SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.1 & IIIA-IV.1	Analytical methods for the determination of pure active substance

			Official use only
		1 REFERENCE	
1.1	**Reference**	CIPAC method coumatetralyl 189, CIPAC Handbook G, pp 24-31 (published), MO-04-001271	
1.2	**Data protection**	No	
1.2.1	Data owner	Published data	
1.2.2	Companies with letter of access		
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINE	
2.1	**Guideline study**	No	
2.2	GLP	No	
2.3	Deviations	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	Sample (about 80 mg of coumatetralyl) is weighed to the nearest 0.1 mg into a volumetric flask (100 ml) and dissolved in, or extracted with acetonitrile using an ultrasonic bath for about 5 min. The mixture is allowed to cool to room temperature and the flask is made up to volume with acetonitrile. 5.0 ml of this solution are pipetted into a volumetric flask (100 ml) which is then made up to volume with solvent mixture I (methanol – water, 50 + 50 (v/v)). Afterwards this solution is mixed well. If necessary, an aliquot of the suspension is filtered or centrifuged to get a clear solution.	
3.1.2	Cleanup	20 µl of the solution are injected into the HPLC and chromatographed in accordance with the indicated conditions. After UV detection at 310 nm, the coumatetralyl concentration is determined using external standardisation.	
3.2	**Detection**		
3.2.1	Separation method	The determination is performed by HPLC under the following chromatographic conditions: Column: stainless steel, 250 x 4 (i.d.) mm, packed with	

		LiChrosorb RP-18 (5 µm); Eluent: 0.5 % acetic acid – acetonitrile, 50 + 50 (v/v); Eluent flow rate: 2 ml/min; Temperature: 50 °C; Injection volume: 20 µl; Retention time: about 5 min; Run time: 8 min;
3.2.2	Detector	UV-detector (wavelength: 310 nm)
3.2.3	Standard(s)	External standard coumatetralyl of known purity
3.2.4	Interfering substance(s)	No data
3.3	**Linearity**	
3.3.1	Calibration range	No data
3.3.2	Number of measurements	No data
3.3.3	Linearity	No data
3.4	**Specifity: interfering substances**	No data
3.5	**Recovery rates at different levels**	No data
3.5.1	Relative standard deviation	No data
3.6	**Limit of determination**	No data
3.7	**Precision**	
3.7.1	Repeatability	Repeatability: 12.1 g/kg at 997 g/kg active ingredient content; Reproducibility: 12.5 g/kg at 997 g/kg active ingredient content
3.7.2	Independent laboratory validation	Not required

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods	The determination of pure active substance coumatetralyl is based on the CIPAC method coumatetralyl 189. The method is based on high performance liquid chromatography with reversed-phase separation of the sample dissolved in acetonitrile. After UV detection at 310 nm, the coumatetralyl concentration is determined using external standardisation.
4.2 Conclusion	Determination of pure active substance coumatetralyl is

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.1 & IIIA-IV.1	Analytical methods for the determination of pure active substance
	performed according to the CIPAC method coumatetralyl 189.
4.2.1 Reliability	1
4.2.2 Deficiencies	No

	EVALUATION BY COMPETENT AUTHORITIES
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	January 2005
Materials and methods	Acceptable
Conclusion	Adopted
Reliability	1
Acceptability	acceptable
Remarks	

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.1 & IIIA-IV.1	Analytical methods for the determination of pure active substance

			Official use only
		1 REFERENCE	
1.1	**Reference**	E. Seidel, 2002, Determination of coumatetralyl in formulations-Assay-HPLC-external standard, Bayer AG, BCS-D Formulation Technology, Monheim, Germany, Report No. 2001-0004804-02E (unpublished), 2002-09-23, MO-02-014117 A. Odenthal, 2002, Validation of HPLC-method 2001-0004804-02-Determination of coumatetralyl in formulations, Bayer AG, BCS-Development/Formulation Technology, Monheim, Germany, Report No. VB1-2001-0004804 (unpublished), 2002-09-10, MO-02-014014	
1.2	**Data protection**	Yes	
1.2.1	Data owner	**Bayer CropScience AG**	
1.2.2	Companies with letter of access		
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No, no guideline available	
2.2	**GLP**	**No**	
2.3	**Deviations**	**No,**	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**	A certain amount of sample is accurately weighed to 0.1 mg into a conical flask. After adding the solvent methanol, the flask is placed into an ultrasonic bath to extract the active ingredient. If necessary a part of the supernatant solution is to be diluted with a methanol-water-mixture. A part of the sample solution is centrifuged. 10 µl of the clear solution are injected into the HPLC and chromatographed in accordance with the indicated conditions. The quantitative evaluation is carried out by comparing the peak areas with those of reference substances, using an external standard.	
3.2	**Detection**		
3.2.1	Separation method	The determination is performed by HPLC under the following chromatographic conditions:	

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.1 & IIIA-IV.1	Analytical methods for the determination of pure active substance

		Column: 250 mm long x 4 mm I.D., packed with LiChrosorb RP 18 Merck, particle size: 5 µm; Eluent: Water and 5 ml acetic acid per litre / acetonitrile (50/50, v/v); Flow rate: 2ml/min; Column temperature: 40 °C; Injection volume: 10 µl
3.2.2	Detector	UV-detector (wavelength 310 nm)
3.2.3	Standard(s)	External standard (coumatetralyl)
3.2.4	Interfering substance(s)	
3.3	**Linearity**	
3.3.1	Calibration range	6 concentrations with single measurements; checked range: 50-150 %. Solutions were prepared by weighing different amounts of a sample with known quantities of analyte (see table A4.1-1).
3.3.2	Number of measurements	Single measurement of 6 concentrations
3.3.3	Linearity	Correlation coefficient: multip. correlation coefficients r_k (first order of calibration function) is given in the report : 0.9999. The function is linear in the operating range.
3.4	**Specificity: interfering substances**	UV-spectra of reference substance and analyte in the sample were compared: The UV-spectra from reference substance and analyte in the sample shows no spectral difference, the retention times are identical. Calibration, sample and blank chromatograms were checked for interferences from excipients: No interferences were found.
3.5	**Recovery rates at different levels**	To determine the accuracy of the method 6 samples of laboratory-prepared synthetic formulation containing known content of analyte were measured. The resulting data pairs (nominal concentration vs. measured concentration) were evaluated by linear regression (see table A4.1-2). Mean value of recovery range: 99.83 % Recovery evaluated by linear regression (100 % · slope): 99.56 % Residual standard deviation: 0.011
3.5.1	Relative standard deviation	No data about relative standard deviation
3.6	**Limit of determination**	No data about the limit of determination or other information concerning the sensitivity of the method
3.7	**Precision**	

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.1 & IIIA-IV.1	Analytical methods for the determination of pure active substance

3.7.1 Repeatability	6 samples from one batch were analysed (single injection, assessment of repeatability). Statistical data: Mean value of the 6-fold determination: 0.7038; Relative standard deviation: 0.41 %
3.7.2 Independent laboratory validation	Not required

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods	A method for the determination of the content of coumatetralyl in formulations was developed. The method is based on high performance liquid chromatography (HPLC) on a reversed-phase using isocratic elution. After UV detection at 310 nm, the quantitative evaluation is carried out by comparing the peak areas with those of reference substances, using an external standard.
4.2 Conclusion	The method was employed with success on the formulation used for validation (Racumin TP, content a.i.: 0.75 %). It may also be applied to other formulations containing the active ingredient coumatetralyl if the absence of chromatographic interferences is ensured. The analytical method was found to be valid.
4.2.1 Reliability	2-3

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.1 & IIIA-IV.1	Analytical methods for the determination of pure active substance
4.2.2 Deficiencies	Yes, purity of the calibration substance used for validation not mentioned; no data about relative standard deviation (recovery); no data about the limit of determination or other information concerning the sensitivity of the method

Section A4	**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**
Annex Point IIA4.1& IIIA-IV.1	Analytical methods for the determination of pure active substance

	EVALUATION BY COMPETENT AUTHORITIES
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	January 2005
Materials and methods	Not relevant
Conclusion	Not relevant
Reliability	4
Acceptability	Not relevant
Remarks	The study does not cover determination of pure active substance in technical material. The method covers determination of pure active ingredient in formulations. The study is evaluated in section B3.

