



# Validation of an analytical method for the determination of Carbadox and Olaquinox in feedingstuffs by Liquid Chromatography coupled to UV and/or DAD

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**Validation of an analytical method for the determination of carbadox and olaquinox in feedingstuffs by liquid chromatography coupled to UV and/or diode array detector**

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## Abstract

The method performance characteristics of an analytical method based on high performance liquid chromatography (HPLC) for the detection of the banned growth promoters carbadox and olaquinox in feedingstuffs were determined through a collaborative study. The relative standard deviation for repeatability ( $RSD_r$ ) ranged from 1.1 % to 5.5 % for carbadox and from 2.5 % to 6.2 % for olaquinox. The relative standard deviation for reproducibility ( $RSD_R$ ) varied between 6.4 % and 10.7 % for carbadox and between 12.8 % and 20.0 % for olaquinox. The HORRAT values were in all cases equal or below the critical value of 1.5. Moreover the trueness in all cases was between the acceptance limits of 80 % and 110 %. Consequently it was concluded that the method is suitable as a quantitative method. When the more specific diode array detector was coupled to the HPLC instrument, the method was also qualitatively examined in terms of correct identification of the target analyte by close examination of the UV spectrum which was obtained. In all cases the percentage rate of correct identification was equal or above 94% for olaquinox and carbadox. Corresponding to this result the percentage rate of false negative result was equal or below 6 % suggesting therefore the extension of the HPLC method from quantitative to confirmatory status when a diode array detector is used.

## Keywords

HPLC-UV, HPLC- Diode Array Detector, validation study, growth promoters, feedingstuffs, olaquinox, carbadox

## Introduction

Following the European ban of some antibiotics (European Communities 1998a) and growth promoters (European Communities 1998b) as feed additives, the European funded research project SIMBAG-FEED (de Jong *et al.* 2006) was launched with the main objective of developing and validating analytical methods for the official control of five antibiotics (spiramycin, tylosin, virginiamycin, avoparcin, zinc bacitracin) and two growth promoters (carbadox and olaquinox). For the simultaneous detection of the two banned growth promoters carbadox and olaquinox a method based on high performance liquid chromatography coupled to spectrophotometric ultraviolet or diode array detection (HPLC-UV or HPLC- Diode Array Detector (DAD)) was developed and subjected to validation by conducting a collaborative study. The minimum target concentrations to be included in the study were set at 4 mg kg<sup>-1</sup> for carbadox and 3 mg kg<sup>-1</sup> for olaquinox, which were 5 times lower than the lowest

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contents formerly fixed by European legislation. A successful validation of this method was envisaged as improving the capability of official feed laboratories to enforce the ban of the target growth parameters, since the former European official methods are only single analyte methods and have not been validated at low concentration levels as included in the present study (European Communities, 1998c; European Communities, 1999)

In this paper, we report on the results of the collaborative study to validate a HPLC method for the detection of carbadox and olaquinox in feed. The purpose of the study was two-fold:-

- a) to test the suitability of the method as a quantitative method, indicating the concentration of these substances in the samples. The lower range of the study was set at 4 mg kg<sup>-1</sup> for carbadox and 3 mg kg<sup>-1</sup> for olaquinox.,
- b) to test the suitability of the method for confirmatory analysis by using information of the full UV spectrum when the measurement has been performed with a diode array detection, especially in cases with limited availability of mass spectrometry instrumentation.

**Methods and materials**

*Design of the study*

The laboratories followed a step-by-step approach involving first a training period on the method through audiovisual material, namely a CD-ROM with the method description and a presentation of the method by the means of media show. In addition the laboratories had to analyse known and blind test samples, reporting the results on an electronic spreadsheet with a feedback questionnaire. This was a specific exercise for the candidate laboratories designed to get them familiar with the method protocol, to evaluate their effective performances on a limited number of samples and to check their rigorous application of the protocol. In total 22 laboratories were selected from the training period to participate in the collaborative study. Out of these 22 laboratories 21 delivered results. In addition, the information from the participants to the training period was utilised to adjust the method protocol in order to facilitate the correct application of the method.

A detailed and revised method protocol, 20 samples to be analysed and electronic forms to report the results of the analyses, the DAD data and to specify details of the execution of the method were sent to

each participant to the collaborative study. In this study the emphasis was placed on two aspects, which were

- a) quantitative analysis, in which the laboratories were asked to report on the content of the carbadox and olaquinox by expressing the response in  $\text{mg kg}^{-1}$  and
- b) the identification of the analytes based on the DAD data.

### Statistics

The design of the study and the target performance characteristics for the evaluation of the LC-UV method were selected according to internationally accepted guidelines for method validation (Horwitz, 1995). The quantitative results submitted by the laboratories were used to estimate average concentrations and standard deviations under repeatability and reproducibility conditions.

Extreme values were not included in the statistical evaluation for different reasons, especially when they were related to obvious experimental deviations from the protocol. Furthermore, as indicated by the IUPAC protocol (Horwitz 1995) the initial reported valid data must be purged of all outliers flagged by the harmonised outlier removal procedure. This procedure basically consists of the sequential application of the Cochran and Grubbs tests (at 2.5% probability level, 1 tail for Cochran and 2 tails for Grubbs) until no further outliers are flagged or until a drop of 22.2% ( $= 2/9$ ) in the original number of laboratories providing valid data would occur.

