Section A4.1 (1) Analytical Methods for Detection and Identification

Annex Point IIA4.1 IIIA-IV.1 Determination of the Active Ingredient content and validation of method

			Official use only
		1 REFERENCE	
1.1	**Reference**	Garofani S. (2002) Difenacoum Technical, Determination of the a.i content: validation of the analytical method. ChemServices. Study No. CH-90/2001	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa	
1.2.2	Companies with letter of access	PelGar International Ltd. (only for use in Annex I listing of difenacoum)	
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**	EPA guidelines OPPTS 830.1800	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	Not required.	
3.1.2	Cleanup	There is no purification stage applied to the analysis of the technical active substance when using this method of analysis for difenacoum technical material	
3.2	**Detection**	Non-entry field	
3.2.1	Separation method	200 mg of the technical substance was dissolved into 10 ml internal standard, 30 ml dichloromethane and 10 ml methanol. This sample was then further diluted 1:100 with methanol before injection. HPLC was performed using a HPLC Column: Lichrospher 5 µm RP18, 200 x 3.0 mm i.d Column Temperature: room temperature Eluent: Methanol/water/acetic acid = 89.2/10/0.8 v/v/v Eluent flow: 0.7ml/min Volume of injection: 10µl Difenacoum: 4.2 min ca. 1,3,5-triphenylbenzene: 106 min ca	
3.2.2	Detector	This method of analysis for difenacoum technical material uses an ultra-violet detector acting at 254 nm	
3.2.3	Standard(s)	This method of analysis for difenacoum technical material uses 1,3,5 – triphenylbenzene as an internal standard	
3.2.4	Interfering	There are no substances currently known which might interfere with this	

	substance(s)	method of analysis for difenacoum technical material
3.3	**Linearity**	
3.3.1	Calibration range	20 – 60 µg/ml
3.3.2	Number of measurements	Linearity tests: 4 injections per concentration level (5 levels). Repeatability test: 2 injections per sample (6 samples).
3.3.3	Linearity	Linearity test on Difenacoum analytical standard (a.i peak area)

	Std 1 20µg/ml	**Std 2 30ug/ml**	**Std 3 40µg/ml**	**Std 4 50 µg/ml**	**Std 5 60µg/ml**
Mean	1118.74	1710.59	2235.05	2916.02	3614.40
Standard deviation	0.43	2.86	3.68	4.18	6.76

R = 0.99839

Linearity test on difenacoum analytical standard (area ratio)

	Std 1 1.250	**Std 2 1.875**	**Std 3 2.500**	**Std 4 3.125**	**Std 5 3.750**
Mean	1.9124	2.9347	3.8449	5.0687	6.3205
Standard deviation	0.0018	0.0035	0.0032	0.0037	0.0041

R = 0.99569

3.4	**Specifity: interfering substances**	Not reported.
3.5	**Recovery rates at different levels**	Not studied.
3.5.1	Relative standard deviation	R.S.D. of Repeatability test = 0.329%
3.6	**Limit of determination**	The calibration range was: 20 – 60 µg/ml. (± 50% of samples for quantitation.)
3.7	**Precision**	

Section A4.1 (1) Analytical Methods for Detection and Identification

Annex Point IIA4.1 IIIA-IV.1

Determination of the Active Ingredient content and validation of method

3.7.1 Repeatability

	Wis (mg)	Ws	As/Ais	F	Difenacoum (% w/w)
Dif Tcn A	81.5	202.6	0.8521	0.3430	99.94
Dif Tcn B	81.5	206	0.8615	0.3430	99.38
Dif Tcn C	81.5	224.8	0.9439	0.3430	99.78
Dif Tcn D	81.5	204.0	0.8588	0.3430	100.03
Dif Tcn E	81.5	203.0	0.8478	0.3430	99.24
Dif Tcn F	81.5	220.7	0.9217	0.3430	99.24
				Mean Value	99.6
				Standard Deviation	0.328
				Precision	0.7

3.7.2 Independent laboratory validation

Not given

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

The determination of the active substance was performed by HPLC with method of the internal standard, using the UV detector. It is based on the comparison between the ratio of the difenacoum analytical standard peak area versus 1.3.5-triphenylbenzene internal standard peak area and the same ratio determined in the sample under examination where a known amount of internal standard (I.S) was added.

The range of linearity tested was from 20 to 60µg/ml of difenacoum. The repeatability test conducted on a sample of technical product gives the precision as 99.6 +/- 0.7% w/w.