Table A4.1-1 Calibration function

Coumatetralyl [mg/100 ml] (per weight)	Coumatetralyl [mg/100 ml] (determined)
4.21	4.23
2.04	2.05
6.05	6.06
2.61	2.58
4.92	4.93
3.68	3.70

Table A4.1-2 Recovery rates

Concentration [mg a.i./100 ml] (per weight)	Concentration [mg a.i./100 ml] (determined)	Recoveries [%]
2.04	2.05	100.49
2.61	2.59	99.23
3.68	3.68	100.00
4.21	4.20	99.76
4.92	4.91	99.80
6.05	6.03	99.67

Mean value of recovery range: 99.83 %

Recovery evaluated by linear regression (100 % · slope): 99.56 %

Residual standard deviation: 0.011

SECTION A4 (4.1-4.3)

See confidential section.

SECTION A4		**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**	
Annex Point IIA4.2 & IIIA-IV.1		4.2.1 SOIL	
		1 REFERENCE	Official use only
1.1	**Reference**	B. Brumhard, 2004, Method 00824 for the Determination of Residues of coumatetralyl in Soil by HPLC-MS/MS, Bayer Crop Science AG-Development-Residues, Operator and Consumer Safety, Monheim am Rhein, Germany, Report No. MR-096/03, Method No. 00824 (unpublished), 2004-01-13. MO-04-000391	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Bayer CropScience AG	
1.2.2	Companies with letter of access		
1.2.3	Criteria for data protection	Data on existing active substance submitted for the first time for entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Method was performed according to EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev.6 of June 20, 2000, BBA Guideline: Residue Analytical Methods for Post-Registration Control Purposes of July 21, 1998 and Commission Directive 96/46/EC amending Council Directive 91/414/EEC of 16 July 1996.	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	Soil samples of 20 g are extracted in a microwave extractor with 40 mL of a mixture of water / acetonitrile. After extraction, subsamples of the extracts are centrifuged to remove fine particles of the soil or sediment. Identification and quantitation of the active ingredient is done by high-performance liquid chromatography using MS/MS detection in the Multiple Reaction Monitoring mode.	
3.1.2	Cleanup		
3.2	**Detection**		

SECTION A4 **ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**

Annex Point IIA4.2 & IIIA-IV.1 4.2.1 SOIL

3.2.1 Separation method

Liquid chromatographic conditions: Column: Supersphere 100 RP-18; length 7.5 cm; i.d. 4.0 mm; Injection volume: 25 µl; Oven temperature: 40 °C;

Mobile phase: A: water /acetonitrile / acetic acid (900/100/0.1, v/v/v)

B: acetonitrile / acetic acid (1000/0.1, v/v)

Gradient:

Time[min]	A[%]	B[%]
0	50	50
2.8	50	50
3	10	90
4	10	90
4.2	50	50
6	50	50

Run time: 6 min; Flow rate: 1.0 ml/min; Retention time: approx. 2.7 min;

Isocratic pump (for flushing the interface):

Mobile phase: water / acetonitrile / acetic acid (500/500/0.1, v/v/v);

3.3.1 Detector

Mass spectrometric detector (MS/MS).

Mass spectrometric parameters:

Mass of parent ion: 293 amu; Mass of product ion: 175 amu;

Dwell: 1000 msec; Ionisation mode: positive ion mode; Ion spray voltage: 5000 V; Focusing potential: 140 V; Entrance potential: 4.5 V; Declustering potential: 31 V; Collision energy: 29 V; Collision cell exit potential: 20 V; Turbo gas: 8 l/min; Curtain gas: 1.39 l/min; Nebuliser gas: 1.31 ml/min; Collision gas: 0.87 ml/min; Turbo gas temperature: 300 °C

3.3.2 Standard(s)

External standard (coumatetralyl)

3.3.3 Interfering substance(s)

For method validation two soil types were used in order to assess a possible influence of different soil characteristics (see table A4.2.1-1).

3.3 Linearity

3.3.1 Calibration range

Standard solutions containing coumatetralyl were measured in order to determine the detector linearity. Concentrations tested were 0.25, 0.5, 2.5, 5.0 and 10 µg/l coumatetralyl corresponding to a concentration in soil of 0.5 to 20 µg/l.

The mass spectrometric detector showed linear response in the range of 0.25 to 10 µg/l of coumatetralyl in solvent.

3.3.2 Number of measurements

Standard solutions were measured in duplicate

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.1 SOIL
3.3.3 Linearity	Correlation coefficient: 0.9995
3.4 Specificity: interfering substances	The analytical results for all control samples were below 0.3 µg/kg coumatetralyl. No significant interferences from the sample matrix were detected at the retention time corresponding to coumatetralyl in the control samples (graphical presentation).
3.5 Recovery rates at different levels	Soil Höfchen and soil Laacher Hof were fortified with coumatetralyl. For method validation, 30 recovery experiments were conducted in the range of 1 to 10 µg/kg. Each sample was injected once into the HPLC instrument. The overall mean recovery of the method was 90% for coumatetralyl as shown in table A4.2.1-2
3.5.1 Relative standard deviation	Relative standard deviation (RSD) of 18.4%. see table A4.2.1-2
3.6 Limit of determination	The limit of quantification is 1 µg/kg for coumatetralyl. The detection limit is 0.3 µg/kg for coumatetralyl.
3.7 Precision	
3.7.1 Repeatability	see recovery rates (3.5)
3.7.2 Independent laboratory validation	not required for soil method

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods	A method was developed and validated for determination of coumatetralyl in soil. After extraction of soil samples with mixtures of water / acetonitrile, subsamples of extracts are centrifuged to remove fine particles of the soil or sediment. Identification and quantification are done by HPLC using MS/MS detection in the Multiple Reaction Monitoring mode.
4.2 Conclusion	The mass spectrometric detector showed linear response in the range of 0.25 to 10 µg/l for coumatetralyl with a correlation coefficient of 0.9995. The method was validated using two different soil types (silt and sandy loam). The overall mean recovery of the method, which was determined in the range of 1 to 10 µg/kg, was 90% for coumatetralyl with a relative standard deviation (RSD) of 18.4%. The limit of quantification is 1 µg/kg and the limit of detection is 0.3 µg/kg for coumatetralyl. The storage stability of the analyte in the extracts was examined by storage of extracts in the freezer at ≤ -18 °C for 11 days. After

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.1 SOIL
	11 days the extracts were reanalysed and showed no decrease of coumatetralyl. The results showed that coumatetralyl residues in the extracts are stable during storage under deep-frozen conditions over a period of at least 11 days.
4.2.1 Reliability	1
4.2.2 Deficiencies	No

	EVALUATION BY COMPETENT AUTHORITIES
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	January 2005
Materials and methods	Acceptable
Conclusion	Adopted
Reliability	1
Acceptability	acceptable
Remarks	

Table A4.2.1-1: Soil types

Soil	Texture of Soil	Organic Matter [%]
Hoefchen	silt (USDA)	3.63
Laacher Hof	sandy loam (USDA)	1.75

TABLE A4.2.1-2 RECOVERY RATES OF COUMATETRALYL

Fortification level [µg/kg]	Soil	Single values [%]					Mean [%]	RSD [%]
1	Hoefchen	122	97	76	75	77	89	20.2
		105	108	78	77	70		
1	Laacher Hof	112	76	88	73	81	81	15.6
		85	68	81	71	73		
mean of all 1 µg/kg single values							85	18.4
10	Hoefchen	81	114	81	99	99	95	14.6
10	Laacher Hof	90	112	112	110	113	107	9.3
mean of all 10 µg/kg single values							101	13.0
mean of all Hoefchen samples							91	18.1
mean of all Laacher Hof samples							90	19.3
overall mean							**90**	**18.4**

RSD: Relative standard deviation; Fortification levels are expressed as concentration of coumatetralyl in wet soil.