The precision data obtained from the collaborative study were then compared with predicted acceptable levels of precision. These levels, as estimated by the Horwitz equation (Horwitz *et al.* 1980), provide an indication as to whether the method is sufficiently precise for the concentration level of the analyte being measured. This indication is expressed by the HORRAT value (Horwitz and Albert 2006). For the between-laboratories reproducibility, the HORRAT value is a comparison of the measured reproducibility precision with the precision calculated by the Horwitz equation for a method measuring at that particular concentration level of analyte:

$$\text{HORRAT} = \frac{RSD_R}{RSD_{R, \text{Horwitz}}}$$

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where  $RSD_R$  is the relative standard deviation of reproducibility obtained in the validation exercise and  $RSD_{R,Horwitz}$  is the calculated standard deviation of reproducibility using the Horwitz equation:

$$RSD_{RHorwitz} = 2^{(1-0.5\log C)}$$

where C is the mass concentration expressed in power of 10 (i.e. 1 mg/g =  $10^{-3}$ ). The target value for the HORRAT value is set at 1, the acceptable limit is 1.5 and the rejection level is 2.

*Test materials*

In this study 10 different materials were utilised as shown in Table 1. The table also indicates the presence and concentration of more banned feed additives that were not measured with the method of this study but with other methods developed within the project SIMBAG-FEED.

(please insert  
Table 1 here)

The test materials were compound feedingstuffs for cattle, pig, poultry and calf containing typical ingredients using a realistic recipe. The banned feed additives were then mixed to the feedingstuffs in two steps. First, the target analytes were added individually to soybean meal to obtain different pre-mixtures each of them containing one of the target analytes at a relatively high concentration, viz 360 mg/kg for carbadox and olaquinox. In a second step the pre-mixtures fortified with the target analytes were mixed with the other ingredients to obtain the final test materials (Kramer *et al.* 2004). The final feedingstuff samples were checked for sufficient homogeneity applying the procedure described elsewhere (Thompson and Wood, 1993). An additional criterion was that if a test material was proven homogeneous for one compound, it would also be for the other compounds. This was possible since the preparation of the samples was identical for all substances and since the suitability of the preparation procedure was confirmed by conducting a pre-study in which two test material were prepared and analysed for all compounds (Vincent *et al.* 2007). In the same study also suitable storage conditions of the test samples were established by conducting stability experiments of the target analytes. The objective of this study was to assure that the concentration of these analytes was stable within the time

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3 frame of the period of the validation study. For the final homogeneity study 10 randomly selected  
4 samples from each material were analysed in duplicates for specific analytes (Table 2) and the results  
5 were subjected to analysis of variance (ANOVA), in order to assess the "between-bottle" variation and  
6 the "within-bottle" variation. Significant differences of both values, indicating possible heterogeneity  
7 were evaluated by using the F-test. More details regarding the homogeneity and the stability study are  
8 given by Vincent *et al.* (2007).  
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15 The results of the homogeneity study are shown in Table 2. The materials 1, 2, 3, 5, 6, 7, 8, 10 were  
16 considered homogeneous, since the calculated F-value was below the critical F-value (F critic), which  
17 was in this exercise 3.02. In contrast, the F-values of materials 4 and 9 were above F critic. However,  
18 also these materials passed the test of sufficient homogeneity since the sampling error representing  
19 possible heterogeneity and which was calculated from the between and within-bottle variation was for  
20 both materials *below* 50% of the target standard deviation of the analytical methods as defined by the  
21 Horwitz equation (Thompson and Wood, 1993). This means that possible heterogeneity of the  
22 materials 4 and 9 as indicated by the F-test did not interfere with the purpose of the collaborative study.  
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32 (Please insert Table 2 here)  
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### 35 *Method*

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39 The two growth promoters are extracted from the feedingstuff sample with a mixture of methanol and  
40 water (1:1 v/v). The extract is or filtered, then purified through a short open glass aluminium oxide  
41 column or by an alumina Solid Phase Extraction (SPE). The final extract is filtered on membrane  
42 syringe filter and analysed by C8 reversed phase HPLC with UV detection at a wavelength of 375 nm.  
43 Alternatively, for confirmation purposes, a DAD, applying a sample wavelength of 375 nm and a  
44 spectrum range of 220 nm to 400 nm can be used.  
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51 Applying the selected isocratic liquid chromatography conditions allows for a simultaneous  
52 determination of olaquinox and carbadox eluting at around 4-6 min and 18-22 min, respectively. A  
53 detailed method description was published by Genouel (2004).  
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The presence of furazolidon can interfere with the determination of carbadox. When using a DAD, many other veterinary drugs or feed additives can be detected such as ronidazol, meticlorpindol, nitrofurazon, dimetridazol, furaltadon, and sulphonamides, without interfering with the determination of the target analytes of this study.