Preliminary tests on difenacoum technical samples were performed to find the best chromatographic conditions and avoid any interference.

Linear regression analysis was performed using the least squared method. By regression analysis the correlation coefficient was calculated. The linearity test was performed with solutions containing 20, 30, 40, 50 and 60ug/ml of difenacoum analytical standard. For each concentration four injections were performed, and a washing methanol solution was injected after the highest standard concentration in order to verify if memory peaks were detected. Mean and standard deviations were assessed with the data of repeated injections.

For repeatability, the same method was performed as above.

1.1 Conclusion

For specificity – both difenacoum and internal standard peaks were well separated and the methanol used as solvent does not present any interference.

The limit of detection of the analytical method was not indicated

Section A4.1 (1) Analytical Methods for Detection and Identification

Annex Point IIA4.1 IIIA-IV.1

Determination of the Active Ingredient content and validation of method

		because it was not an important parameter. The study CH - 90/2001 was a validation for the difenacoum quantitation in technical samples and therefore the concentration of the sample solutions were adjusted to obtain chromatographic peaks with a good integration in order to obtain the better precision for the analytical method. The sensibility of the method must not be considered. The linearity range was from 20 to 60 ppm of difenacoum, corresponding to +/- 50% of the sample solution used for the quantitation (40 ppm). The weight of 200 mg was suggested to have a representative sampling of the technical samples.
1.1.1	Reliability	1
1.1.2	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 FINLAND
Date	30 June 2006
Materials and methods	The determination of the active substance was performed by HPLC with method of the internal standard, using the UV detector. It is based on the comparison between the ratio of the difenacoum analytical standard peak area versus 1.3.5-triphenylbenzene internal standard peak area and the same ratio determined in the sample under examination where a known amount of internal standard (I.S) was added.
Conclusion	For specificity – both difenacoum and internal standard peaks were well separated and the methanol used as solvent does not present any interference. The linearity test was performed with solutions containing 20, 30, 40, 50 and 60µg/ml of difenacoum analytical standard. The slope, intercept and correlation coefficient are reported, but the typical calibration plot is missing. The repeatability test with six replicates and two injections from each replicate gives the precision as 99.6 +/- 0.7% w/w. Mean, standard deviation and variation coefficient are reported. For the reasons listed above, it can be concluded that the analytical method is in compliance with the validation and other criteria required from such method in the SANCO/3030/99 Guidance Document.
Reliability	1
Acceptability	Acceptable
Remarks	-

Section A4.1 (1) Analytical Methods for Detection and Identification

Annex Point IIA4.1 IIIA-IV.1

Determination of the Active Ingredient content and validation of method

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Results and discussion	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant´s summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A4.1 (2) Analytical Methods for Detection and Identification

Annex Point IIA4.1IIIA-IV.1

Difenacoum – Five-batch analysis

The analytical method and the related validation data for the determination of impurity in the difenacoum technical product is considered to be acceptable but is confidential and can be found in Annex for Confidential Data and Information.

Section A4.2 (a) Methods of Identification and Analysis in Soil

Annex Point IIA, IV 4.2 Residues determination of Difenacoum in soil

			Official use only
		1 REFERENCE	
1.1	**Reference**	Morlacchini, M., 2006, Residues determination of Brodifacoum, Difenacoum and Bromadiolone in soil, 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	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
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	40.0g of soil is weighted into a series of 500ml sovirel. The fortified samples, has been prepared adding 1.0ml aliquots of the appropriate spiking solutions, mix B, D,and F approximately from 0.63 to 6.3µg/g. 100ml of 50% acetone/ 50% chloroform extraction solution is added. The soveril is closed and shaken for a minimum of 30 minutes at a rate of approximately 180 movements/ minute on an automatic shaker. The extraction solution is collected in a 500ml raotavapour balloon after filtration on glass fiber. Another 100ml quantity of extraction solution is added and the process repeated again for a further of 30 minutes. The extraction is then filtered again and the process repeated with a further 50ml of extraction solution. The three filtered solutions are combined and evaporated with a rotavapor to 200mm Hg.	
3.1.2	Cleanup	The recovery is made with 10ml of acetone and purified 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 recovery of all solvent in the a flask. The acetone is 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 or stored in a freezer at -20°C if injection doesn't occur immediately.	

