr>
<tr>
<td>405779</td>
<td>4,75</td>
<td>4,56</td>
</tr>
<tr>
<td>405799</td>
<td>2,21</td>
<td>1,62</td>
</tr>
<tr>
<td>405800</td>
<td>5,20</td>
<td>5,74</td>
</tr>
</tbody>
</table>
Annex 4 - Questionnaire

Date(s) of analysis: 8-14 November 1999

Chromatographic conditions:
- Column:
  - ☐ As described in the method
  - ☐ Other: C18 spherical, 5 μm, 3.9 x 15 cm, Waters
- Mobile phase:
  - ☐ As described in the method
  - ☐ Other:
- Flow rate: __________ ml/min
- Injection volume: __________ μl
- Retention time of olaquindox: __________ min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample
Please indicate the olaquindox peak with an arrow.

Recovery results:
- Percentage recovery: __________ %
- Single / duplicate determinations: ☐ single ☐ duplicate
- If duplicate, please give both percentages: __________ % and __________ %
- Spiking level: __________ mg/kg
APPENDIX 7

Result of special requests

of

Masterlab, Putten, The Netherlands
CANFAS COLLABORATIVE STUDIES OCTOBER 2000 - OLAQUINDOX

Annex 4 - Questionnaire

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

Chromatographic conditions:
- Column:
  - ■ As described in the method
  - □ Other: .................................................................
- Mobile phase:
  - ■ As described in the method
  - □ Other: .................................................................
- Flow rate: 1.5........... ml/min
- Injection volume: 20........μl
- Retention time of olaquindox: 6.3.. min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample
Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 89.8%
- Single / duplicate determinations: □ single ■ duplicate
- If duplicate, please give both percentages: 97.7% and 91.9%
- Spiking level: ...... mg/kg
Olaquindox

Instrument: UV_5
Method: vol1\DATA\Elle_Admin\Projects\Olaquindox\Method\Olaquindox.met
File: vol1\DATA\Elle_Admin\Projects\Olaquindox\Dextro\Olaquindox_011226_015
Sequence: vol1\DATA\Elle_Admin\Projects\Olaquindox\Sequence\Olaquindox.seq

User: asc
Run time: 12-01-2000 18:52:21
Input: 24.9698
Dilution: 100

Sequence: vol1\DATA\Elle_Admin\Projects\Olaquindox\Sequence\Olaquindox.seq

Blind positive feed sample

UV-Detector

Results

<table>
<thead>
<tr>
<th>Pk #</th>
<th>Retention Time</th>
<th>Area</th>
<th>Height</th>
<th>ESTD concentration</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.37</td>
<td>30387</td>
<td>1294</td>
<td>4.46817</td>
<td>mg/kg</td>
</tr>
</tbody>
</table>
Olaquindox

Monster: 1 450556

Instrument: UV_5
Method: Method/Olaquindox
File: File_Adm!/DATA\File_Adm!/Project/Olaquindox\Sequence/Olaquindox.seq

Gebruiker: asc
Runtijd: 12-01-2000 17:34:56
Inweg: 25.4734
Verdunning: 100

Area [pk # Retention Time Area Height ESTD concentration Units]

<table>
<thead>
<tr>
<th>Olaquindox</th>
<th>Retention Time</th>
<th>Area</th>
<th>Height</th>
<th>ESTD concentration mg/kg</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00000 BDL</td>
<td>mg/kg</td>
</tr>
</tbody>
</table>
APPENDIX 7

Result of special requests

of

National Veterinary Institute, Uppsala, Sweden
Date(s) of analysis: 00.01.17./00.11.20

Chromatographic conditions:
- Column:
  - As described in the method
  - Other: Hypersil C18 0DS BPS 250 x 4.6 mm
- Mobile phase:
  - As described in the method
  - Other: 
- Flow rate: 1.3 ml/min
- Injection volume: 50 µl
- Retention time of olaquindox: 7.18 min

Chromatograms: Please include representative chromatograms of:
- Blind positive feed samples
- Blind blank feed sample
Please indicate the olaquindox peak with an arrow

Recovery results:
- Percentage recovery: 96.9 %
- Single / duplicate determinations: single
- If duplicate, please give both percentages: 95.7 % and 96.9 %
- Spiking level: 3.5 mg/kg
**EXTERNAL STANDARD TABLE**

| PEAKS NOT FOUND IN THIS RUN | REFERENCE PEAK | 7.77 | bayo |

Areas, times, and heights stored in: D:AYO051.ATB
Data File = D:AYO051.PTS Printed on 11-20-2000 at 09:27:12
Start time: 0.00 min. Stop time: 15.00 min. Offset: 0 min.
Low Value: 0 uv High Value: 133401 uv Scale factor: 5.0

---

**Sample Name:** prov nr 1  
**Method:** D:AYO051  
**Date:** 11-19-2000 15:09:24  
**Operator:** annChannel#: 0  
**Interface:** 0  
**Cycle#: 1**  
**Threshold:** 1  
**Starting Peak Width:** 15  
**Ending Retention Time:** 15.00  
**Amount Injected:** 50.00  
**Sample Weight:** 1.000000  
**Dilution Factor:** 1.00

**TOTAL AMOUNT = 0.0000**
***** EXTERNAL STANDARD TABLE *****

* Sample Name: prov nr 5 *
* Data File: D:BAYO047 *
* Date: 11-19-2000 14:12:14 *
* Interface: 0 Cycle#: 1 Operator ann Channel#: 0 Vial#: *
* Starting Peak Width: 15 Threshold: 1 Area Threshold: 200 *

Starting Delay: 0.00  
Ending retention time: 15.00
Area reject: 200  
One sample per 0.200 sec.
Amount injected: 50.00  
Dilution factor: 1.00
Sample Weight: 1.00000

---

<table>
<thead>
<tr>
<th>NUM</th>
<th>TIME</th>
<th>NAME</th>
<th>CONC</th>
<th>NORMALIZED</th>
<th>AREA</th>
<th>REF</th>
<th>% DELTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.774</td>
<td>bayo</td>
<td>0.4249</td>
<td>100.0000%</td>
<td>28933</td>
<td>1689</td>
<td>17.11</td>
</tr>
</tbody>
</table>

TOTAL AMOUNT = 0.4249

---

Areas, times, and heights stored in: D:BAYO047.ATB
File = D:BAYO047.PTS  Printed on 12-05-2000 at 14:00:32
Start time: 0.00 min. Stop time: 15.00 min. Offset: 0 mv.
Low Value: 0 uv High Value: 47888 uv Scale factor: 5.0
Chromatograms of: M.

84%