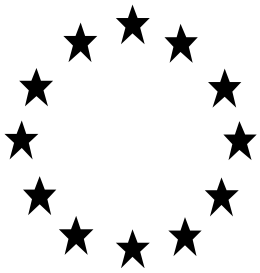
Competent Authority Report



Bromadiolone (PT14)

The Bromadiolone Task Force

DOCUMENT III-A

Section 4: Analytical Methods for Identification and Detection

Rapporteur Member State: Sweden

Final CAR April 2011

KEMI
Kemikalieinspektionen
Swedish Chemicals Agency

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Section A4.1/01	**Determination of the pure active substance and impurities in the technical material**
Annex Point IIA, IV.4.1	**Impurities**

			Official use only
		1 REFERENCE	
1.1	**Reference**	Garofani, S, Bromadiolone technical validation of the analytical method for the impurities determination, ChemService, CH-038/2004, 19 November 2004.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Bromadiolone Taskforce.	
1.2.2			
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	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline Study**	Directive 98/8/EC, 96/46/EC, SANCO/3030/99	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
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3.2.4	Interfering substance(s)	XXXXX	
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Section A4.1/01	**Determination of the pure active substance and impurities in the technical material**
Annex Point IIA, IV.4.1	**Impurities**

3.6	**Limit of determination**	XXXXX
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4.2	**Conclusion**	XXXXX
4.2.1	Reliability	
4.2.2	Deficiencies	

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	XXXXX
Materials and methods	XXXXX
Conclusion	XXXXX
Reliability	XXXXX
Acceptability	XXXXX
Remarks	XXXXX

Section A4.1/02	**Determination of the pure active substance and impurities in the technical material**
Annex Point IIA IV.4.1	**Active substance**

			Official use only
		1 REFERENCE	
1.1	**Reference**	Garofani, S., 2004, BromadioloneTechnical Validation of the Analytical Method for the Active ingredient determination, ChemService S.r.l., CH – 037/2004, 19 November 2004.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa/Bobolna Bromadiolone Taskforce.	
1.2.2			
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	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline Study**	Directive 98/8/EC, 96/46/EC, SANCO/3030/99	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	Not performed.	
3.1.2	Cleanup	Not performed.	
3.2	**Detection**		
3.2.1	Separation method	High performance Liquid Chromatography. Column: Teknokroma 120 OBDS 5 µm, 20 x 0.3 cm i.d. Eluent: Methanol:water:acetic acid (75:25:0.5, v/v/v) Flow rate: 1.0 ml Injection volume: 10 µl Retention times: BRD I: 15.01 minutes BRD II: 17.07 minutes Internal standard: 10.6 minutes	X1
3.2.2	Detector	UV/Vis detection at 254 nm	
3.2.3	Standard(s)	External bromadiolone calibration, and 1,3,5-triphenylbenzene as internal standard.	
3.2.4	Interfering substance(s)	None reported.	
3.3	**Linearity**		
3.3.1	Calibration range	20 – 60 µg/ml	X2
3.3.2	Number of measurements	Linearity test, 4 injections per concentration.	

Section A4.1/02 **Determination of the pure active substance and impurities in the technical material**

Annex Point IIA IV.4.1 **Active substance**

3.3.3 Linearity

Linearity test: $R^2 = 0.997$, $y = 65613x - 36068$

	Concentration (µg/ml)				
injection	20	30	40	50	60
1	1332557	1892866	2666216	3198975	3971264
2	1275021	1926571	2648952	3190950	3951103
3	1268270	1899316	2640714	3154606	3919639
4	1266183	1903688	2626681	3147950	3887631
Mean	1285508	1905610	2645641	3173120	3932409
SD	31592.14	14663.81	16513.13	25577.90	36639.32
RSD	2.46%	0.77%	0.62%	0.81%	0.93%

Ratio between bromadiolone analytical standard and internal standard

Area Ratio:-	Std. 1 0.40	Std. 2 0.70	Std. 3 1.00	Std. 4 1.30	Std. 5 1.60
1st injection	0.3319	0.4934	0.6915	0.8377	1.0289
2nd injection	0.3263	0.5014	0.6938	0.8125	1.0213
3rd injection	0.3245	0.5020	0.6922	0.8375	1.0228
4th injection	0.3257	0.5044	0.6903	0.8377	1.0238
Mean	0.3271	0.5003	0.6920	0.8314	1.0242
S.D.	0.0033	0.0048	0.0015	0.0126	0.0033
RSD (%)	1.00%	0.96%	0.21%	1.51%	0.32%

Linearity test ratio: $R^2 = 0.9975$, $y = 0.5751x + 0.0999$

3.4 Specifity: interfering substances

None stated.

3.5 Recovery rates at different levels

Not studied.

3.5.1 Relative standard deviation

Diastereoisomer BRD I: 0.37%
Diastereoisomer BRD II: 1.48%

X3

3.6 Limit of determination

Not stated.

3.7 Precision

3.7.1 Repeatability

Calculation of the F factor:

X4

Standard	W_{std} (mg)	W_{is} (mg)	A_{std}/A_{is}*	F Factor
1	53.20	15.86	0.8649	0.2604
2	53.10	15.86	0.8681	0.2619
1	53.20	15.86	0.8688	0.2616
2	53.10	15.86	0.8617	0.2600

Mean:	0.2610
Relative Standard Deviation:	0.35%

* Mean of two injections

W_{std} (mg)	W_{is} (mg)	A_{std}/A_{is}*	Bromadiolone content (% w/w)
158.63	416.4	0.6853	100.03
158.63	410.4	0.6746	99.90
158.63	416.2	0.6834	99.81
158.63	406.9	0.8421	125.78
158.63	412.7	0.6768	99.67
158.63	405.0	0.6620	99.35
Mean			99.8
Standard Deviation (S.D.)			0.26
Relative standard deviation (R.S.D.)			0.26%
Precision (2 x S.D.)			0.52

* Mean of two injections

The value in the grey cell was not used in calculations.

Diastereoisomer BRD I:

Area % 1st injection	Area % 2nd injection	Bromadiolone content (% w/w)	BRD I content (% area)
80.17	79.91	99.8	79.88
80.10	80.32	99.8	80.05
78.46	80.35	99.8	79.24
80.03	80.10	99.8	79.90
79.86	79.58	99.8	79.56
79.90	80.18	99.8	79.88
Mean:			79.75
Relative Standard Deviation (R.S.D.)			0.37%
Precision (2 x S.D.)			0.6

Diastereoisomer BRD II:

Area % 1st injection	Area % 2nd injection	Bromadiolone content (% w/w)	BRD II content (% area)
19.83	20.09	99.8	19.92
19.90	19.68	99.8	19.75
21.54	19.65	99.8	20.56
19.97	19.90	99.8	19.90
20.14	20.42	99.8	20.24

20.10	19.82	99.8	19.92
Mean:			20.05
Relative Standard Deviation (R.S.D.)			1.48%
Precision (2 x S.D.)			0.6

3.7.2 Independent laboratory validation: Not performed.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

The internal standard solution was 1.280 g of 1,3,5-triphenylbenzene brought to 200 ml volume (6400 ppm) with methanol and then diluted 1:80 with dichloromethane (80 ppm).

The bromadiolone stock solution for the linearity test was prepared by dissolving 50 mg bromadiolone with 350 mg triethanolamine in 25 ml of methanol. The standard solutions were then prepared from this stock by serial dilution with methanol.

The determination of the separated diastereoisomers is performed by HPLC with a method of area percentage, using UV detection. Determination of the a.i. content was performed by HPLC with UV detection, using both internal and external standard.

4.2 Conclusion

The analytical method was demonstrated to be specific for bromadiolone a.i. in the technical samples. The range tested for the a.i., 20 to 60 µg/ml (± 60% of the solution concentration used for the quantification analysis), was found to be linear. (Each correlation coefficient > 0.99.)

The precision calculated in the repeatability test, can be considered acceptable with respect to the FAO tolerance.

a.i.	99.8 ± 0.5% w/w
1st diastereoisomer (BRD I)	79.75 ± 0.5% area
2nd diastereoisomer (BRD II)	20.05 ± 0.5% area

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 March 2009

Materials and methods X1: The conditions reported are for the analysis of separated diastereomers. The conditions used for the total bromadiolone content were the same except:

Eluent: methanol/water/acetic acid = 84/16/0.05 v/v/v

X2: The calibration range was 0.40-1.60 based on concentration ratios (a.i./i.s.).

Section A4.1/02	**Determination of the pure active substance and impurities in the technical material**
Annex Point IIA IV.4.1	**Active substance**

	The calibration range corresponds approximately to 40-120% of the nominal content in the technical material. X3: The values reported are from the repeatability test (see X4 below). X4: F is the relative response factor (A_{std}/A_{is} /W_{std}/W_{is}). The repeatability data for the total bromadiolone content as well as the data for the individual diastereomers are acceptable (e.g. modified Horwitz value at 20% is 1.71%).
Conclusion	The applicant's version is adopted
Reliability	Reliability indicator 1
Acceptability	The method is acceptable for the analysis of total bromadiolone content as well as the content of the individual diastereomers.
Remarks	No remarks.