Section A4.2 (a) Methods of Identification and Analysis in Soil

Annex Point IIA, IV 4.2 Residues determination of Difenacoum in soil

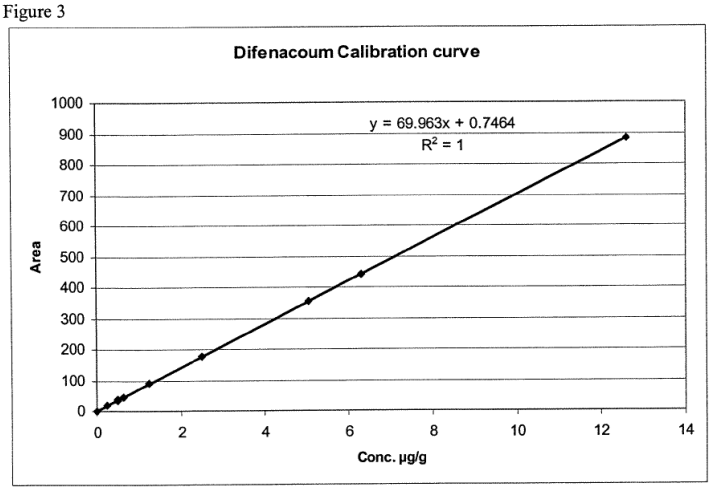
3.2 Detection

3.2.1	Separation method	HPLC UV-Vis Column type 150x4,60 mm/S/N 224016-2 Volume and type of injection 20µl with autosampler Temp of chiller 25ºC Λ of detection 264nm with a window of 4 nm and a reference to 360 with a window of 100nm
3.2.2	Detector	Diode array detector (DAD)
3.2.3	Standard(s)	DIFENACOUM technical grade Lot Nº L13653
3.2.4	Interfering substance(s)	Non detected

3.3 Linearity

Non-entry field

3.3.1	Calibration range	0.252, 0.504, 0.63, 1.26, 2.52, 5.04, 6.3 and 12.6 $\mu g/g^{-1}$ (Conc. Equiv. in soil. 0.006, 0.013, 0.016, 0.032, 0.063, 0.126, 0.158, 0.315 g^{-1})
3.3.2	Number of measurements	4 measurements at fortification levels.
3.3.3	Linearity	Figure 3



For linear regression equations describing the detector response as a function of the standard calibration curve concentrations, the correlation coefficients (R^2) were greater than 0.998

Section A4.2 (a) Methods of Identification and Analysis in Soil

Annex Point IIA, IV 4.2

Residues determination of Difenacoum in soil

3.4 Specifity: interfering substances

Non detected

3.5 Recovery rates at different levels

Table 5

File	Date	Sample name	Conc. Add. µg/g	Conc. equiv. in soil µg/g	Area	Conc. Found µg/g	Recovery %
10190005	18/10/2005	Rec 1	0.63	0.016	40.4	0.57	90.0
10190014	19/10/2005	Rec 4	0.63	0.016	40.2	0.56	89.5
10190018	19/10/2005	Rec 7	0.63	0.016	40.4	0.57	90.0
10190026	19/10/2005	Rec 10	0.63	0.016	40.1	0.56	89.3
10190006	18/10/2005	Rec 2	2.52	0.063	168.4	2.40	95.1
10190015	19/10/2005	Rec 5	2.52	0.063	168.2	2.39	95.0
10190019	19/10/2005	Rec 8	2.52	0.063	168.3	2.39	95.0
10190027	19/10/2005	Rec 11	2.52	0.063	168.0	2.39	94.9
10190007	18/10/2005	Rec 3	6.30	0.158	391.2	5.58	88.6
10190016	19/10/2005	Rec 6	6.30	0.158	409.5	5.84	92.7
10190020	19/10/2005	Rec 9	6.30	0.158	409.3	5.84	92.7
10190028	19/10/2005	Rec 12	6.30	0.158	408.4	5.83	92.5
10190004	18/10/2005	blank	0.00	0.000	n.r.	0.00	
10190013	19/10/2005	blank	0.00	0.000	n.r.	0.00	
10190017	19/10/2005	blank	0.00	0.000	n.r.	0.00	
10190025	19/10/2005	blank	0.00	0.000	n.r.	0.00	
						Average	92.1
						std. Dev.	2.5

3.5.1 Relative standard deviation

2.5%

3.6 Limit of determination

The limit of quantification (LOQ) and detection (LOD) for the determination of Difenacoum in soil was calculated using the standard deviation from the (0.64µg/g Difenacoum) 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 = 0.0214

LOD = 0.0064

3.7 Precision

3.7.1 Repeatability

No data

3.7.2 Independent laboratory validation

No data

Section A4.2 (a) Methods of Identification and Analysis in Soil

Annex Point IIA, IV 4.2 Residues determination of Difenacoum in soil

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 Brodifacoum, Difenacoum and 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 Difenacoum determination in soil is based on extraction from blank and spiked soil (40.0g) using chloroform:acetone 1:1 solution. The extract is concentrated by rotary evaporator and recovery 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. The sorbent traps are extracted and analysed immediately.