SECTION A4	**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**
Annex Point IIA4.2 & IIIA-IV.1	4.2.2 Air

Official use only

1 REFERENCE

1.1	**Reference**	E. Hellpointner, 2003, Coumatetralyl: Enforcement Method No. 00810 for the Determination of coumatetralyl in Air by HPLC-UV and Confirmation via DAD-Spectra Matching, Bayer Crop Science AG, Development Metabolism and Environmental Fate, Monheim, Germany, Report MEF- 102/03, Method No. 00810 (unpublished), 2003-07-25, MO-03-011359
1.2	**Data protection**	Yes
1.2.1	Data owner	Bayer CropScience AG
1.2.2	Companies with letter of access	
1.2.3	Criteria for data protection	Data on existing active substance submitted for the first time for entry into Annex I/IA

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Method was performed according to EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 6 (2000-06-20) and BBA: Guidance: Residue Methods for Enforcement, Status 21st July 1998 (BBA-Reports 43, 1998).
2.2	**GLP**	Yes
2.3	**Deviations**	No

3 MATERIALS AND METHODS

3.1 Preliminary treatment

Sampling of air:

The adsorption tube contains two adsorption layers separated from each other by a plug of glass wool, the larger layer (with 100 mg ®Tenax) facing the inlet of the tube during air sampling. The second smaller adsorption layer (with 50 mg of ®Tenax) is used for detection of a possible breakthrough of active substance during sampling. For sampling air is drawn through the adsorption tube over a period of 6 hours at a flow rate of approx. 2 l/min (vol. = 720 l). After sampling the tubes are directly processed or are closed with plastic caps and are kept cool.

Extraction:

Both layers are processed separately. The glass wool separating both layers and the upper glass wool facing the inlet of the tube are processed together with the first (that is the larger) layer. Each individual layer is removed from the glass tube and passed into a 10 ml vial with snap cover. The glass tube is rinsed with 5 ml of acetonitrile. The first (large) layer is mixed with these 5 ml of solvent; the second (smaller) layer is also mixed with 5 ml of acetonitrile. The active substance is extracted from the adsorption material by using a laboratory shaker (300 rpm) for 15 minutes. For HPLC analysis of the samples in case of the upper level of quantification the extracts were diluted 1:20 (v/v) with acetonitrile prior to injection. The crude extract of the ®Tenax is analysed. After liquid chromatographic separation the content is determined by UV-DAD detection. The UV signal was evaluated against the mean of two linear regression analysis functions.

3.2 Detection

3.2.1 Separation method

Analysis for coumatetralyl was done by liquid chromatographic separation using a ®Lichrospher column (100 RP-18 (Merck), length: 250 mm, inner diameter: 4 mm, particle size: 5 µm) and an UV-DAD detector.

Injection volume: 50 µl ; column temperature: 40 °C;

mobile phase: A: Milli-Q-water (+ 0.1 % phosphoric acid),

B :acetonitrile ; gradient:

Time [min]	A [%]	B [%]
0.0	80	20
3.0	80	20
13.0	0	100
15.0	0	100
16.0	80	20

stop at 15.0 min; post time: 3.0 min; flow rate: 1.0 ml/min

In addition the detection method was confirmed by comparison (matching) of the UV spectra of the respective peaks recorded by a diode array detector (DAD) with that of the reference standard.

3.2.2 Detector

UV-DAD detector (wavelength: 285 nm (BW 10 nm) and reference 450 nm (BW 80 nm))

3.2.3 Standard(s)

External standard (coumatetralyl)

3.2.4 Interfering substance(s)

Substances of the adsorption material may interfere

3.3 Linearity

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.2 Air

3.3.1 Calibration range	The linearity of the HPLC-UV method was confirmed by analysing six different concentrations ranging from 1.295 to 12.954 mg/l. See table A4.2.2-1
3.3.2 Number of measurements	Single measurement of 6 concentrations (see table A4.2.2-1).
3.3.3 Linearity	Correlation coefficient (R^2) = 0.99993
3.4 Specificity: interfering substances	The detection method is highly specific because of using the DAD peak library search mode. One blank sample was investigated during the determination of extraction recovery rates. The chromatograms of the adsorption system (first layer) did not show any chromatographic signal at the retention time of coumatetralyl corresponding to concentrations greater than the limit of detection. When testing the limit of quantification (LOQ) the chromatograms of the blank samples of the adsorption system showed a low chromatographic signal at the retention time of coumatetralyl corresponding to a concentration of 0.069 µg/ml or 0.481 µg a.s. /m^3.
3.5 Recovery rates at different levels	Extraction recovery rates (elution recovery): Defined amounts of dissolved active substance were dropped to the adsorption tubes. The solvent was removed by drawing air through the tube for 10 min (2 l/min). The first layer of ®Tenax, the glass wool and one blank sample were analysed. The mean recovery rate from five individual tests is 93.9% and 102.1% for the concentration of 0.030 to 0.599 mg/m^3 respectively as given in table A4.2-2. A good extraction efficiency has been demonstrated. Desorption recovery rates: Desorption recovery tests at the LOQ and at 20 times the LOQ were performed after spiking the ®Tenax tubes with coumatetralyl dissolved in 50 µl of acetonitrile. Air (mean temperature approx. 35 °C, mean air humidity approx. 80 %) was drawn through the tubes during a period of 6 hours. Five tubes for the test at the LOQ and at 20 times the LOQ were examined and two tubes for non-spiked blank samples test. On average a total recovery of approx. 88.6 % was determined for coumatetralyl within the concentration range of 0.030 to 0.599 mg/m^3 tested. This value proved the sufficient capability of ®Tenax retaining coumatetralyl during air sampling. For detailed results see table A4.2.2-3.

	Breakthrough of active substance: The breakthrough of a.s. was measured by investigating the second (smaller) adsorption layer of the ®Tenax tubes resulting from both the upper and lower LOQ-test series. The chromatograms of the 20 times the LOQ show a low chromatographic signal at the retention time of coumatetralyl.
3.5.1 Relative standard deviation	See table A4.2.2-2 and table A4.2.2-3
3.6 Limit of determination	The limit of quantification was 0.030 mg coumatetralyl/m³ air. The upper level of quantification was 0.599 mg coumatetralyl/m³ air (20 x LOQ). Limit of detection of HPLC-UV: 0.0147 µg/ml corresponding to 0.1022 µg/m³.
3.7 Precision	
3.7.1 Repeatability	See recovery rates (point 3.5)
3.7.2 Independent laboratory validation	Not required for air.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods	An analytical method for the determination of coumatetralyl in air is performed. The capability of ®Tenax tubes for adsorption of the active substance was tested at a temperature of approx. 35 °C and a relative humidity of approx. 80 %. Air is drawn through the adsorption tubes containing active substance with a rate of 2 l/min during a period of six hours. The adsorbed active substance is extracted with acetonitrile and determined by liquid chromatographic separation using UV detection. In addition the detection method was confirmed by comparison (matching) of the UV spectra of the respective peaks recorded by a diode array detector (DAD) with that of the reference standard.
4.2 Conclusion	The method was validated to an upper concentration of 0.599 mg coumatetralyl/m³ air. Sufficient total recoveries could be demonstrated despite using so-called worst-case climatic conditions (approx. 35 °C air temperature and 80 % relative air humidity). Similar recoveries are to be expected under sampling conditions in practice (i.e. under different climate conditions). Due to the highly specific detection method when using the DAD peak library search mode an additional confirmatory method is not regarded necessary.

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.2 Air

4.2.1 Reliability	1
4.2.2 Deficiencies	No

SECTION A4	**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**
Annex Point IIA4.2 & IIIA-IV.1	4.2.2 Air

	EVALUATION BY COMPETENT AUTHORITIES
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	January 2005
Materials and methods	Acceptable
Conclusion	Adopted
Reliability	1
Acceptability	acceptable
Remarks	

Table A4.2.2-1: Linearity for coumatetralyl

Conc. [mg/l]	Area [mAU x s]
12.954	912.1
9.716	678.3
6.477	455.0
4.5340	322.6
2.5908	183.79
1.2954	94.59

Slope: 69.91832

Correlation coefficient: (R^2): 0.99993

Intercept: 3.28285

Table A4.2.2-2 Extraction recovery rates

Concentration [mg a.s./m^3]	Recovery rate [%] (individual figures: n = 5)	Relative standard deviation [%]
0.030	93.9 (94.1; 93.6; 92.0; 94.4; 95.0)	1.2
0.599	102.1 (102.0; 100.9; 103.8; 100.9; 103.1)	1.3

Table A4.2.2-3 Desorption recoveries (first layer of ®Tenax)

Concentration [mg a.s./m^3]	Climatic conditions		Recovery rate [%] (individual figures: n = 5)	Relative standard deviation [%]
	Temp. [°C]	Relative air humidity [%]		
0.030	35	80	91.1 (90.8; 90.9; 90.4; 92.0; 91.3)	0.7
0.599	35	80	86.2 (86.4; 79.6; 88.5; 91.4; 84.9)	5.1