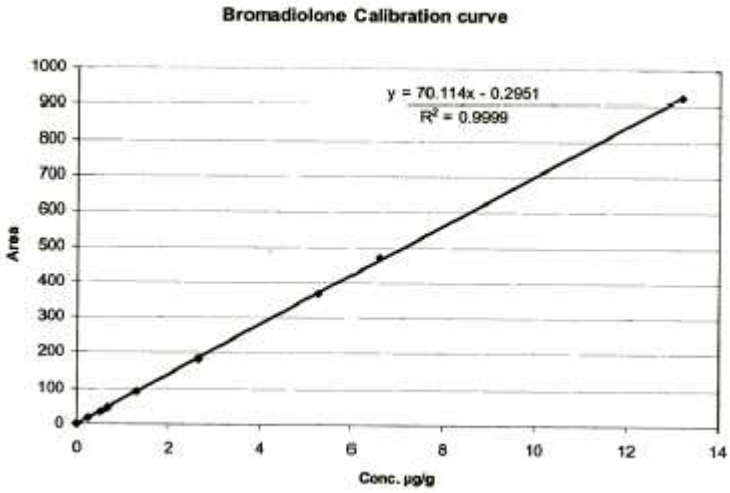
Section A4.2(a)/01 **Determination of residues in soil**

Annex Point IIA, IV.4.2(a) **bromadiolone**

			Official use only
		1 REFERENCE	
1.1	**Reference**	Morlacchini, M. (2006) Residues Determination of Brodificoum, Difenacoum and Bromadiolone in Soil: Final Report for Bromadiolone Residue Determination. CERZOO. Project Code: CZ/05/002/ACTIVA/SOIL	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa/PelGar Bromadiolone Task Force	
1.2.2	Companies with letter of access	PelGar International Ltd. Activa S.r.l.	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing [a.s. / b.p.] for the purpose of its entry into Annex I	
		2	
2.1	**Guideline**	Directive 96/23/EC	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	40.0 g of soil is placed into a series pf 500 ml sovirel, and 100 ml of a 50% acetone/50% chloroform extraction solution is added. The mixture is then shaken for a minimum of 30 minutes at a rate of 180 movements/minute, on an automatic shaker. The solvent is collected after filtration, and then another 100 ml of extraction solution is added and shaken again for a minimum of 30 minutes. This process is repeated for a third time with 50 ml of extraction solution. The three filtered solutions are combined and evaporated with a rotavapor to 200 mm of Hg.	X1
3.1.2	Cleanup	Recovery is made with 10 ml of acetone, and purification in a glass column with 6 g of florisil and 1 g of anhydrous sodium sulphate. The solution is washed with 40 ml of acetone and evaporated with nitrogen. 1 ml of methanol:water (1:1) is added and centrifuged for 5 minutes at 2000 rpm, and the final solution is transferred ready for injection into HPLC.	
3.2	**Detection**		
3.2.1	Separation method	HPLC UV-Vis Instrument: Agilent™ HPLC 1100 binary pump with DAD detector, autosampler, degasser and chiller Column for HPLC: Synergy 4u Fusion RP80A Phenomenes 150 x 4,60 mm S/N 224016-2 Volume and type of injection: 20 µl with autosampler Temperature of chiller: 25°C Software: Agielent ChemStation A7.0	

3.2.2	Detector	Diode Array Detector (DAD) Wavelength of detection: 264 nm with a window of 4 nm and a reference to 360 with a window of 100 nm
3.2.3	Standard(s)	Bromadiolone technical grade Lot Number: L12478
3.2.4	Interfering substance(s)	Non detected

3.3 Linearity

3.3.1	Calibration range	0.264, 0.258, 0.660, 1.320, 2.640, 5.280, 6.60, 13.20 µg/ml (Equivalent concentrations in soil: 0.007, 0.013, 0.017, 0.033, 0.066, 0.132, 0.165, 0.330 µg/g)
3.3.2	Number of measurements	4
3.3.3	Linearity	

Correlation coefficient > 0.998

3.4 Specifity: interfering substances

None detected X2

3.5 Recovery rates at different levels X3

File	Date	Sample name	Conc. Add. (µg/g)	Conc. Equiv. in soil (µg/g)	Area	Conc. Found (µg/g)	Recovery (%)
10190005	18/10	Rec 1	0.66	0.0165	42.5	0.61	92.5
10190014	19/10	Rec 4	0.66	0.0165	42.3	0.61	92.0
10190018	19/10	Rec 7	0.66	0.0165	42.2	0.61	91.8
10190026	19/10	Rec 10	0.66	0.0165	42.6	0.61	92.7
10190006	18/10	Rec 2	2.56	0.064	171.0	2.44	95.4
10190015	19/10	Rec 5	2.56	0.064	171.3	2.45	95.6
10190019	19/10	Rec 8	2.56	0.064	171.0	2.44	95.4
10190027	19/10	Rec 11	2.56	0.064	171.4	2.45	65.7
10190007	18/10	Rec 3	6.60	0.165	412.7	5.89	89.2

10190016	19/10	Rec 6	6.60	0.165	432.1	6.17	93.4
10190020	19/10	Rec 9	6.60	0.165	432.6	6.17	93.5
10190028	19/10	Rec 12	6.60	0.165	431.5	6.16	93.3
10190004	18/10	Blank	0.00	0.000	n.r.	0.00	
10190013	19/10	Blank	0.00	0.000	n.r.	0.00	
10190017	19/10	Blank	0.00	0.000	n.r.	0.00	
10190025	19/10	Blank	0.00	0.000	n.r.	0.00	
* All dates are 2005						Average	93.4
						Std. Dev.	1.9

3.5.1	Relative standard deviation	1.9%	
3.6	**Limit of determination**	The limits of quantisation (LOQ) and detection (LOD) for the determination of bromadiolone in soil was calculated using the standard deviation from the added mix (0.64 µg/g bromadiolone) recovery results. The LOQ was calculated as ten times the standard deviation (10s), and the LOD was calculated as three times the standard deviation (3s) of the results of the analysis of a minimum of 4 samples. LOQ: 0.0260 LOD: 0.0078	X4
3.7	**Precision**		
3.7.1	Repeatability	No data	
3.7.2	Independent laboratory validation	No data	

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	The test method for bromadiolone determination in soil is based on extraction from blank and spiked soil (40.0 g) using a chloroform:acetone 1:1 solution. The extract is concentrated by rotary evaporation and recovered with acetone prior to purification with a florisil-sodium sulphate column. The elutes are dried and reconstituted with methanol:water (1:1) and analysed by HPLC UV-Vis.	
4.2	**Conclusion**	The LOD, LOQ, recovery rates and linearity suggest that the method is valid for identification and analysis of bromadiolone in soil.	X5
4.2.1	Reliability	1	X6
4.2.2	Deficiencies	Yes	

Evaluation by Competent Authorities
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
EVALUATION BY RAPPORTEUR MEMBER STATE

Section A4.2(a)/01 **Determination of residues in soil**

Annex Point IIA, IV.4.2(a) **bromadiolone**

Date

March 2009

Materials and methods

X1: Hazardous solvents like chloroform should be avoided in analytical methods for enforcement purposes as stated in the TNsG on Analytical Methods.

X2: Analysis of untreated soil control samples indicated no interferences. However, HPLC-UV is not a highly specific method as defined by the TNsG on Analytical Methods and a confirmatory method is thus needed.

X3: The characteristics of the soil used in the validation is reported as:

Parameter	Result
pH	5.95
Cationic Exchange capacity (meq/100 g)	36.42
Organic matter (%)	5.04
Cd (mg/kg)	< 0.015
Hg (mg/kg	0.024
Ni (mg/kg)	161.75
Pb (mg/kg)	12.65
Cu (mg/kg)	393.65
Zn (mg/kg)	215.30
B & J Test Cr (IV) (mg/kg)	0.023
Silt (%)	25.84
Clay (%)	26.52
Sand (%)	47,64
Assimilable P (mg/kg)	134.54
Total N Kjeldhall (%)	2227.40

X4: LOQ should be set at the lowest level used for fortification, as long as acceptable accuracy and precision was obtained for that level (i.e. not based on signal to noise ratios)

Conclusion

X5: The method utilises hazardous agents (chloroform), which should be avoided for enforcement methods. Moreover, the technique used (HPLC-UV) is not highly specific, as defined by the TNsG on Analytical methods and a confirmatory method is thus needed.

Reliability

The method itself appears to be sufficiently validated, but the reporting of the study is somewhat incomplete. In addition to this the method utilises chloroform an d the reliability is thus lowered to:

Reliability indicator 3

Acceptability

Due to the use of chloroform, the method is not accepted in this form. Moreover, a confirmatory method is needed.

The applicant has submitted a report for a new method ("Residues determination of BRODIFACOUM, DIFENACOUM and BROMADIOLONE in soil"; Morlacchini, M. 2009) but no summary. The methiod appears to be in compliance with the criteria in the TNsG for Analytical methods, except that the validation was done at three levels (0.066, 0.132 and 6.6) using four samples at each levels. The RMS propses to include the new method in a revised CAR.

Remarks

None

Section A4.2(a)/02 **Determination of residues in soil**

Annex Point IIA, IV.4.2(a) **bromadiolone**

			Official use only
		1 REFERENCE	
1.1	**Reference**	Morlacchini, M., 2009. Residues determination of Brodifacoum, Difenacoum and Bromadiolone in soil. Supplement n.2 to the Final Report: Bromadiolone residue determination. CERZOO (Italy), Study CZ/05/002/Activa/Soil	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force Activa / Babolna / Laboratorios Agrochem S.A. / PelGar Bromadiolone task force	
1.2.2			
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s./ b.p. for the purpose of its entry into Annex I authorisation	
		2	
2.1	**Guideline**	Directive 96/23/EC	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	100.0g of soil was weighed into a series of 500ml sovirel. The fortified samples were prepared by adding 1.0ml aliquots of the appropriate spiking solutions, mix B, D, and F approximately from 0.66 to 6.6µg/g. 150ml of dichloromethane was added to the sovirel. The soveril was closed and shaken for a minimum of 30 minutes at a rate of approximately 180 movements/ minute on an automatic shaker. The solvent was collected in a 500ml rotavapour balloon after filtration on sodium sulphate. Another 100ml quantity of dichloromethane was added and the process repeated again for a further 30 minutes. The extraction was filtered again and the process repeated with a further 100 ml of dichloromethane. The three filtered solutions were combined and evaporated with a rotavapor to 450mm Hg. Dichloromethane was used as the extraction solvent because of its very good extraction capacity.	
3.1.2	Cleanup	The recovery was made with 10ml of dichloromethane, which was evaporated with nitrogen. One ml of methanol: water (1:1) was added and then centrifuged for 5 minutes at 2000 rpm. The final solution was then transferred in to a 2 ml vial cap for injection into HPLC or stored in a freezer at -20°C if injection doesn't occur immediately.	X1
3.2	**Detection**		