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 Difenacoum in soil

4.2.1 Reliability 1

4.2.2 Deficiencies No

Section A4.2 (a) Methods of Identification and Analysis in Soil

Annex Point IIA, IV 4.2

Residues determination of Difenacoum in soil

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE FINLAND
Date	14 September 2006
Materials and methods	The test method for Difenacoum determination in soil is based on extraction from blank and spiked soil (40.0g) using chloroform : acetone 1:1 solution. The extract is concentrated by rotary evaporator and recovery 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-DAD.
Conclusion	The HPLC-DAD is acceptable confirmatory technique and the UV-spectra obtained under the conditions of the determination have been submitted. In the analytical method chloroform has been used in extraction solution. In 3.3 Linearity the equation of calibration line and correlation coefficient have been reported and a typical calibration plot submitted. The calibration has been made by double determinations at eight concentrations (0.252 - 12.6 mg/ml). In 3.5 the recoveries have been reported for three fortification levels (0.63, 2.52, and 6.30 µg/ml, which are equivalent to sample concentrations of 0.016, 0.063 and 0.158 mg/kg, respectively). In 3.6 the limit of quantification is reported to be 0.0214 µg/g. The blank values does not exceed 30% of the LOQ. For the reasons listed above, it can be concluded that the analytical method is in compliance with the validation and other criteria required from such method in the SANCO/3029/99 Guidance Document.
Reliability	2
Acceptability	acceptable
Remarks	Hazardous reagents should be avoided, chloroform must be substituted by less harmful solvent. The analytical method for natural sediment samples could be clarified together with the analytical method for soil.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Results and discussion	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant´s summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2 Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

			Official use only
		1 REFERENCE	
1.1	**Reference**	Martinez M.P. 2005. Difenacoum Technical: Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters, Test Laboratory of ChemService S.r.l. ChemService Study No. CH-288/2005	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.3.1	Companies with Letter of access	PelGar International Ltd. Activa srl	
1.2.2	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 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline**	EEC guideline SANCO/3030/99 rev. 4 Directive 96/46/EC and 98/83/EC	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	1 L of water is extracted with 3 x 50 ml of dichloromethane and the organic extract evaporated to dryness by rotary evaporation at 40º C	
3.1.2	Cleanup	The residue is redissolved in with 0.5ml of methanol.	
3.2	**Detection**		
3.2.1	Separation method	Separation by HPLC/MS/DAD	
3.2.2	Detector	DAD detector with an LCQ advantage ionic trap mass detector	
3.2.3	Standard(s)	Difenacoum standards: 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml	
3.2.4	Interfering substance(s)	Non detected	
3.3	**Linearity**	Non-entry field	
3.3.1	Calibration range	Difenacoum standard range: 0.1 – 0.5 µg/ml	
3.3.2	Number of measurements	4 measurements of each standard	
3.3.3	Linearity	The range tested was from 0.1 to 0.5 $\mu g l^{-1}$, corresponding to concentrations from 0.05 to 0.25 $\mu g l^{-1}$ and was found to be linear. r >0.99	

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2

Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

3.4 Specifity: interfering substances

Non detected

3.5 Recovery rates at different levels

TABLE 4 Drinking water: recovery at fortification level L1 (0.05 µg/L)

Code Number	A_S	C_S (1) (µg/mL)	V_S (mL)	V_W (L)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	0.50	1.0	n.d.	-
Blank 2	0	-	0.50	1.0	n.d.	-
Spike L1-1	28387270	0.09	0.50	1.0	0.0464	92.86
Spike L1-2	26999658	0.09	0.50	1.0	0.0442	88.33
Spike L1-3	30545268	0.10	0.50	1.0	0.0500	99.92
Spike L1-4	29364362	0.10	0.50	1.0	0.0480	96.06
Spike L1-5	27895904	0.09	0.50	1.0	0.0456	91.26
				Mean value :	**0.047**	**93.7**
				Standard deviation (S.D.) :	0.0020	4.00
				Coefficient of Variation (C.V. %) :	*4.3%*	*4.3%*