SECTION A4	**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**
Annex Point IIA4.2 & IIIA-IV.1	4.2.3 WATER

			Official use only
		1 REFERENCE	
1.1	**Reference**	B. Brumhard, 2004, Enforcement Method for the Determination of coumatetralyl in Drinking and Surface Water by HPLC-MS/MS, Bayer Crop Science AG-Development Residues, Operator and Consumer Safety, Monheim am Rhein, Germany, Report No. MR-076/03, Method No. 00820 (unpublished), 2004-01-08, MO-04-000390	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Bayer CropScience AG	
1.2.2	Companies with letter of access		
1.2.3	Criteria for data protection	Data on existing active substance submitted for the first time for entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Method was performed according to EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 6 of June 20, 2000; BBA Guideline: Residue Analytical Methods for Post – Registration Control Purposes of July 21, 1998 and Commission Directive 96/46/EC amending Council Directive 91/414/EEC of 16 July 1996.	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
		Sample preparation: Surface water is filtered using common paper filters. 20 % acetonitrile is added to the water samples. Additionally, acetic acid is added to water samples to a final concentration of 0.1 ml/l. Then the water samples are directly injected into the HPLC instrument and analysed in accordance with the indicated conditions. Identification and quantitative determination are done by means of electrospray MS/MS-detection.	
3.2	**Detection**		
3.2.1	Separation method	Liquid chromatographic conditions:	

Column: Hy Purity Aquastar, length: 150 mm. i.d.: 4.6 mm;

Particle size: 3 µm; Oven temperature: 40 °C; Injection volume: 220 µl; Flow rate: 1 ml/min; Run time: 15 minutes; Mobile phase: A: milli-Q-water / acetonitrile / acetic acid

(900/100/0.1, v/v/v)

B: acetonitrile / acetic acid (1000/0.1, v/v)

Retention time: 8.2 min

Gradient: Time	A [%]	B[%]
0	80	20
1	80	0
8	10	90
10	10	90
0.1	80	20

Mass Spectrometry – Principle of measurement:

Substances introduced into the mass spectrometer are ionised using an electrospray interface. Sample ions are accelerated by an adequate voltage regulation and separated by mass in the first quadrupole (Q1). The most abundant ions (the protonated and deprotonated ions) of the analyte (parent ions) are impulsed with nitrogen in the collision cell (Q2). Fragments of these ions (product ions) are separated by mass in the third quadrupole (Q3) and detected.

Calculation of concentrations is done by comparison of the peak areas of the samples with the peak areas of the external standard solutions.

Mass spectrometric detector (MS/MS).

Mass spectrometer operating parameters:

Detection time: 6.5 – 10.0 min; Principle: electrospray in positive mode; Cone: 50 V; Collision energy: 20 eV;

Mass of parent ion: 293.1 amu; Mass of product ion: 174.8 amu;

The product ion of coumatetralyl (m/z ratio 293.1 → 174.8) was used for identification and quantification.

3.2.3 Standard(s): External standard (coumatetralyl)

3.2.4 Interfering substance(s): Substances of sample matrix may interfere.

Characteristics of the surface water from the river Rhine used for method validation see table A4.2.3-1. Surface water was sampled in Leverkusen-Hitorf.

3.3 Linearity

3.3.1 Calibration range: The linearity of HPLC-MS/MS detection of coumatetralyl was determined in the range from 0.042 µg/L to 10.49 µg/l

	(see table A4.2.3-2). The mass spectrometric detector showed linear response in the concentration range of 0.042 µg/l to 10.49 µg/l for coumatetralyl in surface water.
3.3.2 Number of measurements	Duplicate measurement of 7 concentrations (see table A4.2.3-2)
3.3.3 Linearity	Correlation coefficient r = 1.00000
3.4 Specificity: interfering substances	Coumatetralyl was not detected in the surface water control samples. Influence of matrix: The MS/MS detection of coumatetralyl is affected by the matrix. The peak area of coumatetralyl in a surface water sample containing 0.5 µg/l was reduced to approx. 42 % of the corresponding peak area in milli-Q-water. The matrix effect was determined by 4 injections of each solution.
3.5 Recovery rates at different levels	Method validation for coumatetralyl: Surface water samples were fortified with coumatetralyl at 0.052 µg/l and at 10.49 µg/l. These test solutions were injected ten times each into the HPLC-MS/MS instrument. The peak areas for coumatetralyl were determined and are listed in table A4_2.3-3. The mean peak area of 119 relates to a coumatetralyl concentration of 0.052 µg/l in surface water. The mean peak area of 22663 relates to a coumatetralyl concentration of 10.49 µg/l in surface water. The relative standard deviation for the peak area was 5.0 % and 0.9 %, respectively. The relative standard deviation for the retention time was < 0.1 % for both concentrations. Because of the direct measurement of fortified samples without separate extraction and clean-up steps it is not possible to determine recovery rates and an estimate of the accuracy of the analytical technique was made by an assessment of the linearity of matrix calibration and by determination of the reproducibility of sample analysis (SANCO/3029/99 rev.4, 11/07/00, Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414, 4.1 (iii) (a)). From the single peak area of each sample listed in table A4.2.3-3 the percentages in table A4.2.3-4 were calculated relative to the mean area, which was set as 100 %.

3.5.1 Relative standard deviation	The relative standard deviation of 5.0 % relates to a coumatetralyl concentration of 0.052 µg/l and the relative standard deviation of 0.9 % relates to a coumatetralyl concentration of 10.49 µg/l.
3.6 Limit of determination	The limit of quantification for coumatetralyl is 0.05 µg/l. A validation for drinking water was not necessary because the limit of quantification for surface water was validated at a level below the drinking water limit of 0.1 µg/l.
3.7 Precision	
3.7.1 Repeatability	see recovery rates (point 3.5)
3.7.2 Independent laboratory validation	Not required

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods	A method was developed and validated for determination of coumatetralyl in drinking and surface water samples. Water samples are analysed by direct injection into an HPLC instrument. Identification and quantitative determination are done by means of electropray MS/MS-detection. Calculation of concentrations of coumatetralyl is performed by comparison of the peak areas of the samples with the peak areas of the external standard solutions.
4.2 Conclusion	The mass spectrometric detector showed linear response in the concentration range of 0.042 µg/l to 10.49 µg/l for coumatetralyl with a correlation coefficient of 1.00000. The MS/MS detection of coumatetralyl is affected by the matrix. The peak area of coumatetralyl in a surface water sample containing 0.5 µg/l was reduced to approx. 42 % of the corresponding peak area in milli-Q-water. Reproducibility testing for surface water samples of coumatetralyl at concentrations of 0.05 µg/l and 10.5 µg/l yielded a relative standard deviation for the peak area of 5.0 % and 0.9 %, respectively. The relative standard deviation for the retention time was < 0.1 % for both concentrations. The limit of quantification for coumatetralyl in surface water and drinking water is 0.05 µg/l. A validation for drinking water was not necessary because the limit of quantification for surface water was validated at a level below the drinking water limit of 0.1 µg/l. Because of the high selectivity of the HPLC-MS/MS method, an additional confirmatory method is not required.