**Results and discussion**

*Training phase*

In the training phase the participants were asked to determine the growth promoters carbadox and olaquinox in the training samples, including blind samples of MAT 2 and MAT 7. 22 out of 28 invited laboratories in the training phase reported results. For carbadox, no particular problem was highlighted. For olaquinox, four laboratories were identified as presenting diverging results and were asked for further clarification. The information obtained from the laboratories revealed that they deviated at various places from the protocol. The method protocol was therefore amended in order to clarify the identified potential sources of misinterpretation. In general, no severe problems regarding the implementation and application of the method protocol were reported to the organiser of the study. All laboratories that delivered results were selected to participate in the collaborative study.

*Collaborative study*

In total 21 laboratories delivered results. In detail, carbadox was measured in 7 materials and olaquinox in 6 materials as shown in Table 1. However, after checking the rigorous application of the protocol in the collaborative study, only 19 were considered valid as two laboratories deviated from the protocol. Indeed, two laboratories used gradient elution which was not foreseen in the method protocol. The results from another laboratory were excluded because of evident mismatch of samples. 11 out of the remaining 18 laboratories utilised a DAD, whereas 7 laboratories applied a UV detector. Figure 1 shows the chromatogram and the corresponding DAD spectra submitted by one of the participating laboratories.

Other results were further excluded as outliers after Cochran and Grubbs tests as highlighted in Table 3 and Table 4. For both target analytes not more than 22.2 % of the laboratories were identified as outliers. The reported results and the indication of the outliers are presented in Table 3 and Table 4.

(Please insert [Table 3](#) and [Table 4](#) here)

The values for the relative standard deviation for repeatability ( $RDS_r$ ) and relative standard deviation for reproducibility ( $RDS_R$ ) per test material and per compound are outlined for carbadox and olaquinox in [Table 5](#) and [Table 6](#), respectively.

(Please insert [Table 5](#) and [Table 6](#) here).

The  $RDS_r$  varied between 1.1 % and 5.5 % for carbadox and between 2.5 % and 6.2 % for olaquinox. The corresponding  $RDS_R$  ranged from 6.4 % to 10.7 % for carbadox and from 12.7 % to 20 % for olaquinox. Figure 2 depicts the HORRAT values obtained for the precision data of both target analytes showing that they were always below 1.5 except for olaquinox in material 2 (1.53). Since this value was still very close to the first benchmark of 1.5 and far below the second benchmark of 2, the result for olaquinox in material 2 was considered acceptable. From these results it was concluded that the method is fit for the intended purpose.

(Please insert Figure 2 here)

In addition, the results of the study were also utilised to assess the *trueness* of the method, which was possible, since the target values of the analytes in the test materials were known. Therefore we calculated the percentage ratio of the estimated average values obtained in the validation exercise ([Table 5](#) and [Table 6](#)) to the target concentrations of carbadox and olaquinox ([Table 1](#)) as indicator of the trueness of the method. As shown in Figure 3, for all materials the estimated trueness was comprised between the acceptance limits of 80% and 110% as proposed by Commission Decision 657/2002 (European Communities, 2002) for analyte concentrations above 0.01 mg kg<sup>-1</sup>. This supports the conclusion that the method is fit for quantification purposes of the two growth promoters.

(Please insert Figure 3 here)

*Confirmation criteria – Use of DAD*

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The results from 11 laboratories which have applied a DAD were further evaluated to examine, whether the method can also be used for confirmatory purposes. Therefore appropriate criteria were established in a workshop with all participating laboratories against which the suitability of the DAD for confirmatory purposes was evaluated. This was achieved by calculating the correct identification rate of the target analyte by close examination of the obtained UV spectra..

Unfortunately, presently no general confirmation criteria are officially defined for the multi analyte determination of banned growth promoters in *feedingstuffs* by means of LC-DAD. Only information from the two official LC-UV single analyte methods (European Communities, 1998c; European Communities, 1999) for the determination of the target analytes in feed are available, however at higher levels of concentration, using also DAD for confirmatory aspects. Therefore, four confirmation criteria were defined and described in the method protocol, which were set by analogy with the ones described for specific *food matrices* (European Communities, 2002) and reported in single official methods for coccidiostats and/or growth promoters determination in feeds such as diclazuril (European Communities, 1999) or halofuginone (European Communities, 1993). In detail, the following criteria were applied:

- a) The retention time of the target analytes in the *test samples* shall be equal within a range of  $\pm 5$  % of the retention time of the target analytes in the *standard solution*.
- b) The purity of the peak of the target analytes in the *test sample* is evaluated by comparing baseline corrected UV spectra (difference spectra) measured at the apex, the up-slope and the down-slope inflection points of the peak. For those parts of the spectra with a relative absorption of at least 10 % related to the wavelength with the highest absorption, at each wavelength the relative absorption shall be equal for all spectra. Two different hypothetical criteria were applied which were (1) an acceptable relative deviation of  $\pm 15\%$  and (2) an acceptable relative deviation of  $\pm 25\%$ .

Sufficient agreement of the difference spectra of the target analytes in the *test sample* and the *standard* both measured at the apex of the corresponding peaks were evaluated by comparing those parts of the spectra with a relative absorption of at least 10 % related to the wavelength with the highest absorption. Sufficient agreement between both spectra were confirmed when the following criteria were met:

- c) at each wavelength the relative absorption shall be equal for spectrum from the standard and the test sample. Two different hypothetical criteria were applied which were (1) an acceptable relative deviation of  $\pm 15\%$  and (2) an acceptable relative deviation of  $\pm 25\%$
- d) the same maxima are present within a wavelength margin of 4 nm.