TABLE 5 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)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	1.50	1.0	n.d.	-
Blank 2	0	-	1.50	1.0	n.d.	-
Spike L2-1	57570092	0.26	1.50	1.0	0.3921	78.42
Spike L2-2	61293244	0.29	1.50	1.0	0.4286	85.72
Spike L2-3	56553556	0.25	1.50	1.0	0.3821	76.42
Spike L2-4	61307128	0.29	1.50	1.0	0.4287	85.75
Spike L2-5	62375204	0.29	1.50	1.0	0.4392	87.84
				Mean value :	**0.414**	**82.8**
				Standard deviation (S.D.) :	0.0226	4.53
				Coefficient of Variation (C.V. %) :	*5.5%*	*5.5%*

* corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples L2 and with the lowest standard calibration level for fortified samples L1 and for control samples.
n.d. not detected, lower than L.O.D. (0.025 µg/L)

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2

Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

TABLE 6 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)	DFN (μg/L)	Recovery (%) *
Blank 1	0	-	10.00	1.0	n.d.	-
Blank 2	0	-	10.00	1.0	n.d.	-
Spike L3-1	84636848	0.44	10.00	1.0	4.3836	87.67
Spike L3-2	87127056	0.45	10.00	1.0	4.5464	90.93
Spike L3-3	79744752	0.41	10.00	1.0	4.0638	81.28
Spike L3-4	83651872	0.43	10.00	1.0	4.3192	86.38
Spike L3-5	81426440	0.42	10.00	1.0	4.1737	83.47
				Mean value :	**4.297**	**85.9**
				Standard deviation (S.D.) :	0.1672	3.34
				Coefficient of Variation (C.V. %) :	*3.9%*	*3.9%*

TABLE 7 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)	DFN (μg/L)	Recovery (%) *
Blank 1	0	-	125.00	1.0	n.d.	-
Blank 2	0	-	125.00	1.0	n.d.	-
Spike L4-1	79456152	0.40	125.00	1.0	50.5613	101.12
Spike L4-2	77855672	0.39	125.00	1.0	49.2532	98.51
Spike L4-3	73795376	0.37	125.00	1.0	45.9348	91.87
Spike L4-4	86854064	0.45	125.00	1.0	56.6075	113.21
Spike L4-5	74962512	0.38	125.00	1.0	46.8887	93.78
				Mean value :	**49.849**	**99.7**
				Standard deviation (S.D.) :	3.7583	7.52
				Coefficient of Variation (C.V. %) :	*7.5%*	*7.5%*

* corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples L3 and L4 and with the lowest standard calibration level for control samples.
n.d. not detected, lower than L.O.D. (0.025 μg/L)

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2

Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

TABLE 10 Ground water: recovery at fortification level L1 (0.05 μg/L)

Code Number	A_S	C_S (1) (μg/mL)	V_S (mL)	V_W (L)	DFN (μg/L)	Recovery (%) *
Blank 1	0	-	0.50	1.0	n.d.	-
Blank 2	0	-	0.50	1.0	n.d.	-
Spike L1-1	24592152	0.09	0.50	1.0	0.0469	93.83
Spike L1-2	23225780	0.09	0.50	1.0	0.0443	88.62
Spike L1-3	32831860	0.13	0.50	1.0	0.0626	125.27
Spike L1-4	23948990	0.09	0.50	1.0	0.0457	91.38
Spike L1-5	32891760	0.13	0.50	1.0	0.0627	125.50
				Mean value :	**0.052**	**104.9**
				Standard deviation (S.D.) :	0.0084	16.79
				Coefficient of Variation (C.V. %) :	*16.0%*	*16.0%*

TABLE 11 Ground 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)	DFN (μg/L)	Recovery (%) *
Blank 1	0	-	1.50	1.0	n.d.	-
Blank 2	0	-	1.50	1.0	n.d.	-
Spike L2-1	57367860	0.28	1.50	1.0	0.4221	84.43
Spike L2-2	60575424	0.30	1.50	1.0	0.4517	90.34
Spike L2-3	58746220	0.29	1.50	1.0	0.4348	86.97
Spike L2-4	62500540	0.31	1.50	1.0	0.4695	93.90
Spike L2-5	59027504	0.29	1.50	1.0	0.4374	87.49
				Mean value :	**0.443**	**88.6**
				Standard deviation (S.D.) :	0.0162	3.24
				Coefficient of Variation (C.V. %) :	*3.7%*	*3.7%*

* corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples L2 and with the lowest standard calibration level for fortified samples L1 and for control samples.
n.d. not detected, lower than L.O.D. (0.025 μg/L)

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2

Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

TABLE 12 Ground 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)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	10.00	1.0	n.d.	-
Blank 2	0	-	10.00	1.0	n.d.	-
Spike L3-1	88018144	0.47	10.00	1.0	4.6995	93.99
Spike L3-2	77284504	0.40	10.00	1.0	4.0393	80.79
Spike L3-3	94455576	0.51	10.00	1.0	5.0955	101.91
Spike L3-4	84244864	0.45	10.00	1.0	4.4674	89.35
Spike L3-5	92859336	0.50	10.00	1.0	4.9973	99.95
				Mean value :	**4.660**	**93.2**
				Standard deviation (S.D.) :	0.3814	7.63
				Coefficient of Variation (C.V. %) :	*8.2%*	*8.2%*

TABLE 13 Ground water: recovery at fortification level L4 (50 µg/L)

Code Number	A_S	C_S (1) (µg/mL)	V_S (mL)	V_W (L)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	125.00	1.0	n.d.	-
Blank 2	0	-	125.00	1.0	n.d.	-
Spike L4-1	85577120	0.45	125.00	1.0	56.8674	113.73
Spike L4-2	79513720	0.42	125.00	1.0	52.2053	104.41
Spike L4-3	82096992	0.43	125.00	1.0	54.1915	108.38
Spike L4-4	73344616	0.38	125.00	1.0	47.4618	94.92
Spike L4-5	85721416	0.46	125.00	1.0	56.9783	113.96
				Mean value :	**53.541**	**107.1**
				Standard deviation (S.D.) :	3.5226	7.05
				Coefficient of Variation (C.V. %) :	*6.6%*	*6.6%*

* corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples L3 and L4 and with the lowest standard calibration level for control samples.
n.d. not detected, lower than L.O.D. (0.025 µg/L)

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2

Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

TABLE 16 Surface water: recovery at fortification level L1 (0.05 µg/L)

Code Number	A_S	C_S (1) (µg/mL)	V_S (mL)	V_W (L)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	0.50	1.0	n.d.	-
Blank 2	0	-	0.50	1.0	n.d.	-
Spike L1-1	41390020	0.12	0.50	1.0	0.0616	123.25
Spike L1-2	41508328	0.12	0.50	1.0	0.0618	123.61
Spike L1-3	45706060	0.14	0.50	1.0	0.0681	136.11
Spike L1-4	69121040	0.21	0.50	1.0	0.1029	205.83
Spike L1-5	44890524	0.13	0.50	1.0	0.0668	133.68
				Mean value :	**0.065**	**129.2**
				Standard deviation (S.D.) :	0.0029	5.80
				Coefficient of Variation (C.V. %) :	*4.5%*	*4.5%*

TABLE 17 Surface 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)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	1.50	1.0	n.d.	-
Blank 2	0	-	1.50	1.0	n.d.	-
Spike L2-1	72003008	0.33	1.50	1.0	0.4888	97.76
Spike L2-2	62698172	0.27	1.50	1.0	0.4032	80.64
Spike L2-3	65275024	0.28	1.50	1.0	0.4269	85.38
Spike L2-4	69564680	0.31	1.50	1.0	0.4664	93.27
Spike L2-5	63627932	0.27	1.50	1.0	0.4118	82.35
				Mean value :	**0.439**	**87.9**
				Standard deviation (S.D.) :	0.0328	6.57
				Coefficient of Variation (C.V. %) :	*7.5%*	*7.5%*

* corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples L2 and with the lowest standard calibration level for fortified samples L1 and for control samples.
n.d. not detected, lower than L.O.D. (0.025 µg/L)
The values in the grey cells were not considered in the calculation (Dixon Test)

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2 Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

TABLE 18 Surface 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)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	10.00	1.0	n.d.	-
Blank 2	0	-	10.00	1.0	n.d.	-
Spike L3-1	94549816	0.46	10.00	1.0	4.6406	92.81
Spike L3-2	95200800	0.47	10.00	1.0	4.6805	93.61
Spike L3-3	90860864	0.44	10.00	1.0	4.4145	88.29
Spike L3-4	89416856	0.43	10.00	1.0	4.3260	86.52
Spike L3-5	94345720	0.46	10.00	1.0	4.6281	92.56
				Mean value :	**4.538**	**90.8**
				Standard deviation (S.D.) :	0.1408	2.82
				Coefficient of Variation (C.V. %) :	*3.1%*	*3.1%*