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.3 WATER

	For determination of storage stability of coumatetralyl in water, surface water samples with a concentration of 0.524 µg/l were stored for a period of three weeks. Coumatetralyl was stable for a storage period of at least 21 days under refrigerator as well as freezer conditions. The mean recoveries were in the range of 104 to 105 %.
4.2.1 Reliability	1
4.2.2 Deficiencies	No

SECTION A4 Annex Point IIA4.2 & IIIA-IV.1	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION 4.2.3 WATER	
	EVALUATION BY COMPETENT AUTHORITIES	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	January 2005	
Materials and methods	Acceptable	
Conclusion	Adopted	
Reliability	1	
Acceptability	acceptable	
Remarks		

Table A4.2.3-1: Characteristics of the surface water

Parameter	Value
Total organic carbon (TOC)	3 mg/l
Dissolved organic carbon (DOC)	3 mg/l
Conductivity at 25 °C	535 µS/cm
pH	7.3
Water hardness	11.2 °dH
Dry residue after filtration (mud content)	144 mg/l

Table A4.2.3-2: Linearity of HPLC-MS/MS detection of coumatetralyl

Concentration [µg/l]	1. Area [area counts]	2. Area [area counts]	Area average [area counts]	Area calculated [area counts]
0.0420	106	100	103	106
0.0525	123	124	124	129
0.1049	252	266	259	243
0.5245	1155	1149	1152	1162
1.049	2304	2356	2330	2311
5.245	11550	11384	11467	11498
10.49	23180	22811	22996	22982

Constant (a) = 2189.51
Point of intersection (b) = 13.8
Correlation coefficient = 1.00000

Table A4.2.3-3: Method validation of coumatetralyl (peak areas)

Surface water sample conc. [µg/l]	Peak area						
	Single Values					Mean value [area counts]	Relative standard deviation [%]
0.052	119	129	121	122	124		
	113	116	114	110	124	119	5.0
10.49	22130	22636	22823	22869	22893		
	22640	22649	22693	22603	22696	22663	0.9

Table A4.2.3-4: Method validation of coumatetralyl (percentages found)

Surface water sample conc. [µg/l]	Percentage found						
	Single Values					Mean value	Relative standard deviation [%]
0.052	100	108	102	103	104		
	95	97	96	92	104	100	5.0
10.49	98	100	101	101	101		
	100	100	100	100	100	100	0.9

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (1)

		Official use only
	1 REFERENCE	
1.1 Reference	[redacted] 2003, Method: Determination of coumatetralyl in Liver by HPLC: Methodology and Validation of Analyses; [redacted] Contract Report: LC0304/040 (unpublished), December 2003, MO-04-001349	
1.2 Data protection	Yes	
1.2.1 Data owner	Bayer CropScience AG	
1.2.2 Companies with letter of access		
1.2.3 Criteria for data protection	Data on existing active substance submitted for the first time for entry into Annex I/IA	
	2 GUIDELINES AND QUALITY ASSURANCE	
2.1 Guideline study	No	
2.2 GLP	No	
2.3 Deviations	No	
	3 MATERIALS AND METHODS	
3.1 Preliminary treatment	A sample of liver is chopped and a 2-g subsample ground in anhydrous sodium sulphate. Warfarin internal standard is added and the mixture is homogenized after adding 15 ml chloroform/acetone (1/1). The content is transferred into a centrifuge tube, shaken for 10 minutes and centrifuged for 5 minutes. The supernatant is decanted and the extraction process repeated twice more with chloroform/acetone (1/1). The combined extracts are evaporated and taken up in hexane/chloroform/acetone (GPC Solvent) for application to a gel permeation column, containing Bio-Beads SX-3, for clean-up. The eluent from the column is again evaporated and taken up in mobile phase for HPLC analysis. The HPLC is equipped with an Alltech 250 × 4.6 mm, 5-µm Econosil C18 column, fluorescence detector and post-column reagent pump. The solvent is methanol/water, acidified with acetic acid.	
3.2 Detection		
3.2.1 Separation method	HPLC analysis with post-column reaction is used: Aliquots of the sample are chromatographed on an Alltech 250 x	

		4.6 mm, 5 µm Econosil C18 column (acid) using acidified methanol and water as the mobile phase. The post-column reagent is ammonia/methanol/water (10/10/80). The flow rate of the ammonia solution is adjusted until the effluent has a pH of approximately 10.1. This is equivalent to a micrometer setting of 0.04 on the post column reagent pump of the Waters system, or a flow rate of 0.35 ml/min on the Agilent system. Sparging of this solution is recommended. The HPLC is run at a flow-rate of 1.5 ml/min with helium sparging, using a gradient programme as follows: Initial: 65 % A; 35 % B 6 minutes: 84 % A; 16 % B 10 minutes: 95 % A; 5 % B 15 minutes 65 % A; 35 % B Mobile phase: A. 2.50 ml of glacial acetic acid is added to HPLC methanol to 1 l and filtered through a 0.22-µm filter. B. 1.25 ml glacial acetic acid is added to deionised water to 500 ml and filtered through a 0.45-µm filter.
3.2.2	Detector	Fluorescence detector is used for determination.with an excitation wavelength of 310 nm, an emission wavelength of 390 nm, a gain of x1000 and a filter setting of 1.5 for the Waters system. The retention time is 7.4 and 8.8 minutes for warfarin and coumatetralyl respectively. Greatest detector sensitivity is achieved by keeping the volume of post column reagent to a minimum and instead using stronger solution strength.
3.2.3	Standard(s)	Warfarin, at a concentration 5 x higher than the coumatetralyl standard (to give similar peak areas) is used as the internal standard.
3.2.4	Interfering substance(s)	Substances of the sample matrix may interfere. Matrix effects are eliminated by using an internal standard (warfarin).
3.3	**Linearity**	
3.3.1	Calibration range	The linearity and sensitivity of the detector are determined by analysing five different concentrations ranging from 0.05 µg/ml to 1.00 µg/ml (see table A4.2.4-1).
3.3.2	Number of	Single measurement of five concentrations (see table A4.2.4-1).

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (1)

measurements	
3.3.3 Linearity	No data about the equation of the calibration line and the correlation coefficient.
3.4 Specificity: interfering substances	Matrix effects are eliminated by using an internal standard (warfarin).
3.5 Recovery rates at different levels	Initial method validation: Liver samples are spiked with 0.2, 2.0 and 4.0 µg coumatetralyl in 2 g liver (= 0.1, 1.0 and 2.0 µg/g) and analysed. The precision derived from each set of spiked samples, is calculated as the relative standard deviation (coefficient of variation). The initial method uncertainty at 95 % confidence level is ± 11 % and the recoveries were 92 % at 0.1 µg/g, 99 % at 1.0 µg/g and 99 % at 2.0 µg/g (all n = 6-8). (See table A4.2.4-2). Running method validation: Method precision and uncertainty are measured from the running results of sample duplicates carried out in the course of using the method. The running method uncertainty at 95 % confidence is ± 24 %. (See table A4.2.4-3).
3.5.1 Relative standard deviation	See recovery rates (point 3.5)
3.6 Limit of determination	The method detection limit (MDL) in tissue is 0.02 µg/g, where the tissue sample weighs 2 g. Initial method validation: To calculate the method detection limit (MDL) for the method, eight replicates near the estimated MDL (5 x MDL) are assayed and the standard deviation s is calculated. From a table of one-sided t distribution, the value of t for 8-1 = 7 degrees of freedom was 1.89 at 95 % confidence level. The MDL for the method was taken as 1.89 x s. For the practical quantitation limit (PQL) a value equal to 5 times the method detection limit (MDL) is taken. (See table A4.2.4-2).
3.7 Precision	
3.7.1 Repeatability	See recovery rates (point 3.5)
3.7.2 Independent laboratory validation	Coumatetralyl concentrations measured in sub samples from the same liver tissue by the Landcare Research laboratory and by a second independent laboratory, LincLab, New Zealand were compared.

Six samples of rat liver tissue were analysed.

The LincLab method was based on the Landcare method: Liver was extracted with chloroform/acetone and residues resuspended after drying into acetonitrile. The extract was passed through a C18 solid-phase extraction column to remove co-extractants and the eluant analysed by reversed phase LC with external standards for coumatetralyl and UV diode array detector. Instrument detection limit, IDL = 0.02 ppm (mg/l); method detection limit, MDL = 0.16 µg/g. Recovery data, 90, 92, 92 and 109 % recovery from fortifications at 1 µg/g. The MDL for this method is approximately 10 x higher than the Lancare method due to the relative intensity of the UV detector. Paired t-tests between concentrations measured in the same samples by the different laboratories revealed no significant difference between laboratories for coumatetralyl residue concentrations (t_5 =-0.511, P = 0.631). Landcare Research results: 0.63, 0.67, 0.72, 1.1, 0.77 and 0.60 µg/g.
LincLab results: 0.82, 0.80, 0.70, 1.1, 0.50 and 0.79 µg/g.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

A method for the determination of coumatetralyl in animal liver tissue using HPLC with fluorescence detection has been developed based on the method of Hunter. A post-column pH switching technique using ammonia/methanol/water exploits the natural fluorescence of this compound. Warfarin is used as an internal standard.

The initial method validation was based on the APHA (American Public Health Association) Standard Methods.

Once the method is up and running, method precision and uncertainty are measured from the running results of sample duplicates carried out in the course of using the method (running method validation).