The criterion of 15 % regarding the acceptable deviation was taken from the corresponding single analyte official methods (European Communities, 1998c; European Communities, 1999).

Figure 4 and Figure 5 summarise the percentage rate of correct identification obtained for the above mentioned criteria. For the criteria b) and c) the results for both acceptability options ( $\pm 15\%$  and  $\pm 25\%$ ) are shown. The statistical treatment has been carried out with all matrices separately and no major influence of the matrix nature (poultry, cattle or pig) could be identified. Therefore, the results from all materials were pooled, regardless of the specific matrix or analyte concentration, but the statistical assessment was done individually for carbadox and olaquinox. However, as for another collaboratively studied method within the SIMBAG-FEED which is based on thin layer chromatography (Vincent *et al.* 2006), the calf feedingstuff has been identified as being a problematic matrix also in this case for the analysis of olaquinox. The results of percentage rate of correct identification obtained for olaquinox are therefore represented with and without inclusion of the calf feedingstuff data.

As can be seen from Figure 4, for the two criteria (a) and (d) the results obtained for the percentage rate of correct identification for carbadox and olaquinox were equal to or above the acceptance limit of 95 %. For the criterion (c) applying the  $\pm 15\%$  acceptable variation, the corresponding method performance characteristics were for carbadox about 87 % and for olaquinox 80%, when including the data from the calf feed or 85 % without the data from the calf feed. However, intrinsically, it is well known in UV absorbance measurements that a slight difference in solvent composition and/or matrix composition could lead to significant differences into the spectra obtained, e.g. a shift at the apex. The criterion (c) in particular could therefore be too strictly defined because not taking into account the specific characteristics of feed analysis (e.g. matrix effects)

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When the decision threshold was set at 25% (Figure 5) for criteria (b) and (c), the situation dramatically improves for carbadox hence ensuring percentage rate of correct identification far above the 95% acceptance limit. For olaquinox, the same method performance characteristic is also increased and is of the order of magnitude of 94% for both criteria b and c. Furthermore, there are no significant differences whether the calf feedingstuff data are included or not. These results demonstrate that the LC-DAD method is suitable for the confirmation of both the presence of carbadox and olaquinox in feedingstuffs. The confirmation criteria were hence refined in the validated method protocol (Genouel 2005).

*Conclusion*

A HPLC method coupled to UV detection for the simultaneous determination of the concentration of the banned growth promoters olaquinox and carbadox has been validated via a collaborative study. The results of the validation experiment confirmed fitness for purpose of the method as a quantitative method, since all HORRAT values were below or at the critical limit of 1.5 and the trueness was in all cases between the acceptance limits of 80 % and 110 %.

When utilising a DAD detector instead of measuring exclusively at *one* wavelength, an additional evaluation demonstrated that the method lends itself also for confirmatory purposes. Based on the result from this study, appropriate decision criteria for the evaluation of the UV spectrum have been proposed. Therefore it can be concluded that the analytical method validated in this study is suitable for official control purposes.

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Table 1. Target concentrations of the analytes in the samples used in the this study (all concentrations are expressed on a gravimetric basis, in mg kg<sup>-1</sup>)

	Mat 1	Mat 2	Mat 3	Mat 4	Mat 5	Mat 6	Mat 7	Mat 8	Mat 9	Mat 10
Feed matrix	Pig	Pig	Poultry	Poultry	Calf	Cattle	Cattle	Calf	Pig	Poultry
Avoparcin		3.1	4.1		3.1	2.0		1.0	5.1	
Bacitracin	3.2		1.9	0.6	1.3	1.9	2.6		6.4	
Spiramycin	0.7	3.6				1.4	2.9	2.2	5.0	7.2
Tylosin			1.1	4.4	3.3	2.2	5.5		11.0	7.7
phoshate										
Virginiamycin	4.0	2.0		1.0			5.0	3.0	10.0	7.0
Olaquinox	10.0	5.0		12.0	3.0		7.0		15.0	
Carbadox		8.0	4.0		6.0	16.0		12.0	10.0	20.0

**Table 2.** Summary of the homogeneity study of the samples used in the SIMBAG-Feed project (all concentrations are expressed on a gravimetric basis in mg kg<sup>-1</sup>). LC-UV: Liquid chromatography coupled to UV detector; LC-MS/MS: Liquid chromatography coupled to a triple quadrupole mass spectrometer. Critical F-value: 3.02, RSD (%): Relative standard deviation of the analytical error measured in the homogeneity exercise

Material	Feed	Compound analysed	Analytical method applied	F-value	Target concentration (mg kg <sup>-1</sup> )	Measured concentration (mg kg <sup>-1</sup> )	RSD (%)
1	Pig	Zinc	LC-MS/MS	1.15	3.2	3.7	4.9
		Bacitracin					
2	Pig	Spiramycin	LC-MS/MS	1.86	3.6	3.8	6.3
3	Poultry	Carbadox	LC-UV	2.60	4.0	4.6	2.5
4	Poultry	Olaquinox	LC-UV	8.90	12.0	12.0	2.0
5	Calf	Olaquinox	LC-UV	1.10	3.0	3.0	2.5
6	Cattle	Spiramycin	LC-MS/MS	0.33	1.4	1.3	8.4
7	Cattle	Spiramycin	LC-MS/MS	1.72	2.9	2.5	4.4
8	Calf	Spiramycin	LC-MS/MS	0.22	2.2	1.8	2.4
9	Pig	Carbadox	LC-UV	8.80	10.0	9.3	1.9
10	Poultry	Carbadox	LC-UV	3.13	20.0	20.3	3.3