Section A4.2(a)/02	**Determination of residues in soil**
Annex Point IIA, IV.4.2(a)	**bromadiolone**

3.2.1	Separation method	The analysis was performed with a HPLC MS. Column type: Thermo Electron Corporation Hypersil Gold C18 5µm, 100x4,60 mm/S/N 0572062N Volume and type of injection: 10µl with autosampler Temp of column holder: 25ºC Ions of identification and detection: 509.60 -510.70 m/z The solvents utilised were water with 0.1% of formic acid and acetonitrile with the following solvent gradient program, carrier gas was nitrogen: Table 1. Solvent gradient program of HPLC MS:	X2
3.2.2	Detector	Mass spectrometry detector. Detector settings: MSQ: ESI+, SIM m/z 510.0 [M+H) +, dwell time 0.5 sec, span0, 5 amu. T (ESI): 550°C. Cone voltage: 70V.	X3
3.2.3	Standard(s)	Bromadiolone 98.5% Lot.60921. Dr. Ehrenstorfer GmbH	
3.2.4	Interfering substance(s)	Non detected	
3.3	**Linearity**	Non-entry field	
3.3.1	Calibration range	0, 0.06, 0.09, 0.14, 0.15, 0.34, 0.67, 1.65, 3.34, 5.52, 6.80 and 13.03 µg/g (Conc. Equiv. in soil. 0, 0.00066, 0.000825, 0.00132, 0.00165, 0.0033, 0.0066, 0.0165, 0.033, 0.0528, 0.066 and 0.132 µg/ g).	X4
3.3.2	Number of measurements	4 measurements at fortification levels.	
3.3.3	Linearity	Fig 1: Bromadiolone calibration curve 0-1.65 µg/g	

Time (min)	Flow (ml/min)	Water 0.1% formic ac. (%)	Acetonitrile (%)
0.00	0.5	50.0	50.0
5.00	0.5	50.0	50.0
10.00	0.5	10.0	90.0
15.00	0.5	10.0	90.0
18.00	0.5	50.0	50.0
22.00	0.5	50.0	50.0

X5

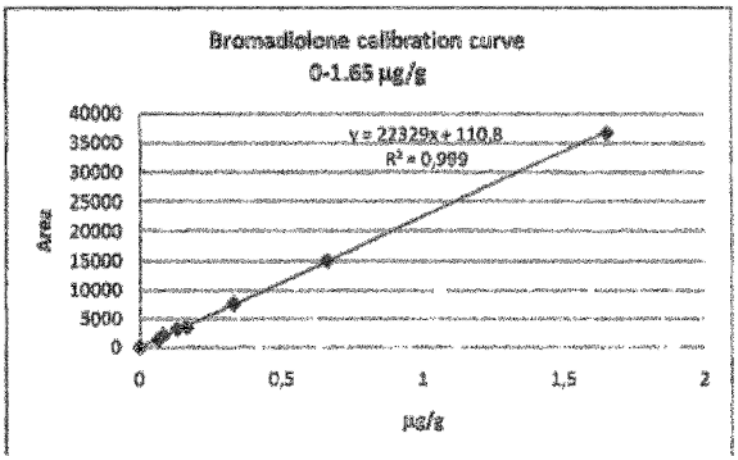
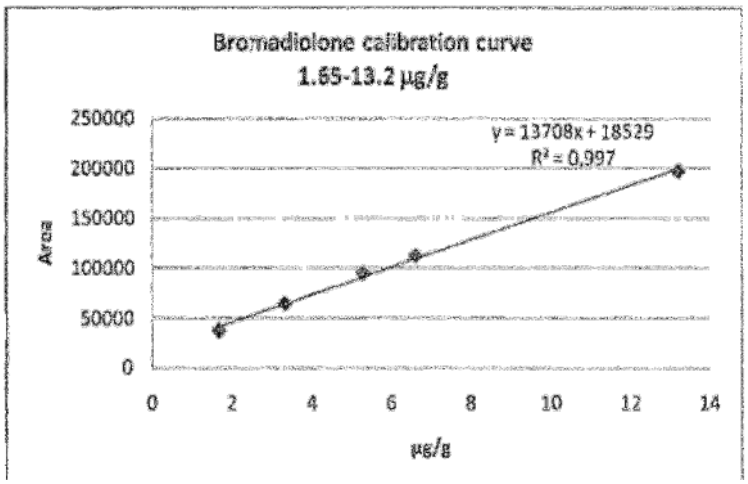
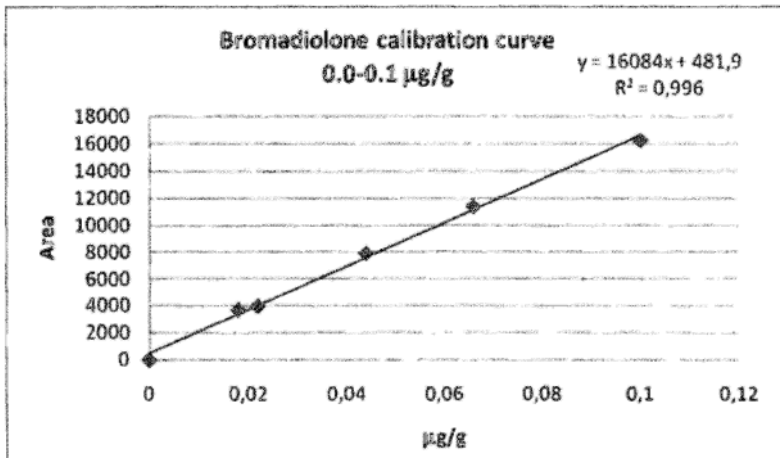


Fig 2: Bromadiolone calibration curve 0-1.65 µg/g



For linear regression equations describing the detector response as a function of the standard calibration curve concentrations, the correlation coefficients (R^2) was greater than 0.997.

Fig 3: Bromadiolone calibration curve 0.0-0.1 µg/ g



3.4 Specifity: interfering substances

Non detected

3.5 Recovery rates at different levels

The average recovery for 12 spiking soil of Bromadiolone was 88.9±8.8. In the second test (added concentration of 0.22 µg/g) performed the average recovery was 97.1±0.7.

X6

Table 2: Recovery tests with added concentration 0.066, 0.132 and 6.6µg/g.

080515_15	16/05/2008	Rec 1	0,066	0,00088	1248	0.05081	77.2
080516_C_02	16/05/2008	Rec 2	0,066	0,00088	1245	0.05079	77.0
080516_C_15	16/05/2008	Rec 3	0,066	0,00086	1256	0.05124	77.6
080521_02	21/05/2008	Rec 4	0,066	0,00085	1261	0.05151	78.0
080515_17	16/05/2008	Rec 11	0.132	0.00152	3002	0.12948	98.1
080516_C_04	16/05/2008	Rec 12	0.132	0.00132	2986	0.12876	97.6
080516_C_17	16/05/2008	Rec 13	0.132	0.00132	2984	0.12779	96.8
080521_04	21/05/2008	Rec 14	0.132	0.00132	2974	0.12822	97.1
080515_18	16/05/2008	Rec 21	6,60	0.088	100826	6.00399	91.1
080516_C_05	16/05/2008	Rec 22	6,60	0.088	1001125	6.00904	89.4
080516_C_18	16/05/2008	Rec 23	6,60	0.088	1012053	6.041182	91.5
080521_05	21/05/2008	Rec 24	6,60	0.088	1011578	6.038299	91.3
080516_C_01	16/05/2008	blank	0,00	0,000	n.r.	0,00	
080516_C_14	16/05/2008	blank	0,00	0,000	n.r.	0,00	
080516_C_27	16/05/2008	blank	0,00	0,000	n.r.	0,00	
080521_01	21/05/2008	blank	0,00	0,000	n.r.	0,00	
						Average	88.9
						std. Dev.	8.8

Table 3 Average recovery and standard deviation about the range 0.066-6.6 µg/g

Compound	Validation Range (µg/g)	Number of Samples (n)	Average Percent Recovery	Standard Deviation (s)
Bromadiolone	0.066–6.6	12	88.9	8.8

Table 4: Recovery test performed with added concentration 0.022 µg/g.

File	Date	Sample name	Conc. µg/g	Conc. Equivalent in soil µg/g	Area	Conc. Found µg/g	Recovery %
090929_B_06	29/09/2009	Rec 31	0.022	0.00022	3922	0.02139	97.2
090929_B_07	29/09/2009	Rec 32	0.022	0.00022	3924	0.02140	97.3
090929_B_08	29/09/2009	Rec 33	0.022	0.00022	3942	0.02151	97.8
090929_B_09	29/09/2009	Rec 34	0.022	0.00022	3874	0.02109	95.9
090929_B_10	29/09/2009	Rec 35	0.022	0.00022	3927	0.02142	97.4
						Average	97.1
						Std dev	0.7

3.5.1 Relative standard deviation

8.8% - first test

0.73% - second test

X6

3.6 Limit of determination

The limit of quantification (LOQ) and detection (LOD) for the determination of Bromadiolone in soil was calculated using the standard deviation from the added mix M (0.066µg/ g Bromadiolone) recovery results. The LOQ was calculated as ten times the standard deviation (10*s*) and the LOD was calculated as three times the standard deviation (3*s*) of the results of the analysis of a minimum of 4 samples.

LOQ = 0.003543

LOD = 0.001063

Table 5. LOD and LOQ at the spiked concentration of 0.066 µg/g

Compound	Average Recovery (µg/g)	Standard Deviation (*s*)	Limit of Detection (3*s*)	Limit of Quantitation (10*s*)
Bromadiolone	0.060275	0.000354	0.001063	0.003543

This value of calculated LOQ was below the lowest standard and so the LOQ will be 0.004 µg/ g (lowest spiked concentration 0.066 µg/g). In a similar manner, the calculated LOD for the analyte was below 0.001 µg/g.