4.2 Conclusion

Chromatography of standard solutions gave a linear calibration curve for coumatetralyl over the range 0.05 µg/ml to 1.00 µg/ml.

The method detection limit (MDL) in tissue is 0.02 µg/g, where the tissue sample weighs 2 g.

The initial method uncertainty at 95 % confidence level is ± 11 % and the recoveries were 92 % at 0.1 µg/g, 99 % at 1.0 µg/g and 99 % at 2.0 µg/g (all n = 6-8). The running method uncertainty at 95 % confidence is ± 24 %.

Interlaboratory tests revealed no significant difference between

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (1)

	laboratories for coumatetralyl residue concentrations (t_5 =-0.511, P = 0.631). Results obtained using this analysis for coumatetralyl are expected to be quite typical and suitable to accurately measure concentrations in liver tissue.
4.2.1 Reliability	2
4.2.2 Deficiencies	Yes, no data about purity of coumatetralyl and warfarin used as standards; no data about the equation of the calibration line and the correlation coefficient

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
ANNEX POINT IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (1)
	EVALUATION BY COMPETENT AUTHORITIES
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	January 2005
Materials and methods	Acceptable
Conclusion	Adopted. LOQ cannot be defined lower than the lowest fortification level: 0.1 mg/kg
Reliability	2
Acceptability	acceptable The study is acceptable despite that no information about the correlation coefficient and the equation of the linearity is submited. The linearity curve, which is submitted seems to be linear.
Remarks	

Table A4.2.4-1: Linearity for coumatetralyl

Concentration [µg/ml]	Peak area
0.05	813581
0.10	1402053
0.20	2679935
0.50	6723566
1.00	12270363

Table A4.2.4-2: Initial method validation (method bias, precision, uncertainty and detection limits)

Replicate	Fortification level [µg/g]		
	0.1	1.0	2.0
1	0.1	1.0	2.04
2	0.09	1.04	2.06
3	0.1	1.06	1.96
4	0.09	0.91	2.12
5	0.08	0.94	1.85
6	0.09	1.02	1.85
7	0.09	-	2.02
8	0.1	-	1.99
Bias [%]:	-8	-1	-1
Precision [%]:	8	6	5
Method uncertainty (95 % c.l.) [%]:	11	8	7
Method detection limit [µg/g]:	0.0134		
Practical quantitation limit [µg/g] :	0.0668		

The method bias is equivalent to recoveries of 92 %, 99 % and 99 % at 0.1, 1.0 and 2.0 µg/g respectively. The method uncertainty is taken as ± 11 %, and the method detection limit 0.02 µg/g.

Table A4.2.4-3: Running method validation (analysis of duplicates)

1st result of analysis	2nd result of analysis
0.15	0.15
0.11	0.09
0.1	0.11
0.12	0.12
0.08	0.09

0.14	0.13
0.162	0.235
19.475	16.094
Mean method precision = 17 % **Mean method uncertainty (95 % c.l.) = 24 %**	

SECTION A4	**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**
Annex Point IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (2)

Official use only

1 REFERENCE

1.1	**Reference**	D. E. Mundy and A. F. Machin, 1982, The multi-residue determination of coumarin-based anticoagulant rodenticides in animal materials by high-performance liquid chromatography, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, Great Britain, Journal of Chromatography, 234 (1982), 427 – 435 (published), MO-03-003654
1.2	**Data protection**	No
1.2.1	Data owner	publication
1.2.2	Companies with letter of access	
1.2.3	Criteria for data protection	No data protection claimed

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	No
2.2	**GLP**	No
2.3	**Deviations**	Not applicable

3 MATERIALS AND METHODS

3.1 Preliminary treatment

Extraction:
Liver and stomach contents (10 g) were homogenised with anhydrous sodium sulphate (20 mg) and chloroform (30 ml). The extract was filtered through sintered glass, the residue was homogenised with a further 15 ml of chloroform and filtered. The filtrates were combined. Serum or urine (10 ml) was acidified with hydrochloric acid (5 N, 2 ml) and extracted with 15 and 10 ml of chloroform. The combined extracts were dried with anhydrous sodium sulphate and filtered. Filtrates were concentrated under a stream of nitrogen at 35 °C.

Clean-up:
The chloroform extract was concentrated to 10.0 ml, a 2-ml aliquot was injected on to a Sep-Pak cartridge and elution was performed with 4 ml of methanol-chloroform (15:85, v/v). The

eluate was taken to dryness at 35 °C under nitrogen and the residue was dissolved in methanol (0.1 – 2.0 ml, according to the expected rodenticide content). For a more selective clean-up the chloroform extract was concentrated to about 1 ml and transferred to the cartridge, which was eluted with chloroform (4 ml).

3.2 Detection

3.2.1 Separation method

The determination in animal relicta is based on high performance exclusion chromatography. The first three compounds, including coumatetralyl, are not separated, but are subsequently differentiated by adsorption or reversed-phase high-performance liquid chromatography of the appropriate eluate fraction collected from the exclusion column, with UV detection.

Chromatographic conditions:

The HPLC columns were of stainless steel, 5 mm I. D., internally polished. Exclusion and adsorption columns were 250 mm long, slurry packed with Magnusil 5 µm porous silica; reversed-phase columns were100 mm long, packed with Magnusil 8H C_{22}.

Determination:

Duplicate aliquots (20 µl) of extracts and of standard solutions in methanol were chromatographed on the exclusion column with methanol as eluent at a flow rate of 1 ml/min.

Quantification followed by reference to standard solutions (see point 3.2.3). If a peak was detected at the retention time of the three compounds, including coumatetralyl, the eluate fraction was collected for identification by adsorption or reversed-phase chromatography.

Identification:

The eluate fraction was taken to dryness under nitrogen and the residue was dissolved in 50 µl of cyclohexanone-dichlormethane-acetic acid (75:25:0.6, v/v) for adsorption chromatography or methanol-water-acetic acid (80:20:0.8, v/v) for reversed-phase chromatography. In either case the solvent was used as the mobile phase for HPLC, at a flow-rate of 1 ml/min. If more than one of the three rodenticides was identified, quantification was by reversed-phase chromatography with reference to suitable standards.

3.2.2 Detector

Exclusion column: fluorescence detector, operated at excitation and emission wavelengths of 315 and 410 nm, respectively.

Adsorption or reversed-phase chromatography : UV detector, detection at 260 nm.

SECTION A4	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (2)

3.2.3 Standard(s)	External standards (mixtures of rodenticides (Warfarin and one of the other three compounds (coumatetralyl, Brodifacoum and Difenacoum) for the exclusion chromatography).
3.2.4 Interfering substance(s)	Substances of the sample matrix (co-extractives) and other coumarin-based rodenticides may interfere.
3.3 Linearity	
3.3.1 Calibration range	Chromatography of standard solutions gave a linear calibration curve for coumatetralyl over the range 300 pg to 2.5 µg.
3.3.2 Number of measurements	No number of measurements mentioned in the report.
3.3.3 Linearity	No data about correlation coefficient in the report.
3.4 Specificity: interfering substances	Coumatetralyl was eluted well before the co-extractive fraction and the coumarin-based rodenticide Warfarin by using exclusion chromatography and after this it could be differentiated/separated in the collected eluate fraction from other coumarin-based rodenticides by adsorption or reversed-phase chromatography.

3.5 Recovery rates at different levels

Fortification experiments for coumatetralyl were performed over the range from 0.05 mg/kg to 1.0 mg/kg. Five separate samples of substrate (pig liver) at each fortification level were analysed.

Recoveries from spiked liver:

Fortification level [mg/kg]	Mean recovery [%](*)	± 95 % confidence interval
1.0	90	± 7.6
0.5	91	
0.1	87	
0.05	93	

(*) Results are presented as the mean recovery at each fortification level.

3.5.1 Relative standard deviation

No relative standard deviation mentioned in the report.

3.6 Limit of determination

The lower limit of determination is about 0.02 mg/kg. A more selective clean-up lowers the limit for coumatetralyl to about 1 µg/kg or below.