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Table 3. Single results form the collaborative study on the determination of *carbadox* in the test samples abbreviated as MAT. The concentrations are given in mg kg<sup>-1</sup> and the composition of the materials is shown in

Table 1. Results identified as outliers (<sup>a</sup> due to the Cochran test and <sup>b</sup> due to the Grubbs test) are given with grey background and were not included in the statistical assessment of the method performance characteristics. n.r.: no result due to an interference from another peak

Lab	MAT 2		MAT 3		MAT 5		MAT 6		MAT 8		MAT 9		MAT 10	
No.	a	b	a	b	a	b	a	b	a	b	a	b	a	b
1	8.3	8.4	4.5 <sup>b</sup>	4.5 <sup>b</sup>	10.6 <sup>b</sup>	10.3 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	16.1 <sup>b</sup>	15.4 <sup>b</sup>	9.6	9.5	19	18.9
5	6.8	6.8	3.3	3.3	4.1	4	13.1	12.9	7.9	9.2	8.3	8.2	17.1	16.8
8	7.5	7.2	3.5	3.5	5.6	5.6	14	14	11	11	8.7	8.6	18	18
10	7.3	7.8	3.6	3.6	5.7	5.7	14.5 <sup>a</sup>	2.6 <sup>a</sup>	11.1	11.4	9.2	8.9	18.4	18.4
12	0 <sup>a</sup>	6.8 <sup>a</sup>	3.4	3.4	4.5	5	13	13.1	9.5	7.3	8.1	8.1	16.1	16.6
14	7.4	7.2	3.5	3.5	5.5	5.6	14.8	14.8	11.7	11.6	9.2	9	19.1	19.1
16	6	6	3 <sup>a</sup>	3.2 <sup>a</sup>	4.7	4.9	12.4	13.3	9.3	9	8.2	7.2	14	15.7
19	7.6	7.5	3.6	3.7	5.8	5.8	14.5	14.4	11	11	9.5	9.3	19.1	19.4
20	7.8	7.7	3.8	3.8	6	6	15.2	15.2	11.3	11.6	10.1	9.8	20.1	20.6
25	8	7.6	3.9	3.8	5.8	5.8	14.5	14	11.3	10.7	9.2	9.1	18.6	18.1
28	7.3	7.4	3.6	3.5	6.1	5.8	15.1	13.8	10.7	11.5	8.4	8.7	17.6 <sup>a</sup>	0 <sup>a</sup>
29	7.3	7	3.6	3.6	5.8	5.7	13.5	13.5	10.6	10.8	8.8	8.6	17.9	17.9
30	8.3	7.8	4.6 <sup>b</sup>	4.2 <sup>b</sup>	7.1 <sup>a</sup>	11.7 <sup>a</sup>	15.4	15.3	17.1 <sup>b</sup>	17.2 <sup>b</sup>	8.4	10.3	19.6	17.5
31	6.9	7.5	3.8	3.8	5.1	5.3	14	13	10	11	8.4	8	17	18
32	7.4	8.1	3.7	3.7	5.6	5.8	14.5	14.5	10.9	11.4	9.2	8.9	18	18.6
33	6.1	6	3	3	7 <sup>a</sup>	8.1 <sup>a</sup>	11.8	11.9	10.6	10.5	7.4	7.2	15.3	15.6
37	7.8	7.9	3.4	3.5	5.6	5.4	15	16	12	11	9.4	9.6	20	20
41	7.3	6.8	0 <sup>b</sup>	0 <sup>b</sup>	n.r.	n.r.	18 <sup>a</sup>	15 <sup>a</sup>	n.r.	n.r.	11	9.3	24	22

**Table 4.** Single results from the collaborative study on the determination of *olaquinox* in the test samples abbreviated as MAT. The concentrations are given in mg kg<sup>-1</sup> and the composition of the materials is shown in **Table 1**. Results identified as outliers (<sup>a</sup> due to the Cochran test and <sup>b</sup> due to the Grubbs test) are given with grey background and were not included in the statistical assessment of the method performance characteristics.

