Section A4.3/01 Analytical Methods for Detection and Identification

Annex Point IIA, IV.4.1 *Residues in Food and feedstuff*

Official use only

1 REFERENCE

1.1	**Reference**	Baltussen IE, 2006, Development and Validation of an Analytical Method for the Analysis of 2-(n-octyl)-4-isothiazolin-3-one (OIT) (Active Ingredient in ACTICIDE OIT) in Altromin Pelleted Rat and Dog Diet, Notox [redacted], unpublished.
1.2	**Data protection**	Yes
1.2.1	Data owner	THOR GmbH, Germany
1.2.2	Companies with letter of access	
1.2.3	Criteria for data protection	Data submitted on existing A.S. for the purpose of its entry into Annex I.

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes
2.2	**GLP**	Yes
2.3	**Deviations**	No

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Extraction	Methanol extraction of test diet samples followed by 0.45 µm membrane filtration.
3.1.2	Cleanup	-
3.2	**Detection**	-
3.2.1	Separation method	RP-HPLC using LiChrosorb RP-select B column and 80/20 (v/v) methanol/Milli-Q water containing 0.2% Acetic acid.
3.2.2	Detector	Spectrophotometric (280 nm)
3.2.3	Standard(s)	OIT-Standard ([redacted]), THOR GmbH, Speyer
3.2.4	Interfering substance(s)	-
3.3	**Linearity**	
3.3.1	Calibration range	
3.3.2	Number of measurements	Altromin pellet rat and dog diet samples were analysed in three different series of analysis. In each series, samples were defrosted at room temperature and ground. From each ground diet sample, two 1 gram sub-samples were extracted with 50 ml methanol (ultrasonication for 4 hours at 4P ± 2°G). Thereafter, the extracts were filtered through a 0.45 µm membrane filter (Spartan 30/0.45 RC), diluted to mobile phase composition, filtered again and injected into the HPLC (single injection). Accuracy was calculated using the six results for each diet.

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3.3.3	Linearity	A linear relationship between response and active ingredient concentration was found over a concentration range of 0.0988 - 9.98 mg a.i./L (in end solution) representing the working range of the method.
3.4	**Specifity: interfering substances**	One peak was observed in chromatograms of analytical standard solutions. It was assumed that the peak derives from the active ingredient. The area of the active ingredient peak was used as response in the calculations during the validation tests. In chromatograms of blank Altromin pellet rat diet, no interfering peaks were observed. In chromatograms of blank Altromin pellet dog diet, a peak at the active ingredient position was detected. The highest level at which this peak was detected corresponded to 3.9 mg a.i./kg. Maximum contribution to the peak area at LOQ level was 12.5%. Because this was less than 30% as required by the guideline, the interference was considered not significant. In conclusion, the analytical method was found to be specific for the analysis of OIT in Altromin pellet rat and dog diets.
3.5	**Recovery rates at different levels**	Mean recoveries were between 33 and 86 %. The recovery criterion in the guideline (70-110%) was only met for the two highest levels in the rat diet (1600 and 3200 mg t.s./kg) and the three highest levels in the dog diet (1600,3200 and 6000 mg t.s./kg). During method development it was concluded that the lower recoveries were most likely due to reaction of OIT with sulphur containing compounds in the diet and/or effects of irreversible binding of OIT. Despite the low recoveries for the lower concentration levels, extraction was complete with regard to the extractable OIT. Therefore, the method was considered acceptable for determination of OIT in Altromin pellet rat diets in the OIT concentration range of 95.5 - 3070 mg/kg which corresponds to an ACTICIDE OIT concentration range of 100 - 3200 mg/kg and in Altromin pellet dog diets in the OIT concentration range of 95.5 - 5753 mg/kg which corresponds to an ACTICIDE OIT concentration range of 100 - 6000 mg/kg. The results are summarized in the next table 1
3.5.1	Relative standard deviation	-
3.6	**Limit of determination**	The LOQ is defined as the lowest concentration level at which an accuracy in the range 70-110% and a repeatability of less than 20% is demonstrated. The repeatability criterion was met at all concentrations tested. Recovery however was not in the required range at the lower concentrations due to reactivity of OIT with sulfur containing compounds in the diets and/or effects of irreversible binding of OIT. Because extraction was reproducible and complete with regard to the extractable OIT, it was decided to report the LOQ for ACTICIDE® OIT to be 100 mg/kg. The, limit of detection for the active ingredient was determined to be 0.008 mg a.i./I (in end solution) at an injection volume of 20 µI. Taking a dilution factor of 1.25, a sample amount of 1 gram and an extraction solvent volume of 50 ml into account, this corresponds to a limit of detection for diet Samples of 0.5 mg a.i./kg.
3.7	**Precision**	
3.7.1	Repeatability	Repeatability between series Based on the results for the accuracy samples, it was concluded that at all concentration levels the coefficient of variation was < 20%. The repeatability between series was therefore considered acceptable.