Since the calculated LOQ was lower than 0.066 µg/ g, a recovery test was performed at 0.022 µg/ g (equivalent to 0.00022 µg/ g in soil).

Table 6: LOD and LOQ at the added concentration of 0.022 µg/ g.

Compound	Average Recovery (µg/g)	Standard Deviation (*s*)	Limit of Detection (3*s*)	Limit of Quantitation (10*s*)
Bromadiolone	0.021362	0.000169	0.000480	0.001600

X7

3.7 Precision

3.7.1 Repeatability

No data

3.7.2 Independent laboratory validation

No data

Section A4.2(a)/02	**Determination of residues in soil**
Annex Point IIA, IV.4.2(a)	**bromadiolone**

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	The aim of the study was to develop and validate an analytical method for the determination of Bromadiolone residues in soil in order to meet European Directive requirements. The analytical method is based according to the directive 96/23/EC. The test method for Bromadiolone determination in soil was based on extraction from blank and spiked soil (100.0g) using dichloromethane and on a filtration on sodium sulphate. The extract was concentrated by rotary evaporator and recovered with dichloromethane. The elutes were dried under nitrogen flux, resumed with methanol: water 1:1 and analysed by HPLC MS.	
4.2	**Conclusion**	The limit of detection, limit of quantification, recovery rates and linearity suggest that the method is valid for identification and analysis of Bromadiolone in soil	
4.2.1	Reliability	1	X7
4.2.2	Deficiencies	No	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date April 2010

Materials and methods

X1: The description of the clean-up stage is not completely clear. It is assumed that the extract (see 3.1.1) was evaporated to 10 ml by the rotavaopor and then completely by purging with nitrogen. Then 1 mL methanol:water (1:1) was added which means that the volume of the final injected extract was 1 mL.

X2: The quantification is based on the area for both peaks of bromadiolone (1 per diasteromer)

X3: It should be noted that it appears that only one mass fragment was used for quantification/confirmation. However, the supporting chromatograms show the typical pattern for the bromine isotopes which is considered sufficient.

X4: The calibration was done using matrix matched standards in the range 0.066-13.2 µg/mL which corresponds to 100% of LOQ and 200% of the highest fortification level (i.e. 0.66 µg/kg-130.2 µg/kg). In addition as the LOQ was calculated (see X below) to be lower than the chosen lowest level of 0.66 µg/kg an additional recovery test was done at 0.22 µg/kg and for that purpose a calibration curve was also generated in the range 0-1 µg/kg (0-0.1 µg/ml)

X5: The reported range of 0-1.65 µg/g corresponds to 0-1.65 µg/ml in the injected extract (corresponds to 0-16.5 µg/kg soil). It should be noted that the blank was included in the calibration. When the blank is excluded a r^2 of 0.9997is obtained (n=7). In the same way the second and the third calibration curves corresponds to 16.5-132 µg/kg soil and 0-1 µg/kg soil respectively. If the blank is omitted for the third calibration curve a r^2 of 0.9981 is obtained (n=5).

X6: The characteristics of the soil used in the validation were as follows:

Section A4.2(a)/02 **Determination of residues in soil**

Annex Point IIA, IV.4.2(a) **bromadiolone**

Parameter	Result
pH	5.95
Cationic exchange cap. (meq/100 g)	36.42
Organic matter (%)	5.04
Cd (mg/kg)	< 0.015
Hg (mg/kg)	0.024
Ni (mg/kg)	161.75
Pb (mg/kg)	12.65
Cu (mg/kg)	393.65
Zn (mg/kg)	215.30
B & J test Cr (IV) (µM/g)	0.023
Silt (%)	25.84
Clay (%)	26.52
Sand (%)	47.64
Assimilable P (mg/kg)	134.54
Total N Kjeldhall (%)	0.25
Assimilable K (mg/kg)	2227.40

The validation could be summarized as (including the recovery experiments at 0.22 µg/kg):

Fortification level (µg/kg)	N	Recovery		%RSD
		Range	Mean	
0.22	5	95.9-97.8	97.1	0.7
0.66	4	77.0-78,0	77.5	0.7
1.32	4	96.8-98.1	97.4	0.6
66	4	91.1-92.4	91.7	0.6
blank	4	n.d.	-	-

n.d. = not detected.

X7: It should be noted that the LOQ should be set at 0.22 µg/kg which appears to be exceptionally low (i.e. the requirement is ≤50 µg/kg)

Conclusion The applicant's version is adopted.

Reliability X7: Since the reporting of the study is somewhat unclear and as only 4 samples were used per fortification level except at LOQ the reliability is lowered to:

Reliability indicator 2

Acceptability The method is considered acceptable for analysing bromadiolone in soil

Remarks None

Section A4.2(b)/01 **Determination of residues in air**

Annex Point IIA, IV.4.2(b) **bromadiolone**

	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	As the active substance has a vapour pressure of < 0.05 mPa (Section A3.2, Annex Point IIA, III.3.2.) it is considered to be of low volatility and therefore, in accordance with the TNsG on Data Requirements for the Biocidal Products Directive, analytical methods in air are not required.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	March 2009	
Evaluation of applicant's justification	Given the low vapour pressure of bromadiolone and as the representative product will not form dust/mists/aerosols under the use envisaged no analytical method for air is considered required.	
Conclusion	The applicant's justification is accepted.	
Remarks	None	

Section A4.2(c)/01 **Determination of residues in water**

Annex Point IIA, IV.4.2(c) **bromadiolone**

			Official use only
		1 REFERENCE	
1.1	**Reference**	Martinez, M. P. (2005) Bromadiolone Technical: Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface Waters. ChemService S.r.l. ChemService Study No. CH-290/2005	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Bromadiolone Task Force	
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	
		2 GUIDELINE AND QUALITY ASSURANCE	
2.1	**Guidelines**	EEC Guideline SANCO/3030/99 rev.4 11/07/00	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	1 litre of water is extracted with 3 x 50 ml of dichloromethane and the organic extract evaporated to dryness at 40°C.	
3.1.2	Cleanup	The residue is re-dissolved with 0.5 ml of methanol.	
3.2	**Detection**		
3.2.1	Separation method	HPLC/MS	
3.2.2	Detector	Mass (Scan in Selected Ion Monitoring (SIM) and Selected Reaction Monitoring (SRM))	X1
3.2.3	Standard(s)	Bromadiolone standards: 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml	X2
3.2.4	Interfering substance(s)	None	
3.3	**Linearity**		
3.3.1	Calibration range	0.1 – 0.5 µg/ml	X2
3.3.2	Number of measurements	4 measurements of each standard.	
3.3.3	Linearity	Correlation coefficient >0.99.	
3.4	**Specificity: interfering substances**	None specified	X3
3.5	**Recovery rates at different levels**	Drinking water: Recovery at fortification level L1 (0.05 µg/l)	X4

Code	A_s	C_s (1)	V_s	V_w	BDF	Recovery

Number		(µg/ml)	(ml)	(l)	(µg/l)	(%)*
Blank 1	0	-	0.50	1.0	n.d.	-
Blank 2	0	-	0.50	1.0	n.d.	-
Spike L1-1	20988560	0.10	0.50	1.0	0.0502	100.45
Spike L1-2	17841970	0.09	0.50	1.0	0.0427	85..39
Spike L1-3	20809060	0.10	0.50	1.0	0.0498	99.59
Spike L1-4	20717290	0.10	0.50	1.0	0.0496	99.15
Spike L1-5	16772310	0.08	0.50	1.0	0.0401	80.27
				Mean value:	0.046	93.0
				Standard deviation (S.D.):	0.0042	8.4464
				Coefficient of variation (C.V. %):	9.1	9.1

Drinking water: Recovery at fortification level L2 (0.5 µg/l)

Code Number	A_s	C_s (1) (µg/ml)	V_s (ml)	V_w (l)	BDF (µg/l)	Recovery (%)*
Blank 1	0	-	1.50	1.0	n.d.	-
Blank 2	0	-	1.50	1.0	n.d.	-
Spike L2-1	36670460	0.24	1.50	1.0	0.3627	72.54
Spike L2-2	38562630	0.26	1.50	1.0	0.3908	78.16
Spike L2-3	40144390	0.28	1.50	1.0	0.4143	82.85
Spike L2-4	40928040	0.28	1.50	1.0	0.4259	85.18
Spike L2-5	37724800	0.25	1.50	1.0	0.3783	75.67
				Mean value:	0.394	78.9
				Standard deviation (S.D.):	0.0231	4.62
				Coefficient of variation (C.V. %):	5.9	5.9

Drinking water: Recovery at fortification level L3 (5.0 µg/l)

Code Number	A_s	C_s (1) (µg/ml)	V_s (ml)	V_w (l)	BDF (µg/l)	Recovery (%)*
Blank 1	0	-	10.00	1.0	n.d.	-
Blank 2	0	-	10.00	1.0	n.d.	-
Spike L3-1	49330820	0.37	10.00	1.0	3.6710	73.42
Spike L3-2	56984300	0.44	10.00	1.0	4.4285	88.57
Spike L3-3	54783080	0.42	10.00	1.0	4.2107	84.21

Spike L3-4	55898500	0.43	10.00	1.0	4.3211	86.42
Spike L3-5	47742050	0.35	10.00	1.0	3.5138	70.28
				Mean value:	4.029	80.6
				Standard deviation (S.D.):	0.3665	7.33
				Coefficient of variation (C.V. %):	9.1	9.1

Drinking water: Recovery at fortification level L4 (50 µg/l)

Code Number	A_s	C_s (1) (µg/ml)	V_s (ml)	V_w (l)	BDF (µg/l)	Recovery (%)*
Blank 1	0	-	125.00	1.0	n.d.	-
Blank 2	0	-	125.00	1.0	n.d.	-
Spike L4-1	49181300	0.37	125.00	1.0	45.7028	91.41
Spike L4-2	54331770	0.42	125.00	1.0	52.0749	104.15
Spike L4-3	46162380	0.34	125.00	1.0	41.9679	83.94
Spike L4-4	54848580	0.42	125.00	1.0	52.7143	105.43
Spike L4-5	44074550	0.32	125.00	1.0	39.3849	78.77
				Mean value:	46.369	92.7
				Standard deviation (S.D.):	5.3181	10.64
				Coefficient of variation (C.V. %):	11.5	11.5