Lab No.	MAT 1		MAT 2		MAT 4		MAT 5		MAT 7		MAT 9	
	a	b	a	b	a	b	a	b	a	b	a	b
1	10.1	10.2	5	5.2	12.5	12.6	3	2.9	7.1	7	15.3	14.9
5	7.3	6.7	3.3	3.3	8.4	7.6	0 <sup>b</sup>	0 <sup>b</sup>	4.8	4.7	9.7	9.9
8	9.7	9.6	4.5	4.5	11.7	12	2.3	2.3	6.6	6.6	14.9	14.8
10	9.3	9.3	4.8	5	11.8	11.7	3	2.8	6.9	7	15.7	15.4
12	8.8	9	4.1	5.1	10.4	9.9	1.9	2.3	7.1	6.9	13.6	13.5
14	10.3	10.3	5	5.3	12.4	12.2	3.1	2.7	7.4	7.1	15.2	15.2
16	7.5	8.3	3.7	3.7	9.5	9.2	2.5	2.6	6.2	6.3	13.2 <sup>a</sup>	11.1 <sup>a</sup>
19	11.7	11.4	6.3	6.3	14.1	14	3.6	3.7	8.5	8.9	19.4	18.3
20	9.3	9.5	4.6	4.4	11.6	11.4	2.2	2.5	6.4	6.5	13.9	14.7
25	9.8	9.1	5.4	4.4	10.9	11.2	2.3	2.5	6.3	5.6	14.2	14.1
28	9.5	8.8	4.8	4.4	10.2	11.7	2.5	3	0 <sup>a</sup>	6.3 <sup>a</sup>	13.9	14.2
29	9.9	9.8	5	4.9	12	11.8	2.9	2.9	6.8	6.8	14.8	14.4
30	10.2	10.2	5	5.1	12	12	2.3	2.4	14 <sup>b</sup>	14.9 <sup>b</sup>	14.5 <sup>a</sup>	17.4 <sup>a</sup>
31	9.6	9.2	4.8	4.5	11	11	2.7	2.6	9 <sup>a</sup>	6.3 <sup>a</sup>	14	14
32	9.5	9.1	4.2	4	11.2	10.6	2.2	2.1	5.9	6.5	13.8	13.3
33	6.6	7.6	3.4	3.2	9.4	9.7	1.4	1.4	5.1	4.6	11.2	10.8
37	13 <sup>a</sup>	16 <sup>a</sup>	7	6.8	16	15	3	2.9	7.9	8.3	17	18
41	11	10	4.4	4.2	21 <sup>a</sup>	15 <sup>a</sup>	3.7 <sup>a</sup>	0 <sup>a</sup>	7.9	7.3	23 <sup>a</sup>	20 <sup>a</sup>

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**Table 5.** Results from the statistical evaluation for *carbadox* in feedingstuffs.  $S_r$ : standard deviation for repeatability;  $RSD_r$  (%): relative standard deviation for repeatability;  $S_R$ : standard deviation for reproducibility;  $RSD_R$  (%): relative standard deviation for reproducibility. "No. of laboratories" indicates the number of laboratories retained after removal of outliers. The number in brackets indicates the number of laboratories identified as outliers.

	MAT 2	MAT 3	MAT 5	MAT 6	MAT 8	MAT 9	MAT 10
No. of laboratories	17 (1)	14 (4)	14 (4)	15 (3)	15 (3)	18 (0)	17 (1)
Target concentration (mg/kg)	8	4	6	16	12	10	20
Average (mg/kg)	7.3	3.6	5.4	14	11	9	18
$S_r$ (mg/kg)	0.24	0.04	0.14	0.40	0.58	0.48	0.65
$RSD_r$ (%)	3.3	1.1	2.5	2.9	5.5	5.4	3.6
$S_R$ (mg/kg)	0.65	0.23	0.56	1.1	1.1	0.83	1.9
$RSD_R$ (%)	8.9	6.4	10.3	7.7	10.6	9.4	10.7
HORRAT value	0.7	0.5	0.8	0.7	0.9	0.8	1.0

**Table 6.** Results from the statistical evaluation for *olaquinox* in feedingstuffs.  $S_r$ : standard deviation for repeatability;  $RSD_r$  (%): relative standard deviation for repeatability;  $S_R$ : standard deviation for reproducibility;  $RSD_R$  (%): relative standard deviation for reproducibility. No. of laboratories indicates the number of laboratories retained after removal of outliers. The number in brackets indicates the number of laboratories identified as outliers.

	MAT 1	MAT 2	MAT 4	MAT 5	MAT 7	MAT 9
No. of laboratories	17 (1)	18 (0)	17 (1)	16 (2)	15 (3)	15 (3)
Target concentration (mg/kg)	10	5	12	3	7	15
Average (mg/kg)	9.4	4.7	11.4	2.6	6.7	14.4
$S_r$ (mg/kg)	0.36	0.27	0.38	0.16	0.26	0.36
$RSD_r$ (%)	3.9	5.7	3.4	6.2	3.8	2.5
$S_R$ (mg/kg)	1.2	0.9	1.8	0.5	1.1	2.2
$RSD_R$ (%)	12.7	19.4	15.4	20.0	16.0	15.2
HORRAT value	1.1	1.5	1.4	1.4	1.3	1.4

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**Legends**

Figure 1: Chromatogram and DAD spectrum from laboratory 19 obtained on material 2

Figure 2. Fitness for purpose of the relative standard deviation for reproducibility indicated by the HORRAT value. The target value is set at 1, the acceptable limit is 1.5 and the rejection level is 2

Figure 3. Estimation of the trueness of the method: The average concentration for carbadox and olaquinox obtained in the study related to the target values (Table 1). Error bars represent the expanded uncertainty which is two times the standard error.