		Repeatability within one series Repeatability within one series was tested at three concentrations by analysis of six samples per concentration in one series of analysis. The results are summarized in the next table 2
3.7.2	Independent laboratory validation	-

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	A high performance liquid chromatographic (HPLC) method for quantitative analysis of OIT (i.e. 2-(n-Octyl)-4-isothiazolin-3-one, the active ingredient in the test substance ACTICIDE® OIT) in Altromin pellet rat and dog diet was developed. A LiChrosorb RP-select B column was used with 80(20 (v/v) methanol/ Milli-Q water containing 0.2% Acetic acid as the mobile phase, and a spectrophotometric detector set to read the absorbance at 280 nm. For validation of the analytical method, standard solutions were prepared in methanol at exactly known concentrations between 2735 mg a.i./I and 4803 mg a.i./I using the analytical standard. Calibration solutions for the validation tests were obtained by dilution of these standard solutions with mobile phase. End solution for all samples was in mobile phase. All concentrations were corrected for the purity of the analytical standard. For determination of accuracy, repeatability and storage stability, Altromin pellet rat and dog diets W13re prepared covering the concentration range to be used in toxicity studies with rat and dog. From these diets, samples were taken for use in this study. The samples were stored at -20°C until pre-treatment.
4.2	**Conclusion**	The method is valid.
4.2.1	Reliability	1
4.2.2	Deficiencies	No

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Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	03/11/2009
Materials and methods	Not applicable
Conclusion	Not applicable
Reliability	Not applicable
Acceptability	Not applicable
Remarks	This method has been submitted as additional data and is not strictly required due to the use pattern of this PT. Therefore, the method has not been evaluated as it is not required.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Results and discussion	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant´s summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Tabelle 1

ACTICIDE® OIT NOTOX [redacted]

Altromin pellet diet for	Concentration target [mg t.s./kg]	Concentration prepared[1] (n=6) [mg a.i./kg]	Mean Concentration analysed (3 series) [mg a.i./kg]	Mean Recovery [%]	Coefficient of variation [%]
rat	100	96.0	31.3	33	15
	200	192	85.1	44	5.2
	400	384	193	50	9.6
	800	767	469	61	1.7
	1600	1534	1146	75	3.3
	3200	3070	2470	80	2.1
dog	100	95.5	44.6	47	16
	200	191	113	59	7.6
	400	384	244	64	5.4
	800	767	519	68	2.0
	1600	1533	1153	75	3.4
	3200	3069	2653	86	2.0
	6000	5753	4506 [2]	78	1.7

[1] Concentration in mg a.i./kg = 0.959 x concentration in mg t.s./kg

[2] The 6000 mg t.s./kg group was added as an extra group to this validation in a later stage. Therefore for this group only 1 series was measured

t.s. Test substance (i.e. ACTICIDE® OIT)

a.i. Active ingredient (i.e. OIT)

Tabelle 2

ACTICIDE® OIT NOTOX [redacted]

Altromin pellet diet for	Concentration prepared [1] [mg t.s./kg]	Concentration prepared [1] (n=6) [mg a.i./kg]	Mean Concentration analysed (1 series) [mg a.i./kg]	Mean Recovery [%]	Coefficient of variation [%]
rat	100	96.0	32.1	33	17
	400	384	196	51	9.5
	3200	3070	2422	79	1.7
dog	100	95.5	42.9	45	11
	400	384	254	66	5.7
	3200	3069	2585	84	1.3
	6000	5753	4506	78	1.7

[1] Concentration in mg a.i./kg = 0.959 x concentration in mg t.s./kg.

t.s. Test substance (i.e. ACTICIDE® OIT)

a.i. Active ingredient (i.e. OIT)