3.6 Limit of determination

50% of the lowest validated level i.e. 0.05 µg/ml, corresponding to 0.025 µg/l in the water matrix samples. X5

3.7 Precision

3.7.1 Repeatability

X6

Drinking water: Repeatability and recovery tests.
Linear calibration with working standard solutions

Bromadiolone (BDL) (m/z 527)	Standard 1 0.1 µg/ml (Peak area)	Standard 2 0.3 µg/ml (Peak area)	Standard 3 0.5 µg/ml (Peak area)
1st injection	20315380	45043050	61748890
2nd injection	20085300	45804420	62829020
3rd injection	20589540	45831680	61119610
4th injection	22080390	46187320	61854660
5th injection	21401070	44383140	58991960
Mean	20894336	45449922	61308828
SD	741098	650958	1281113
CV (%)	3.55	1.43	2.09
	Parameter m (slope)	Parameter q (intercept)	Parameter R (correlation)
	101036230	12240160	0.99237

Ground water: Repeatability and recovery tests.
Linear calibration with working standard solutions

Bromadiolone (BDL) (m/z 527)	Standard 1 0.1 µg/ml (Peak area)	Standard 2 0.3 µg/ml (Peak area)	Standard 3 0.5 µg/ml (Peak area)
1st injection	15791430	41951968	60512520
2nd injection	17459968	42683108	64342040
3rd injection	18454028	48053548	57223176
4th injection	18644500	41882680	60604248
Mean	17587482	43642826	60575496
SD	1130293	2565767	2526893
CV (%)	6.43	5.88	4.16
	Parameter m (slope)	Parameter q (intercept)	Parameter R (correlation)
	107720036	8319257	0.99277

Surface water: Repeatability and recovery tests
Linear calibration with working standard solutions

Bromadiolone (BDL) (m/z 527)	Standard 1 0.1 µg/ml (Peak area)	Standard 2 0.3 µg/ml (Peak area)	Standard 3 0.5 µg/ml (Peak area)

1st injection	19381392	43315204	64026140
2nd injection	22480358	46771520	62181400
3rd injection	23010416	49474848	68093060
4th injection	21724540	47628536	64556370
5th injection	23438180	45529232	64285460
Mean	22006977	46543868	64628486
SD	1431977	2062655	1922352
CV (%)	6.51	4.43	2.97
	Parameter m (slope)	Parameter q (intercept)	Parameter R (correlation)
	106553772	12426979	0.99620

3.7.2 Independent laboratory validation: None

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

4.2 Conclusion: The analytical method was shown to be specific for bromodiolone residues in each type of water sample. X7

The range tested was from 0.1 to 0.5 µg/ml, corresponding to concentrations ranging from 0.05 to 0.25 µg/l in the water samples and was found to be linear.

For precision, the SANCO guideline requires a RSD % lower than 20% for each fortification level; therefore the precision of the analytical method can be considered acceptable.

For accuracy, the SANCO guideline requires individual recovery values in the range 70-110% with a mean value of 80-100% at each level; some deviation obtained can be accepted because of the very low water solubility of the test substance, and the very particular and complicated method of analysis; therefore the accuracy of the analytical method can be considered acceptable.

4.2.1 Reliability: 1

4.2.2 Deficiencies: No

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date: March 2009

Materials and methods: X1: There are some inconsistencies in the description of the method in the study.

At some places it is described as a HPLC-MS method and at other places it appears to be a LC-MS/MS method. However, when this was communicated with the applicant it was clarified that the accuracy and repetability data was generated using the SIM-mode (molecular ion 527 m/z), whereas specificity and linearity data is available from both the SIM and SRM-mode (m/z 527 → 509).

X2: The volume of the extract of the fortified water samples are adapted (see table below) to be within the linear range tested (0.1-0.5 µg/ml):

Fortification level (µg/L)	Volume of water sample (L)	Amount bromadiolone (µg)	Volume of extract (mL)	Conc. injected sample (µg/mL)
0.05	1	0.05	0.5	0.10
0.5	1	0.5	1.50	0.33
5.0	1	5.0	10	0.5
50	1	50	125	0.4

X3: All validation data (except linearity data) was generated in the SIM –mode. However, the applicant has provided chromatograms of fortified samples (0.05 µg/L) for all waters, a standard solution (1 µg/ml) and blank samples for all waters generated using both the SIM-mode and the SRM-mode. No interferences are shown and the requirements for specificity is thus considered met, even though accuracy and precision data was not generated using a highly specific method as outlined in the TNsG for Analytical methods.

X4: Recovery and precision was tested for drinking, ground and surface water. The sources of the water was:
drinking: natural mineral water Fiuggi in glass bottles
ground: water sampled from well
surface: water from Italy's lake Garda sampled at Desenzano

The applicant was asked to provide the characteristics of the water samples used in the validation and they are in the process of gather this from the performing laboratory. The RMS proposes to include the data in a revised CAR.

The validation data for all waters, derived in the SIM-mode are summarized in the table below.

Type of water	Fortification level (µg/L)	Recovery (%)		N	%RSD
		Range	Mean		
drinking	0.05	80-100	93	5	9.1
	0.5	73-85	79	5	5.9
	5.0	70-89	80	5	9.1
	50	79-105	93	5	11.5
ground	0.05	63-87	70	5	13.0
	0.5	84-92	87	5	4.8
	5.0	81-97	88	5	6.1
	50	90-107	97	5	7.0
surface	0.05*	89-113	106	5	9.2
	0.5*	80-90	86	5	4.7
	5.0	76-84	81	5	3.2
	50	107-120	114	5	4.7

* These results were only reported in the summary of the study (i.e. no raw data tables were given).

X5: LOQ is 0.05 µg/l and LOD is defined in the report as 50% of LOQ (i.e. 0.025 µg/l). The LOQ is sufficient for surface water as the lowest relevant effect level is 1.14 mg/l (algae E_rC_{50}). The LOQ is even sufficient with respect to the PNEC for water which is set to 0.38 µg/l.

X6: The reported data is for the calibration data generated during the validation

Section A4.2(c)/01	Determination of residues in water
Annex Point IIA, IV.4.2(c)	bromadiolone

	for the different types of water. The relevant repeatability data is reported in X4 above.
Conclusion	The applicant's version is adopted adding that the requirements for specificity is considered met even though accuracy data and precision data was generated in the SIM-mode, as appropriate chromatograms generated in the SRM-mode and SIM-mode is available.
Reliability	Reliability indicator 1
Acceptability	The method is acceptable for monitoring of bromadiolone in drinking, ground and surface water.
Remarks	None

Section A4.2(d)/01 **Determination of residues in animal and human body fluids and tissues**

Annex Point IIA, IV.4.2(d) **bromadiolone**

Official use only

1 REFERENCE

1.1	**Reference**	Papa, P., et al, 2001, Methods of analysis of the rodenticide residues in human and animal body fluids and tissues: Bromadiolone, IRCCS Policlinico San Matteo of Matteo of Pavia: Analytical Clinical Toxicology Laboratory, June 2001.
1.2	**Data protection**	Yes
1.2.1	Data owner	Bromadiolone Taskforce.
1.2.2	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

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline Study**	None
2.2	**GLP**	No
2.3	**Deviations**	N/A

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Enrichment	10 g of tissues (liver, spleen, lung, kidney, etc.) were homogenised with 10 ml water. NaOH N (0.05 ml) was then added to 2 ml serum/plasma/blood, which contained 100 ng of difenacoum as internal standard. The sample was extracted with 4 ml of ethyl acetate, vortexing for 3 minutes. The mixture is centrifuged and the organic layer evaporated to dryness in a gentle stream of nitrogen.
3.1.2	Cleanup	The residue is reconstituted with 0.1 ml of methanol: water mixture (1:1) and injected into the HPLC system.
3.2	**Detection**	
3.2.1	Separation method	Identification and quantification were performed by reversed-phase high performance liquid chromatography using UV detection. Agilent Liquid Chromatograph: Model: 1100 Column: Merck Lichrosorb RP Select B, 250 mm x 4.6 mm i.d., particles 5 µm (end capped.) Mobile Phase: acetonitrile:methanol:water (70:30:10), 1% D4 Waters reagent (dibutylamine phosphate) Flow: 0.8 ml/min to 1.5 ml/min in 20 minutes. Under these conditions bromadiolones *cis* and *trans* isomers are eluted as a single peak.
3.2.2	Detector	UV diode array, detection at 265 nm.
3.2.3	Standard(s)	100 ng of difenacoum as internal standard.
3.2.4	Interfering substance(s)	None stated.