Figure 4. Sensitivity calculated for each confirmation criteria of the LC-DAD method. For the criteria (b) and (c) the  $\pm 15\%$  limit was applied. Criterion (a): Correspondence of the retention times between standard and test sample. Criterion (b): purity of the UV-spectrum in the test sample. Criterion (c): correspondence of spectra between standard and test sample, comparing the absorbance characteristics. Criterion (d): correspondence of spectra between standard and test sample, comparing the wavelengths. Details are given in the text

Figure 5. Percentage rate of correct identification calculated for each confirmation criteria of the LC-DAD method. For the criteria (b) and (c) the  $\pm 25\%$  limit was applied.

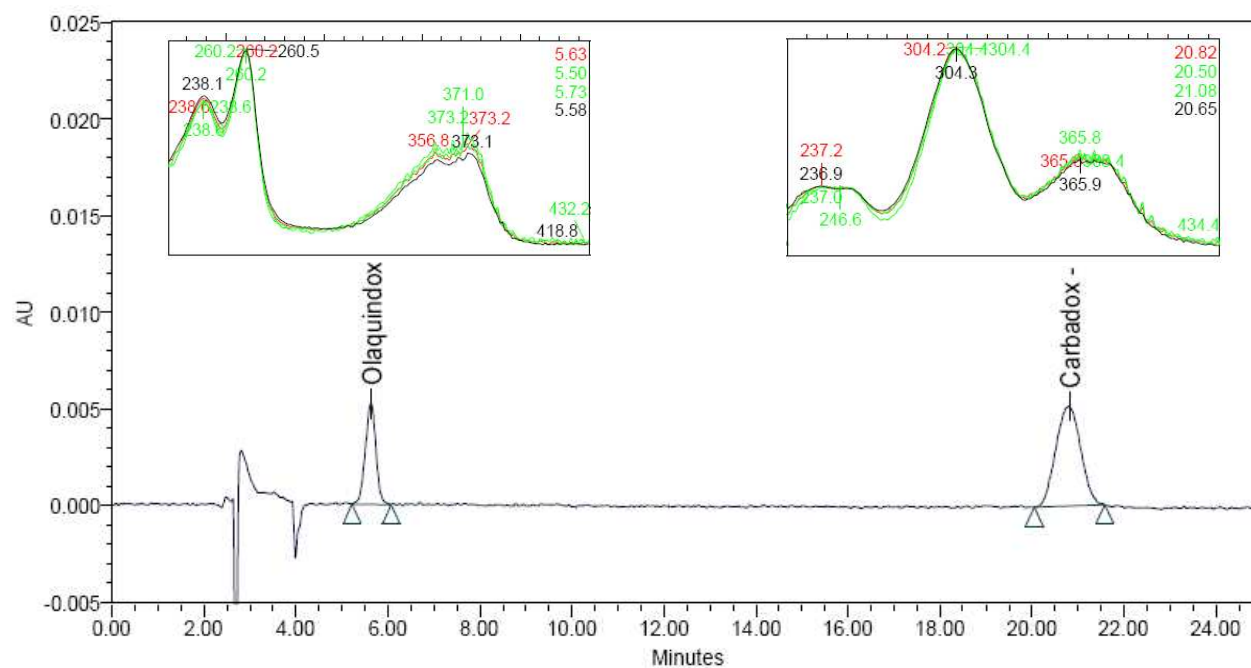


Figure 1: Chromatogram and DAD spectrum from laboratory 19 obtained on material 2

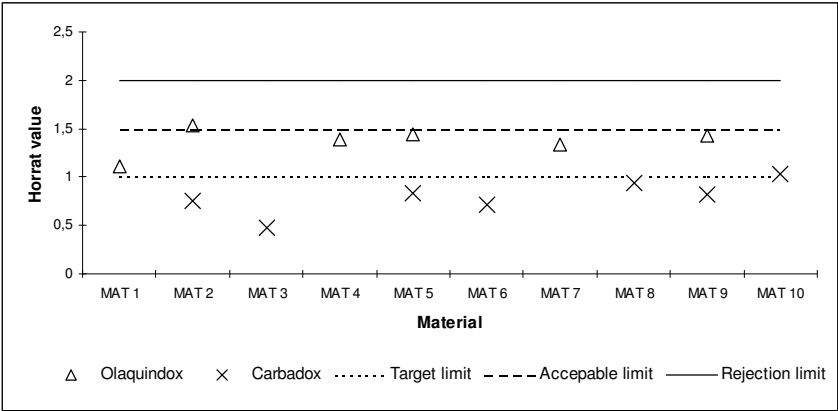
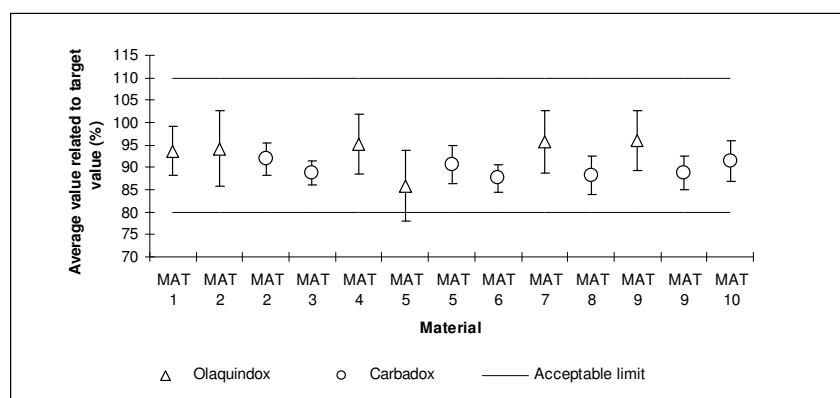