3.7 Precision

Non-entry field

3.7.1 Repeatability

see recovery rates (point 3.5)

3.72 Independent laboratory validation

No data

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

The purpose of this study was to examine the applicability of an analytical method for the determination of the rodenticide coumatetralyl (and three other rodenticides) in animal relicta by high-performance exclusion chromatography. Extracts are cleaned-up on silica cartridges and chromatographed on a porous silica exclusion column, from which the rodenticides are eluted before co-extractives. Three of the compounds, including coumatetralyl, are not separated, but are differentiated by HPLC of collected eluates on an adsorption or reversed-phase column, with UV detection.

4.2 Conclusion

Coumatetralyl can be rapidly and sensitively determined in animal materials by exclusion HPLC on porous silica. The method appears to be applicable to wide range of substrates.

	The lower limit of determination is about 0.02 mg/kg. A more selective clean-up lowers the limit for coumatetralyl to about 1 µg/kg or below. The method can be extended to determine sub-µg/kg levels. Mean recoveries from spiked substrate were generally above 80 % at levels of 0.05 – 1.0 mg/kg.
4.2.1 Reliability	3
4.2.2 Deficiencies	Yes, test substance not specified; incomplete information about the Linearity (number of measurements, equation of calibration line and correlation coefficient not mentioned), only calibration range mentioned in the report instead of the single concentrations used for calibration; no graph no data about the range of the recovery rates at each fortification level (only mean values of recoveries for each level mentioned) and about the relative standard deviation;

SECTION A4	**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION**
Annex Point IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (2

	EVALUATION BY COMPETENT AUTHORITIES
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	January 2005
Materials and methods	acceptable
Conclusion	Adopted
Reliability	3
Acceptability	not acceptable
Remarks	The method seems to fulfill all criterias (recovery 70-110% and RSD < 20%) but the documentation for the validation is not sufficient. A fully validation of method 2 or a new method must be submitted

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
Annex Point IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (3)

			Official use only
		1 REFERENCE	
1.1	**Reference**	R. Bacher, 2007, Method: Validation of an analytical method for the determination and confirmation of coumatetralyl in blood by LC/MS/MS; PTRL Europe, D-89081 Ulm, Germany, PTRL Europe Report No. B 1262 G (unpublished), May 2007, M-288425-01-1	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Bayer CropScience AG	
1.2.2	Companies with letter of access		
1.2.3	Criteria for data protection	Data on existing active substance submitted for the first time for entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes Method was performed according to EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7 of March 17, 2004; and Commission Directive 96/46/EC amending Council Directive 91/414/EEC of 16 July 1996.	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**	1.0 mL of whole blood was extracted using 1.5 mL of acetone. After centrifugation the supernatant was cleaned-up using a disposable kieselguhr column. The column was eluted using ethyl acetate, followed by dichloromethane and hexane. The combined eluate was evaporated to dryness. The residue was re-dissolved in 10 mL of acetonitrile/water (1/1, v/v) for LC/MS/MS determination. This method was developed and validated based on a multi-residue method for blood published by Frenzel et al. (T. Frenzel, H. Sochor, K. Speer, and M. Uihlein, Journal of Analytical Toxicology, Vol. 24, July/August 2000: Rapid Multimethod for Verification and Determination of Toxic Pesticides in Whole Blood by Means of Capillary GC-MS). The method was adapted to LC/MS/MS detection owing to the polarity of coumatetralyl.	

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
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3.2 Detection

3.2.1 Separation method

RP-HPLC conditions are used:

Aliquots of the sample are chromatographed on an Phenomenex Luna C18, 50 mm length, 2 mm i.d., 5 µm particle size with a pre-column (Phenomenex C18 RP, 4 x 2 mm) using acidified water and acidified acetonitrile as the mobile phase.

The HPLC is run at a flow-rate of 250 µl/min using a gradient programme as follows:

Initial:	50 % A; 50 % B
1 minutes:	50 % A; 50 % B
4 minutes:	5 % A; 95 % B
7 minutes:	5 % A; 95 % B
7.1 minutes:	50 % A; 50 % B
10 minutes	50 % A; 50 % B

Mobile phase:

A. 0.1 % formic acid in water.
B. 0.1 % formic acid in acetonitrile.

Retention time: ca. 2.8 min

3.2.2 Detector

Mass spectrometric detector (MS/MS):
Applied Biosystems MDS Sciex API 4000 triple quadrupole LC/MS/MS system with Turbolonspray (ESI) source, detection of negative ions.

Electrospray Ion Source Conditions:

Source temperature:	550°C
Curtain gas (CUR):	20
Nebulizer gas (GS1):	40
Turbo gas (GS2):	70
Ion spray voltage (IS):	-4500 V
Collision gas (CAD):	5
Entrance potential (EP):	-10 V
Resolution Q1 and Q3:	Unit
Dwell times:	250 msec

Mass spectrometer operating parameters:
Mass of parent ion: 291 amu; Mass of product ions: 141 and 135 amu.

The product ion of m/z 141 was used for quantitation, the product ion of m/z 135 was used for confirmation.

3.2.3 Standard(s)

External standard (coumatetralyl)

3.2.4 Interfering

Substances of the sample matrix may interfere.

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	substance(s)	However, acceptable recoveries were achieved by using external calibration with solvent standards..
3.3	**Linearity**	
3.3.1	Calibration range	The linearity and sensitivity of the mass spectrometric detector was determined by analysing eight different concentration levels in the range from 0.5 ng/ml to 100 ng/ml. (see table A4.2.4-4).
3.3.2	Number of measurements	Single and double measurements, respectively, of eight concentrations (see table A4.2.4-4).
3.3.3	Linearity	Linear regression equations (1/x weighted) and correlation coefficient: 1st Transition 291 m/z -> 141 m/z: Y = 2.6 x 105 + 8.3 x 104 (r = 0.9982) 2nd Transition 291 m/z -> 135 m/z: Y = 5.85 x 104 + 1.55 x 104 (r = 0.9987)
3.4	**Specificity: interfering substances**	Coumatetralyl was not detected in the blood control samples. Influence of matrix: The MS/MS detection of coumatetralyl was not significantly affected by the matrix which is confirmed by the recovery data obtained from external calibration using solvent standards.
3.5	**Recovery rates at different levels**	Method validation for coumatetralyl: The analytical method was validated for whole blood at fortification levels corresponding to 0.05 mg/L (LOQ) and 0.5 mg/L (10-times LOQ). The precision derived from each set of spiked samples, is calculated as the relative standard deviation (coefficient of variation). Extraction of whole blood resulted in acceptable average recoveries of 85% for both mass transitions at both fortification levels. The relative standard deviations were ≤ 4%. (n = 5, both levels). (See table A4.2.4-5).
3.5.1	Relative standard deviation	See recovery rates (point 3.5)
3.6	**Limit of determination**	The limit of quantitation for coumatetralyl in whole blood is 0.05 mg/L, where the blood sample is 1 mL. The limit of detection was determined to be 0.005 mg/L.
3.7	**Precision**	
3.7.1	Repeatability	See recovery rates (point 3.5)

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
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3.7.2 Independent laboratory validation	Not required

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods	A method for the determination of coumatetralyl in whole blood using HPLC-MS/MS detection has been developed based on the multi residue method of Frenzel et al. (2000). Whole blood samples were extracted using acetone followed by solid phase clean-up using kieselguhr. The original method was successfully adapted to LC/MS/MS detection of coumatetralyl. LC/MS/MS is demonstrated for unambiguous identification and confirmation of residues. The MS/MS instrument was operated in the Multiple Reaction Monitoring mode (MRM) using the MS/MS transition 291 m/z → 141 m/z for quantitation, and the MS/MS transition 291 m/z → 135 m/z for confirmation
4.2 Conclusion	Linear LC/MS/MS calibration functions were established for quantitation and quantitative confirmation of the analyte. Calibration levels ranged from 0.50 ng/mL to 100 ng/mL (8 levels). Calibration functions calculated by regression analysis gave always correlation coefficients r of > 0.998. The analytical method was validated for whole blood at fortification levels corresponding to 0.05 mg/L (LOQ) and 0.50 mg/L (10-times LOQ). Extraction of whole blood resulted in acceptable average recoveries of 85%. The relative standard deviations were ≤ 4%. The limit of quantitation for the LC/MS/MS method was established at 0.05 mg/L. It is concluded that this method fulfills the reproducibility requirements as defined in the EU Directive 91/414/EEC Annex II, Commission Directive 96/46/EC, and EC Guidance document on residue analytical methods (SANCO/825/00 rev. 7 17/03/04) and is, therefore, applicable as enforcement method.
4.2.1 Reliability	1
4.2.2 Deficiencies	No