Section A4.2(d)/01	**Determination of residues in animal and human body fluids and tissues**
Annex Point IIA, IV.4.2(d)	**bromadiolone**

3.3	**Linearity**		
3.3.1	Calibration range	Not stated.	
3.3.2	Number of measurements	Not stated.	
3.3.3	Linearity	r^2 = 0.9996 in the range 10 – 500 µg/l	X1
3.4	**Specifity: interfering substances**	Not stated.	X2
3.5	**Recovery rates at different levels**	Over 65% for serum and plasma, over 50% for tissues.	X3
3.5.1	Relative standard deviation	Not stated.	
3.6	**Limit of determination**	Sensitivity limit: 5 µg/l in serum, plasma and blood. 10 µg/l in tissues	
3.7	**Precision**	Non-entry field	
3.7.1	Repeatability	CV % of intrarun and interrun data for serum and tissue at different concentrations range from 10% to 25%.	X3
3.7.2	Independent laboratory validation	Not performed.	
		4 APPLICANT'S SUMMARY AND CONCLUSION	
4.1	**Materials and methods**	Bromadiolone was extracted from serum/plasma and tissues with liquid-liquid extraction, and determined by reverse phase HPLC with UV detection.	
4.2	**Conclusion**	The limits of determination, recovery rates and linearity are reported.	
4.2.1	Reliability	2	X4
4.2.2	Deficiencies	No	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	March 2009
Materials and methods	X1: No raw data is given for the calibration (i.e. neither calibration graph nor numbers of calibration points are reported). X2: The proposed method (i.e. HPLC-UV) is not highly specific as defined by the TNsG on Analytical Methods and a confirmatory method is thus needed. X3: The figures for recovery and precision are not supported by any raw data (i.e. number of samples used for fortification is not reported). Moreover, the reported recovery and precision are outside the range generally accepted according to the

Section A4.2(d)/01	**Determination of residues in animal and human body fluids and tissues**
Annex Point IIA, IV.4.2(d)	**bromadiolone**

	TNsG on Analytical Methods (i.e. mean recovery 70-110% and %RSD ≤20%)
Conclusion	The applicant's version is adopted
Reliability	X4: Since the reporting is poor and the stated recoveries and precision is partly outside the acceptable range, the reliability is lowered to: Reliability indicator 3
Acceptability	The method is not acceptable due to the deficiencies described above.
Remarks	None

Section A4.2(d)/02	**Determination of residues in animal and human body fluids and tissues**
Annex Point IIA, IV.4.2(d)	**bromadiolone**

Official use only

1 REFERENCE

1.1	**Reference**	Marshall L (2010) Method validation for the determination of bromadiolone in animal matrices (liver and blood), CEM Analytical Services Limited (CEMAS), Report No.CEMS-4550.
1.2	**Data protection**	Yes
1.2.1	Data owner	Bromadiolone Task Force
1.2.2	Companies with letter access	
1.2.3	Criteria for data protection	

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline**	SANCO/825/00 rev. 7, 17/03/04
2.2	**GLP**	Yes. OECD Principles of Good Laboratory Practice (ENV/MC/CHEM/(98)17) and UK S.I.1999/3106 as amended by SI 994 2004.
2.3	**Deviations**	No

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Extraction	For blood and liver matrices, samples were extracted with acetone/hexane (80/20, v/v). After shaking and centrifugation an aliquot of the extract was evaporated to dryness and redissolved in acetonitrile/water (50/50, v/v). Finally the concentration of bromadiolone in bovine blood and bovine liver was determined using LC-MS/MS. The limit of quantitation (LOQ) for this method is 0.01 mg/kg.
3.1.2	Cleanup	The final solution was filtrated through a 0.45µ LCR filter prior to analysis.
3.2	**Detection**	
3.2.1	Separation method	Agilent 1100 series Liquid chromatography system comprising of a binary pump, degrasser, autosampler, column oven. Chromatography Conditions: Column : Luna Phenyl-Hexyl (5µm 50 mm x 3.0 mm) Column Temperature: 30°C Injection volume: 5µL Mobile phase: A: 10mM ammonium acetate, B: HPLC grade acetonitrate Table 1 Mobile phase composition:

Total Time (min)	Flow rate (µL/min)	%A	%B
0.2	800	80	20
0.5	800	80	20
3.0	800	10	90
5.5	800	10	90
5.6	800	80	20
6.0	800	80	20

Table 2:

Time (min)	Position
0.0	B
0.5	A
5.8	B

A: to mass spectrometer

B: to waste

3.2.2 Detector

LC-MS-MS (primary ion m/z: 79)

X1

Scan Type: MRM
Polarity: Negative
Ion Source: Turbo Spray (TIS)
Resolution Q1: Unit
Resolution Q3: Unit

Table 3 Quantitation Transition:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP	EP
525.1	250.2	200	-50	-30	-5.0

Table 4 Confirmatory Transition:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP	EP
527.2	250.20	200	-50	-30	-5.0

All Transitions:
TEM :450.0
CAD:7.0
CUR:20
GS1:30

GS2:30
Ion Spray -4500.0:
DP:-100
Interface: On

3.2.3 Standard(s)

Fortification Standards:

1000 µg/mL Standard Solution
1.0 µg/mL Standard Solution
0.1µg/mL Standard Solution

Calibration Standards:

1000 µg/mL standard solution: 100 mg of bromadiolone was weighed into a 100 mL volumetric flask and made up to volume with acetone

10µg/mL standard solution: serial dilution of the 1000 µg/mL standard solution as appropriate in acetone.

Preparation of matrix- matched calibration standard solution:

The prepared calibration standards were diluted to produce calibration solutions in acetonitrile/ water (50/50, v/v) as follows;

1.0 µg/mL standard solution : Serial dilution of the 10 µg/mL standards solution as appropriate in acetonitrile/ HPLC water (50/50, v/v)

0.1 µg/mL standard solution: Serial dilution of the 1.0 µg/mL standards solution as appropriate in acetonitrile/ HPLC water (50/50, v/v)

0.01 µg/mL standard solution: Serial dilution of the 0.1 µg/mL standards solution as appropriate in acetonitrile/ HPLC water (50/50, v/v)

Table 5 Matrix-matched calibration standards were made from these solutions.

Parent Concentration (µg/mL)	**Volume Taken. (mL)**	**Made to Final Volume with Control. (mL)**	**Final Standard concentration µg/mL**
1	0.025	1.0	0.025
0.1	0.1	1.0	0.01
0.1	0.05	1.0	0.005
0.1	0.025	1.0	0.0025
0.01	0.1	1.0	0.001
0.01	0.05	1.0	0.0005

3.2.4 Interfering substance(s)

The effect of crop matrices on the LC-MS/MS response was assessed by preparing standards in the presence of matrix and comparing the peak areas of bromadiolone against non-matrix standards at an equivalent concentration.

No significant enhancement or suppression of detector response was observed in the presence of bovine blood matrix; the measured matrix effects were less than 10%. It is therefore appropriate to use non-matrix standards for calibration and quantitation.

No significant enhancement or suppression of detector response was observed in the presence of bovine liver matrix; the measured matrix effects were less than 10%. However, this level of matrix effect still took the recoveries outside the acceptable limits and therefore it was appropriate to use matrix standards for calibration and quantitation.

An assessment of matrix effects is given in the Table 6 below:

Matrix	Matrix Effect for Bromadiolone (%)	
	Quantitation Transition (525.1-250.2)	Quantitation Transition (527.2-250.2)
Bovine Blood	-5.4	-3.2
Bovine Liver	2.3	2.1

3.3 Linearity

3.3.1	Calibration range	0.0005 to 0.025 µg/mL	X2
3.3.2	Number of measurements	Six	
3.3.3	Linearity	The linearity of response of the analytical instrumentation over the range 0.0005 to 0.025 µg/mL for bromadiolone during the validation procedure was acceptable with a correlation coefficient (r) of greater than 0.995	X3

3.4 Specifity: interfering substances

The analytical method developed for the determination of bromadiolone in bovine blood and bovine liver matrices has been shown to be highly specific due to the instrumentation used (LC-MS/MS) and the detection of two separate ion transitions.

3.5 Recovery rates at different levels

Table 7 Summary of Recovery Values of Bromadiolone in Bovine Blood X4

Transition/ m/z	Fortification Level (mg/kg)	Number of Replicates (n)	Mean Recovery (%)	Recovery Range (%)	S D
Quantitation /	0.01	5	97	89 – 110	

525.1 → 250.2	0.10	5	101	93 – 105
	Overall	10	99	89 – 110
Confirmatory/ 527.2 → 250.2	0.01	5	96	89 – 106
	0.10	5	102	97 – 109
	Overall	10	99	89 – 109

Transition/ m/z	**Fortification Level (mg/kg)**	**Number of Replicates (n)**	**Mean Recovery (%)**	**Recovery Range (%)**
Quantitation / 525.1 → 250.2	0.01	5	101	92 – 110
	0.10	5	105	102 – 110
	Overall	10	103	92 – 110
Confirmatory/ 527.2 → 250.2	0.01	5	103	94 – 113
	0.10	5	107	104 – 113
	Overall	10	105	94 – 113

Table 8 Summary of Recovery Values of Bromadiolone in Bovine Liver

3.5.1 Relative standard deviation

Table 9 RSD values were as follows:

Matrix	**Fortification level (mg/kg)**	**RSD (%)**
Bovine Blood (m/z =525.1-250.2)	0.01	9.0
Bovine Blood (m/z =525.1-250.2)	0.1	5.0

Bovine Blood (m/z =527.2-250.2)	0.01	7.8
Bovine Blood (m/z =527.2-250.2)	0.1	5.1
Bovine Liver (m/z =525.1-250.2)	0.01	8.9
Bovine Liver (m/z =525.1-250.2)	0.1	2.9
Bovine Liver (m/z =527.2-250.2)	0.01	8.5
Bovine Liver (m/z =527.2-250.2)	0.1	3.3

3.6 Limit of determination

The limit of determination is 0.01 mg/L (defined as the lowest concentration at which acceptable recovery has been demonstrated). X5

3.7 Precision

3.7.1 Repeatability

RSD values are presented above in section 3.5.1.

3.7.2 Independent laboratory validation

N/A

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

Specimens were analysed using CEMAS SOP CEM-3442 (draft 1) 'Analytical Method for the Determination of Bromadiolone in Blood and Liver'.

For blood and liver matrices, samples were extracted with acetone/hexane (80/20, v/v). After shaking and centrifugation an aliquot of the extract was evaporated to dryness and redissolved in acetonitrile/water (50/50, v/v).

Quantitation was performed by the external standardisation with linearity.

The limit of quantitation (LOQ) for this method is 0.01 mg/kg.

4.2 Conclusion

The method CEMAS SOP CEM-3442 (draft) has been successfully validated for bromadiolone in control specimens of bovine blood and bovine liver fortified at 0.01 mg/kg and 0.10 mg/kg.