Figure 2. Fitness for purpose of the obtained relative standard deviation for reproducibility indicated by the HORRAT value. The target value is set at 1, the acceptable limit is 1.5 and the rejection level is

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*Figure 3.* Estimation of the trueness of the method: The average concentration for carbadox and olaquinox obtained in the study related to the target concentrations. The ratio is expressed in terms of percentage. Error bars represent the expanded uncertainty which is two times the standard error of the average.



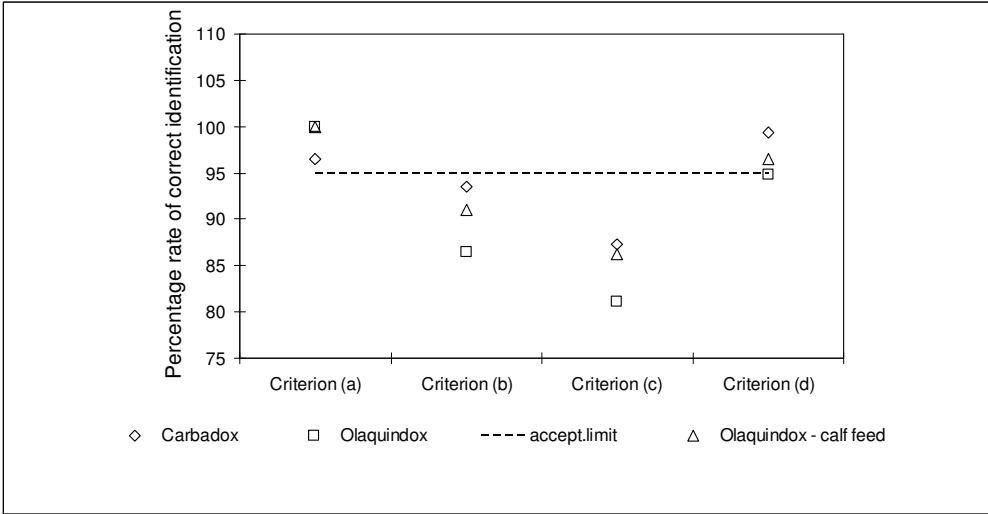


Figure 4. Percentage rate of correct identification calculated for each confirmation criteria of the LC-DAD method. For the criteria (b) and (c) the  $\pm 15\%$  limit was applied. Criterion (a): correspondence of the retention times between standard and test sample. Criterion (b): purity of the UV-spectrum in the test sample. Criterion (c): correspondence of spectra between standard and test sample, comparing the absorbance characteristics. Criterion (d): correspondence of spectra between standard and test sample, comparing the wavelengths. Details are given in the text

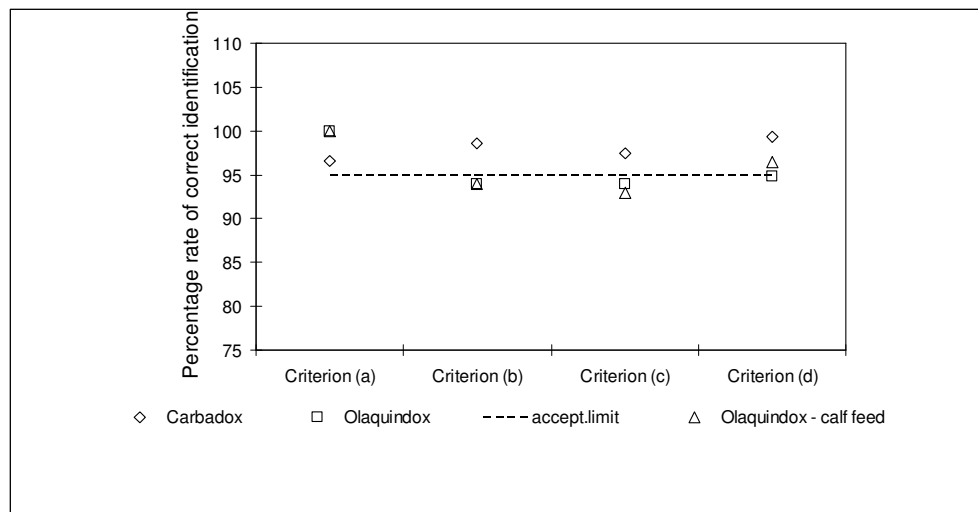


Figure 5. Percentage rate of correct identification calculated for each confirmation criteria of the LC-DAD method. For the criteria (b) and (c) the  $\pm 25$  % limit was applied. Criterion (a): correspondence of the retention times between standard and test sample. Criterion (b): purity of the UV-spectrum in the test sample. Criterion (c): correspondence of spectra between standard and test sample, comparing the absorbance characteristics. Criterion (d): correspondence of spectra between standard and test sample, comparing the wavelengths. Details are given in the text

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