TABLE 19 Surface water: recovery at fortification level L4 (50 µg/L)

Code Number	A_S	C_S (1) (µg/mL)	V_S (mL)	V_W (L)	DFN (µg/L)	Recovery (%) *
Blank 1	0	-	125.00	1.0	n.d.	-
Blank 2	0	-	125.00	1.0	n.d.	-
Spike L4-1	101392500	0.51	125.00	1.0	63.2511	126.50
Spike L4-2	90175552	0.44	125.00	1.0	54.6561	109.31
Spike L4-3	92223480	0.45	125.00	1.0	56.2253	112.45
Spike L4-4	86123296	0.41	125.00	1.0	51.5510	103.10
Spike L4-5	84881736	0.40	125.00	1.0	50.5997	101.20
				Mean value :	**53.258**	**106.5**
				Standard deviation (S.D.) :	2.2770	4.55
				Coefficient of Variation (C.V. %) :	*4.3%*	*4.3%*

* corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples L3 and L4 and with the lowest standard calibration level for control samples.
n.d. not detected, lower than L.O.D. (0.025 µg/L)
The values in the grey cells were not considered in the calculation (Dixon Test)

3.5.1	Relative standard deviation	See tables above	
3.6	**Limit of determination**	The limit of detection (LOD) of this method is defined as 50% of the lowest validated level, i.e. 0.05µgml^{-1} corresponding to 0.025µgl^{-1} in the water matrix sample.	x
3.7	**Precision**	Non-entry field	

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2 Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

3.7.1 Repeatability

TABLE 3 Drinking water: Repeatability and Recovery Tests. Linear calibration with working standard solutions

Difenacoum (DFN) (m/z 443)	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	30395288	68033392	93004536
2nd injection	33096762	76188536	99044384
3rd injection	33350924	67878008	86395208
4th injection	28987308	65079648	83742504
Mean	**30568333**	**68109822**	**91746422**
S.D.	2421456	4404033	5840758
C.V. (%)	*7.92%*	*6.47%*	*6.37%*
	Parameter m (slope)	Parameter q (intercept)	Parameter R (correlation)
	152945223	17591292	0.99150

TABLE 9 Ground water: Repeatability and Recovery Tests. Linear calibration with working standard solutions

Difenacoum (DFN) (m/z 443)	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	26115312	61791472	89538128
2nd injection	25372506	61799296	88665208
3rd injection	27734480	70136850	95966224
4th injection	25615526	61148724	90780216
Mean	**26209456**	**63719086**	**91237444**
S.D.	920299	3714692	2831717
C.V. (%)	*3.51%*	*5.83%*	*3.10%*
	Parameter m (slope)	Parameter q (intercept)	Parameter R (correlation)
	162569970	11617671	0.99609

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2 Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

TABLE 15 Surface water: Repeatability and Recovery Tests. Linear calibration with working standard solutions

Difenacoum (DFN) (m/z 443)	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	28023680	64232340	97731688
2nd injection	32895660	71174536	102283568
3rd injection	34738410	75108872	98411616
4th injection	35731840	73253648	96909440
5th injection	30958470	68052176	95800432
Mean	**33581095**	**70942349**	**98834078**
S.D.	1824354	3859084	2207232
C.V. (%)	*5.43%*	*5.44%*	*2.23%*
	Parameter m (slope)	Parameter q (intercept)	Parameter R (correlation)
	163132458	18846103	0.99651

3.7.2 Independent laboratory validation

None

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2 Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

The objective of the study was to adjust and validate the analytical method for the determination of difenacoum residues in drinking, ground and surface water samples. The analytical conditions were suitably adapted to obtain the best results on the difenacoum residues in the three types of water. The validation of the analytical procedure was performed following the SANCO/3029/99 rev. 4 guideline.

Both repeatability and recovery test were performed using freshly fortified control samples of all three types of water (drinking, ground and surface)

4.2 Conclusion

The range tested was from 0.1 to 0.5 $\mu g ml^{-1}$, corresponding to concentrations from 0.05 to 0.25 $\mu g l^{-1}$ 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 70-100% with a mean value 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 complex method of analysis; therefore the accuracy of the analytical method can be considered acceptable.