The validation of the method is deemed to have been successful and is appropriate for the determination of bromadiolone in bovine blood and bovine liver matrices

Section A4.2(d)/02	**Determination of residues in animal and human body fluids and tissues**
Annex Point IIA, IV.4.2(d)	**bromadiolone**

4.2.1	Reliability	1
4.2.2	Deficiencies	No

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date April 2010

Materials and methods

X1: The reporting of a primary ion of m/z 79 must be erroneous (i.e. the transitions used were 525.1 →250.2 and 527.2→250.2 for the quantification and confirmation respectively).

X2: The calibration range corresponds to 50% of LOQ (0.5 ng/ml) to 250% x the highest fortification level (25 ng/ml).

X3: The linearity data can be summarized as follows:

Standard	Transition	Range (ng/ml)	R^2
s	q	0.5-25	0.9995
mm (l)	q	0.5-25	0.9996
s	c	0.5-25	0.9995
mm (l)	c	0.5-25	1.0000

s: solvent standard
mm (l): Matrix matched standard (liver)
q: quantification
c: confirmation

X4: It is assumed that mg/kg blood can be translated into mg/L. Matrix-matched standards were used for the generation of the data for liver whereas solvent standards were used for blood. Two blanks were assessed in each experiment.
X5: The LOQ should be 0.01 mg/L and 0.01 mg/kg for blood and tissues respectively.

Conclusion The applicant's version is adopted.

Reliability Reliability indicator 1

Acceptability The method is acceptable for analysing bromadiolone in blood and tissues.

Remarks None

Section A4.3/01	**Determination of residues in/on food or feedstuffs**
Annex Point IIIA, IV.1.	**bromadiolone**

			Official use only
		1 REFERENCE	
1.1	**Reference**	Turnbull, G. (2005). Validation of Analytical Methodology to Determine Rodenticides in Food Matrices. Central Science Laboratory unpublished report number PGD-180, 16 June 2005.	
1.2	**Data protection**	Yes.	
1.2.1	Data owner	Bromadiolone Task Force	
1.2.2	Companies with letter of access	None.	
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.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	SANCO/825/00 rev. 6.	
2.2	**GLP**	Yes.	
2.3	**Deviations**	No.	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Extraction	<u>Cucumber</u> Bromadiolone is extracted from cucumber by blending with ethyl acetate. The filtered extract is purified by SPE cartridge and determination is by LC-MS-MS. <u>Wheat</u> Bromadiolone is extracted from wheat by blending with ethyl acetate. The filtered extract is purified by gel permeation chromatography (GPC) prior to determination by LC-MS-MS. <u>Meat</u> Bromadiolone is extracted from meat by shaking with a mixture of dichloromethane and acetone. The filtered extract is purified by GPC prior to determination by LC-MS-MS. <u>Oilseed rape</u> Bromadiolone is extracted from oilseed rape by blending with acetone. The filtered extract is partitioned with hexane and purified by GPC prior to determination by LC-MS-MS. <u>Lemon</u> Bromadiolone is extracted from lemon by blending with ethyl acetate. The extract is partitioned with water and purified by SPE cartridge prior to determination by LC-MS-MS.	
3.1.2	Cleanup	Gel permeation chromatography or SPE catridge.	
3.2	**Detection**		

3.2.1	Separation method	HPLC, Phenomenex Luna 150 mm x 2 mm i.d. column packed with 5 µm Phenyl-Hexyl with mobile phase: 10 mM ammonium acetate and methanol.	
3.2.2	Detector	MS-MS (primary ion m/z: 250).	X1
3.2.3	Standard(s)	External standard.	X2
3.2.4	Interfering substance(s)	Analysis of control samples demonstrated that there were no substances which interfered with the detection of bromadiolone. There were no chromatographic peaks above 30% of the LOQ at the retention time of bromadiolone.	
3.3	**Linearity**		
3.3.1	Calibration range	0.03 to 1.2 µg/mL.	X3
3.3.2	Number of measurements	Eight.	
3.3.3	Linearity	R^2 = 0.9433 to 0.9963.	X3
3.4	**Specifity: interfering substances**	Analysis of control samples showed that there were no substances which interfered with the detection of bromadiolone. The use of LC/MS-MS is considered to be highly specific and self-confirmatory. There were no chromatographic peaks above 30% of the LOQ at the retention time of bromadiolone.	
3.5	**Recovery rates at different levels**	Recoveries from fortified cucumber, wheat, meat, oilseed rape and lemon were as follows:	X4

Matrix	Fortification level (mg/kg)	Recovery (%)		
		range	mean	n
Cucumber	0.01	87 – 106	100	5
	0.10	82 – 94	91	5
	overall	82 – 106	95	10
Wheat	0.01	77 – 102	87*	4
	0.10	72 – 96	83	5
	overall	72 – 102	85*	9
Meat	0.01	35 – 58	47	5
	0.10	40 – 77	54	5
	overall	35 – 77	51	10
Oilseed rape	0.01	116 – 137	128	5
	0.10	99 – 121	112	5
	overall	99 – 137	120	10
Lemon	0.01	56 – 85	71	5
	0.10	47 – 68	55	5
	overall	47 – 85	63	10

*One recovery value excluded from calculations as an outlier (Dixon's test)

3.5.1 Relative standard deviation

RSD values were as follows:

Matrix	Fortification level (mg/kg)	RSD (%)	Overall RSD (%)
Cucumber	0.01	7.5	8.1
	0.10	5.7	
Wheat	0.01	12.9*	11.2*
	0.10	10.5	
Meat	0.01	18.0	27.5
	0.10	33.5	
Oil-seed rape	0.01	7.9	10.3
	0.10	8.1	
Lemon	0.01	17.5	21.4
	0.10	17.3	

*One recovery value excluded from calculations as an outlier (Dixon's test)

3.6 Limit of determination

The limit of determination is 0.01 mg/L (defined as the lowest concentration at which acceptable recovery has been demonstrated).

3.7 Precision

3.7.1 Repeatability

RSD values are presented above under 3.5.1.

3.7.2 Independent laboratory validation

Not applicable.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

Cucumber

Bromadiolone is extracted from cucumber by blending with ethyl acetate. The filtered extract is purified by SPE cartridge and determination is by LC-MS-MS.

Wheat

Bromadiolone is extracted from wheat by blending with ethyl acetate. The filtered extract is purified by GPC prior to determination by LC-MS-MS.

Meat

Bromadiolone is extracted from meat by shaking with a mixture of dichloromethane and acetone. The filtered extract is purified by GPC prior to determination by LC-MS-MS.

Oilseed rape

Bromadiolone is extracted from oilseed rape by blending with acetone. The filtered extract is partitioned with hexane and purified by GPC prior to determination by LC-MS-MS.

Lemon

Bromadiolone is extracted from lemon by blending with ethyl acetate. The extract is partitioned with water and purified by SPE cartridge prior

		to determination by LC-MS-MS.	
4.2	**Conclusion**	The methods for determination of residues of bromadiolone in cucumber and wheat have been adequately validated. The methods were successfully evaluated and meet the EU criteria with respect to specificity, linearity and accuracy according to the guidance given in SANCO/825/00. For oilseed rape the mean recovery exceeds the guideline criteria of 70 to 110%. For meat and lemon, mean recovery is less than 70% and the overall RSD is slightly higher than the guideline acceptance criterion of 20%. However, the reports concludes that the methods are acceptable for monitoring purposes on the basis that these are multi-residue methods that allow eight analytes to be determined in the same extract. The method requires equipment and instrumentation which is commonly available in most well-equipped laboratories. Therefore, the methods are suitable for enforcement purposes.	X6
4.2.1	Reliability	1	X7
4.2.2	Deficiencies	For oilseed rape the mean recovery exceeds the guideline criteria of 70 to 110%. For meat and lemon, mean recovery is less than 70% and the overall RSD is slightly higher than the guideline acceptance criterion of 20%. These deviations are not considered to significantly affect the suitability of the method for monitoring purposes.	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date March 2009

Materials and methods

X1: The primary transition is 527 →250 and the qualifier transition 525 →250. Only the primary transition was used to generate the validation data.

X2: External calibration relative to internal standard(s) (coumatetralyl and diphacinone; not evident which is used for which analyte) was employed.

X3: The fortification levels for all matrices correspond to 0.1-1.0 µg/ml injected onto the column (i.e. calibration range covers 30% of LOQ to 120% of 10 x LOQ). It is stated in the analytical method that the matrix matched standards should be used for all matrices for the quantification. However, only one example calibration curve is provided for bromadiolone (i.e. for the lemon analysis) and it is not evident if this relates to matrix matched standards.

In the example calibration curve a quadratic fit is applied using four calibration standards and duplicate injections which gave a r^2 of 0.9916. The reported range for the r^2 (i.e. 0.9433 to 0.9963) is only presented in the result section of the study, but it indicates a poor correlation for some of the matrices. It is stated that a linear fit was employed for all determinations except for lemon (as stated above) and for the low level fortification in meat.

X4: The recovery data for meat (both levels), oilseed rape (LOQ) and lemon (10 x LOQ) are outside the range accepted by the TNsG on Analytical methods (proposed as 70-120% in the range 0.01-0.1 mg/kg).