SECTION	ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION
ANNEX POINT IIA4.2 & IIIA-IV.1	4.2.4 ANIMAL AND HUMAN BODY FLUIDS AND TISSUES (3)
	EVALUATION BY COMPETENT AUTHORITIES
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	July 2007
Materials and methods	Acceptable
Conclusion	Adopted
Reliability	1
Acceptability	Acceptable
Remarks	

Table A4.2.4-4: Linearity for coumatetralyl

Concentration [ng/ml]	Peak area (counts) 291 -> 141 m/z	Peak area(counts) 291 -> 135 m/z
0.5	1.54E+05	3.40E+04
1.0	3.29E+05	6.86E+04
2.0	6.38E+05	1.41E+05
5.0	1.56E+06	3.51E+05
5.0	1.56E+06	3.42E+05
10	2.99E+06	6.56E+05
20	5.67E+06	1.26E+06
50	1.32E+07	2.97E+06
50	1.31E+07	2.94E+06
100	2.48E+07	5.65E+06

Table A4.2.4-5: Method validation (precision, uncertainty and detection limits)

	Fortification level [mg/L]			
Replicate	0.05 mg/L 291 -> 141 m/z	0.05 mg/L 291 -> 135 m/z	0.5 mg/L 291 -> 141 m/z	0.5 mg/L 291 -> 135 m/z
1	81%	83%	90%	89%
2	85%	84%	87%	86%
3	88%	87%	82%	81%
4	86%	87%	83%	82%
5	85%	84%	84%	84%
Average (n = 5)	85%	85%	85%	85%
Precision [Relative standard deviation %]:	3	2	4	4
Method detection limit LOD [mg/L]:	0.005			
Practical quantitation limit LOQ [mg/L] :	0.05			

SECTION A4 (4.3) **ANNEX POINT IIIA-IV.1**	**ANALYTICAL METHODS FOR DETECTION AND IDENTIFICATION** 4.3 RESIDUES IN/ON FOOD OR FEEDSTUFFS AND OTHER PRODUCTS	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	An analytical method for the determination of coumatetralyl residues in/on food or feedstuffs and other products is not required because the active substance is not used in a manner which may cause contact with food or feedstuffs. Under normal use conditions the biocidal product which is sachet of Racumin Paste is placed in bait boxes or comparable equipments or inaccessible rats "feeding place". Because of this use pattern no contact of coumatetralyl with food or feedstuffs and other products is possible	
Undertaking of intended data submission [ ]		
	EVALUATION BY COMPETENT AUTHORITIES	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	January 2005	
Materials and methods	acceptable	
Conclusion	Adopted	
Reliability	0	
Acceptability	acceptable	
Remarks		

Document III-A

Section A8 and A9

Study Summaries, active substance

COUMATETRALYL

CAS No. 5836-29-3

for use in Rodenticides (Product Type 14)

Applicant Bayer Environmental Science

Raporteur Member State: Denmark

January 2009

Section A8 **Annex Point IIA VIII8.1-8.6 & IIIA VIII.1**	**MEASURES NECESSARY TO PROTECT MAN, ANIMALS AND THE ENVIRONMENT**	
REFERENCE	Bayer Crop Science, 2004, Safety Data sheet Racumin techn. S, Bayer Crop Science, SDS No. 102000006018, 2004-02-23, MO-04-002516	**Official use only**
8.1 Recommended methods and precautions concerning handling, use, storage, transport or fire	Handling, use and storage: Suitable protective clothing, including protective PVC or other plastic material gloves for chemicals and respiratory protection (full mask with filter, ABEK-P3) must be worn if product is handled while not enclosed, and if skin contact may occur. Contact with product must be avoided. Working clothes must be kept separate. Soiled or soaked clothing must be removed immediately. It must be cleaned separately, taking suitable precautions, or must be incinerated if necessary. Hands must be washed after work. A shower must be taken at end of work. The product should be used only in area provided with appropriate exhaust ventilation. Product must be kept away from heat and from sources of ignition (no smoking). The product is sensitive to dust explosivity. Container must be kept tightly closed, under lock and key or accessible only to specialists or people who are authorized. The product should be kept dry only in the original container at temperature not exceeding 50 °C. The rules contained in the VCI concept for separate/common storage must be observed (VCI storage class: 6.1AS; TRGS 514). It must be kept away from food, drink and animal feeding stuffs. Transport: Information concerning transport: Toxic. Product must be kept dry and separated from foodstuffs.UN No.: 3027	
8.1.1 case of fire, nature of reaction products, combustion gases, etc	In case of fire, water and carbon monoxide and dioxide could be formed.	

Section A8 **Annex Point IIA VIII8.1-8.6 & IIIA VIII.1**	**MEASURES NECESSARY TO PROTECT MAN, ANIMALS AND THE ENVIRONMENT**	
8.1.2 emergency measures in case of an accident	Accidental release measures: The recommended precautions mentioned above (handling and storage) must be considered and the personal protection equipment mentioned should be used. Product mustn't be emptied into drains or waters. Contact with spilled product or contaminated surfaces should be avoided. It should be kept away sources of ignition. Personal protective equipment should be worned. Unprotected persons must be kept away. Mechanical handling equipment should be used. Contaminated floors and objects should be cleaned thoroughly, observing environmental regulations. Spilled material should be packed in suitable containers for recovery or disposal. Spilled material should not be discharged into the drains/surface water/groundwater.	
8.2 Possibility of destruction or decontamination following release in or on the following: (a) air (b) water, including drinking water (c) soil	Destruction or decontamination following release in the air, water and on soil should be done by incineration.	
8.3 Procedures for waste management of the active substance for industry or professional users	The product should be disposed of by incineration in an authorised special waste incineration plant complying with local legislation. For larger quantities contact manufacturer. The waste key for the unused product is the following : 020108 agrochemical waste containing dangerous substances	
8.3.1 Possibility of re-use or recycling	No recycling	
8.3.2 Possibility of neutralisation of effects	No neutralisation	
8.3.3 Conditions for controlled discharge including leachate qualities on disposal	No discharge	

Section A8 **Annex Point IIA VIII8.1-8.6 & IIIA VIII.1**	**MEASURES NECESSARY TO PROTECT MAN, ANIMALS AND THE ENVIRONMENT**	
8.3.4 Conditions for controlled incineration	Temperature min. 1050 °C, reaction time min. 2 sec, oxygen content min. 6%	
8.4 Observations on undesirable or unintended side-effects, e.g. on beneficial and other non-target organisms	See elements given in primary and secondary poisoning risk assessment , Doc II-B- Appendix I: EXPOSURE SCENARIOS FOR PRIMARY AND SECONDARY POISONING	
8.5 Identification of any substances falling within the scope of List I or List II of the Annex to Directive 80/68/EEC on the protection of ground water against pollution caused by certain dangerous substances	Biocides and compounds derived from them are covered by List II of the Annex to Directive 80/68/EEC.	

Section A9 Annex Point IX	**CLASSIFICATION AND LABELLING**		
	CURRENT CLASSIFICATION AND LABELLING		
Class of danger	T+ Very toxic		
Hazard symbol			
R phrases	R27/28	Very toxic in contact with skin and if swallowed.	
	R48/24/25	Toxic: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed.	
	R52/53	Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.	
S phrases	S28	After contact with skin, wash immediately with plenty of soap and water.	
	S36/37	Wear suitable protective clothing and gloves.	
	S45	In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).	
	S61	Avoid release to the environment. Refer to special instructionslsafety data sheets.	

Section A9 Annex Point IX	**CLASSIFICATION AND LABELLING**	
	PROPOSED CLASSIFICATION AND LABELLING	
Class of danger	T+ Very toxic	
Hazard symbol	The symbolT+ Very Toxic	
R phrases	R61: May cause harm to the unborn child. R 26/28 Very toxic by inhalation and if swallowed. R 24 Toxic in contact with skin R 48/23/24/25 Toxic: danger of serious damage to health by prolonged exposure through inhalation, in contact with skin and if swallowed. R52/53 Harmful to aquatic organisms may cause long-term adverse effects in the aquatic environment.	
S phrases	S53 Avoid all contact S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible). S61 Avoid release to the environment. Refer to special instructions safety data sheets.	