X5: The %RSD for meat at the higher level is too high compared to the criteria in

	the TNsG on Analytical methods (i.e. ≤20% in the range 0.01-0.1 mg/kg).
Conclusion	X6: Since the validation data was not acceptable for meat, oilseed rape and lemon further validation data is needed for these matrices for the method to be fully acceptable. Moreover, the linearity data/calibration data is not sufficiently reported.
Reliability	X7: Due to the somewhat incomplete reporting of the linearity data the reliability for cucumber and wheat, the is set to: Reliability indicator 2 However, due to the unacceptable validation data for meat, oilseed rape and lemons the reliability for that analysis is set to: Reliability indicator 3
Acceptability	Acceptable validation data for meat, oilseed rape and lemon is needed for the method to be considered acceptable for monitoring of bromadiolone in food. This is in line with the requirements for the other applicant for Annex I-inclusion of bromadiolone
Remarks	None

Section A4.3/02 **Determination of residues in/on food or feedstuffs**

Annex Point IIIA, IV.1. **bromadiolone**

			Official use only
		1 REFERENCE	
1.1	**Reference**	Marshall L (2010) Method validation for the determination of Bromadiolone in crop matrices (oilseed rape seed and lemon), CEM Analytical Services Limited (CEMAS), Report No. CEMR-4559.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Bromadiolone Task Force	
1.2.2	Companies with letter of access	None	
1.2.3	Criteria for data protection	None	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	SANCO/825/00 rev. 7, 17/03/04	
2.2	**GLP**	Yes. OECD Principles of Good Laboratory Practice (ENV/MC/CHEM/(98)17) and UK S.I.1999/3106 as amended by SI 994 2004	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Extraction	For whole lemon matrix, samples were extracted with acetone/hexane (80/20, v/v). After shaking and centrifugation an aliquot of the extract was evaporated to dryness and redissolved in acetonitrile/water (50/50, v/v). For oilseed rape seed matrix, samples were extracted with acetone/hexane (80/20, v/v). After shaking and centrifugation an aliquot of the extract was purified on MAX SPE cartridges and eluted with ethyl acetate/methanol/formic acid (90/8/2, v/v/v). The samples were dried and re-dissolved in acetonitrile/water (50/50, v/v).	
3.1.2	Cleanup	Analyte solution was put through a MAX SPE cartridge and then the final solution was filtered through a 0.45µm filter prior to analysis.	
3.2	**Detection**		
3.2.1	Separation method	Agilent 1100 series Liquid chromatography system comprising of a binary pump, degrasser, autosampler, column oven. Chromatography Conditions: Column : Luna Phenyl-Hexyl (5µm 50 mm x 3.0 mm) Column Temperature: 30°C Injection volume: 5µL	

Mobile phase: A: 10mM ammonium acetate, B: HPLC grade acetonitrate

Table 1 Mobile phase composition:

Total Time (min)	Flow rate (µL/min)	%A	%B
0.2	800	80	20
0.5	800	80	20
3.0	800	10	90
5.5	800	10	90
5.6	800	80	20
6.0	800	80	20

Table 2:

Time (min)	Position
0.0	B
0.5	A
5.8	B

A: to mass spectrometer

B: to waste

3.2.2 Detector

LC-MS-MS (primary ion m/z: 79) X1
Scan Type: MRM
Polarity: Negative
Ion Source: Turbo Spray (TIS)
Resolution Q1: Unit
Resolution Q3: Unit

Table 3 Quantitation Transition:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP	EP
525.1	250.2	200	-50	-30	-5.0

Table 4 Confirmatory Transition:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP	EP
527.2	250.20	200	-50	-30	-5.0

All Transitions:
TEM :450.0
CAD:7.0

CUR:20
GS1:30
GS2:30
Ion Spray -4500.0:
DP:-100
Interface: On

3.2.3 Standard(s) Fortification Standards:

1000 µg/mL standard solution
1.0 µg/mL standard solution
0.1µg/mL standard solution

Calibration Standards:

1000 µg/mL standard solution : 100 mg of bromadiolone was weighed into a 100 mL volumetric flask and made up to volume with acetone

10µg/mL standard solution : serial dilution of the 1000 µg/mL standard solution as appropriate in acetone.

Preparation of matrix- matched calibration standard solution :

The prepared calibration standards were diluted to produce calibration solutions in acetonitrile/ water (50/50, v/v) as follows;

2.0 µg/mL standard solution : Serial dilution of the 10 µg/mL standards solution as appropriate in acetonitrile/ HPLC water (50/50, v/v)

0.2 µg/mL standard solution : Serial dilution of the 1.0 µg/mL standards solution as appropriate in acetonitrile/ HPLC water (50/50, v/v)

0.02 µg/mL standard solution : Serial dilution of the 0.1 µg/mL standards solution as appropriate in acetonitrile/ HPLC water (50/50, v/v)

Table 5 Matrix-matched calibration standards were made from these solutions.

Parent Concentration (µg/mL)	**Volume Taken. (mL)**	**Made to Final Volume with Control. (mL)**	**Final Standard concentration µg/mL**
1	0.025	1.0	0.025
0.1	0.1	1.0	0.01
0.1	0.05	1.0	0.005
0.1	0.025	1.0	0.0025
0.01	0.1	1.0	0.001
0.01	0.05	1.0	0.0005

3.2.4	Interfering substance(s)	The effect of crop matrices on the LC-MS/MS response was assessed by preparing standards in the presence of matrix and comparing the peak areas of bromadiolone against non-matrix standards at an equivalent concentration. No significant enhancement or suppression of detector response was observed in the presence of whole lemon matrix; the measured matrix effects were less than 10%. It is therefore appropriate to use non-matrix standards for calibration and quantitation. Significant enhancement or suppression of detector response was observed in the presence of oilseed rape seed matrix; the measured matrix effects were greater than 10%. However, as the recovery determinations are within the acceptable range when non-matrix standards are used for calibration and quantitation, it was appropriate to use non-matrix standards. An assessment of matrix effects is given in the Table 6 below

Matrix	Matrix Effect for Bromadiolone (%)	
	Quantitation Transition (525.1-250.2)	Quantitation Transition (527.2-250.2)
Oilseed Rape Seed	14.8	19.2
Whole Lemon	-0.6	1.7

3.3	**Linearity**		
3.3.1	Calibration range	0.0005 to 0.025 µg/mL	X2
3.3.2	Number of measurements	Six	
3.3.3	Linearity	The linearity of response of the analytical instrumentation over the range 0.0005 to 0.025 µg/mL for bromadiolone during the validation procedure was acceptable with a correlation coefficient (r) of greater than 0.995	X3
3.4	**Specifity: interfering substances**	Analysis of control samples showed that there were no substances which interfered with the detection of Bromadiolone. The use of LC/MS-MS is considered to be highly specific and self-confirmatory.	
3.5	**Recovery rates at different levels**		

Table 7 Summary of Recovery Values of Bromadiolone in Oilseed Rape Seed

Transition / m/z	Fortification Level (mg/kg)	Number of Replicates (n)	Mean Recovery (%)	Recovery Range (%)	Relative Standard Deviation (RSD %)

Quantitation/ 525.1 → 250.2	0.01	5	90	82 – 99	8.3
	0.10	5	98	89 – 116	11.4
	Overall	10	94	82 – 116	10.5
Confirmatory/ 527.2 → 250.2	0.01	5	92	86 – 102	7.3
	0.10	5	102	94 – 115	9.1
	Overall	10	97	86 – 115	9.6

Table 8 Summary of Recovery Values of Bromadiolone in Whole

Transition/ m/z	**Fortification Level (mg/kg)**	**Number of Replicates (n)**	**Mean Recovery (%)**	**Recovery Range (%)**
Quantitation/ 525.1 → 250.2	0.01	5	94	88 – 99
	0.10	5	95	91 – 97
	Overall	10	94	88 – 99
Confirmatory/ 527.2 → 250.2	0.01	5	89	87 – 93
	0.10	5	92	89 – 96
	Overall	10	91	87 – 96

Lemon

3.5.1 Relative standard deviation

Table 9 RSD values were as follows:

Matrix	**Fortification level (mg/kg)**	**RSD (%)**
Oilseed Rape Seed 525.1 → 250.2	0.01	8.3
	0.10	11.4
	0.01	7.3

Oilseed Rape Seed 527.2 → 250.2	0.10	9.1
Whole Lemon 525.1 → 250.2	0.01 0.10	 5.4 2.5
Whole Lemon 527.2 → 250.2	0.01 0.10	2.6 3.0

3.6 Limit of determination

The limit of quantitation was established to be 0.01 mg/kg

3.7 Precision

3.7.1 Repeatability

RSD values are presented above in section 3.5.1.

3.7.2 Independent laboratory validation

N/A

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

Specimens were analysed using CEMAS SOP CEM-3443 (draft 1) 'Analytical Method for the Determination of Bromadiolone in Oilseed Rape Seed and Whole Lemon.

For whole lemon matrix, samples were extracted with acetone/hexane (80/20, v/v). After shaking and centrifugation an aliquot of the extract was evaporated to dryness and redissolved in acetonitrile/water (50/50, v/v). For oilseed rape seed matrix, samples were extracted with acetone/hexane (80/20, v/v). After shaking and centrifugation an aliquot of the extract was purified on MAX SPE cartridges and eluted with ethyl acetate/methanol/formic acid (90/8/2, v/v/v). The samples were dried and re-dissolved in acetonitrile/water (50/50, v/v).

Quantitation was performed by the external standardisation with linearity.

The limit of quantitation (LOQ) for this method is 0.01 mg/kg

4.2 Conclusion

The method CEMAS SOP CEM-3443 (draft) has been successfully validated for bromadiolone in control specimens of oilseed rape seed and whole lemon fortified at 0.01 mg/kg and 0.10 mg/kg.

Section A4.3/02 **Determination of residues in/on food or feedstuffs**

Annex Point IIIA, IV.1. **bromadiolone**

The validation of the method is deemed to have been successful and is appropriate for the determination of bromadiolone in oilseed rape seed and whole lemon matrices.

4.2.1 Reliability 1

4.2.2 Deficiencies

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date March 2009

Materials and methods

X1: The reporting of a primary ion of m/z 79 must be erroneous (i.e. the transitions used were 525.1 →250.2 and 527.2→250.2 for the quantification and confirmation respectively).

X2: The calibration range corresponds to 50% of LOQ (0.5 ng/ml) to 250% x the highest fortification level (25 ng/ml).

X3: The linearity data can be summarized as follows:

Standard	Transition	Range (ng/ml)	R^2
s	q	0.5-25	0.9992
s	c	0.5-25	0.9992

s: solvent standard
q: quantification
c: confirmation

Conclusion The applicant's version is adopted

Reliability Reliability indicator 1

Acceptability The method is considered acceptable for whole lemon and oil-seed rape. Since acceptable data is available for meat (liver) in A4.2(d)/02 above acceptable data is available for all required food and feeding stuffs.

Remarks None