4.2.1 Reliability: 1

4.2.2 Deficiencies: No

Section A 4.2 (c) Analytical Methods for Detection and Identification

Annex Point IIA4.2

Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE FINLAND
Date	4 August 2006
Materials and methods	The test method for Difenacoum determination in drinking, ground and surface waters is based on extraction by dichloromethane. The quantification is done by LC-MS/MS (both SIM and SRM mode). 3.6 The successfully validated LOQ is 0.5 µg/L, because the mean recovery at the level of 0.05 µg/L is 129% and exceeds the required limit.
Conclusion	The validation study and the method seem to be acceptable. The method ensures a specific determination of residues of difenacoum in surface water. The LC-MS/MS method used for identification and quantification is highly specific. In 3.3 Linearity slope, intercept, and correlation coefficient have been reported, but a typical calibration plot is missing. The calibration has been made by four determinations at five concentrations (0.1 – 0.5 µg/ml) in both SIM and SRM mode. The range of calibration corresponds to 0.05 to 0.25 µg/L in the water samples. In 3.5 the recoveries have been reported for four fortification levels in the range of LOQ and 1000 LOQ. The recovery rates were within the required range 70-110% except for surface water where the mean recovery for LOQ was 129%. The relative standard deviations were below 20 %. In 3.6 the limit of determination is 0.5 µg/l. The blank values does not exceed 30% of the LOQ. In 3.7 Precision the repeatability of recovery is reported for each fortification level. Five determinations have been made at each fortification level. The overall relative standard deviation is within the limit (≤ 20%) in every level. For the reasons listed above, it can be concluded that the analytical method is in compliance with the validation and other criteria required from such method in the SANCO/3029/99 Guidance Document.
Reliability	2
Acceptability	acceptable
Remarks	
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Results and discussion	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant´s summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*

Section A 4.2 (c)	**Analytical Methods for Detection and Identification**
Annex Point IIA4.2	Validation of the Analytical Method for the Determination of the Residues in Drinking, Ground and Surface waters

Remarks

Section A4.2 (d) Analytical Methods for Detection and Identification

Annex Point IIA4.2 & IIIA-IV.1

Methods of analysis in human and animal body fluids and tissues.

			Official use only
		1 REFERENCE	
1.1	**Reference**	Papa, P and Rocchi, L (2001) Methods of Analysis of the Rodenticide Residues in Human and Animal Body Fluids and Tissues.: Difenacoum. IRCCS Policlinico San Matteo of Pavia: Analytical Clinical Toxicology Laboratory.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with letters of access	PelGar International Ltd. Activa srl	
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 GLP	
2.1	**Guideline**	None	
2.2	**GLP**	No	
2.3	**Deviations**	N/A	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	Difenacoum is extracted from serum/plasma/blood and tissues by liquid-liquid extraction. This sample is extracted using ethyl acetate.	
3.1.2	Cleanup	N/A	
3.2	**Detection**		
3.2.1	Separation method	Identification by HPLC- reverse phase mode.	
3.2.2	Detector	The apparatus used was an angilent liquid chromatograph, modek 1100, consisting of a pump for quaternary gradient, a UV diode array detedtor and a fluorinetric. <u>Chromatographic conditions</u> Column: Merck Lichrospher 100 RP-18, 25cm x 4.6mm D.I., particles 5µm (end capped) Mobile phase: acetonitrile, water (80:20) containing 1% D4 Waters reagent (dibutylamine phosphate). Flow: programme from 0.8 ml/min to 1.5 ml/minin 20 minutes. Detection: UV diode array, χ 265 nm and fluoresence, χ excitation 265 nm, χ emission 400 nm,	
3.2.3	Standard(s)	Brodifacoum used as an internal standard	
3.2.4	Interfering substance(s)	N/A	

Section A4.2 (d) Analytical Methods for Detection and Identification

Annex Point IIA4.2 & IIIA-IV.1

Methods of analysis in human and animal body fluids and tissues.

3.3	**Linearity**	
3.3.1	Calibration range	UV linearity in the range 10-500ng/ml Fluorescence detection linearity in the range 10-500ng/ml
3.3.2	Number of measurements	
3.3.3	Linearity	UV detection: $r^2 = 0.9997$, regression line y= 0.0091x +0.0434 Fluorescence detection: r^2=0.9997, regression line y = 0.0134x + 0.0368
3.4	**Specifity: interfering substances**	N/A
3.5	**Recovery rates at different levels**	Recovery in serum and plasma: >65% Recovery in tissue > 50%
3.5.1	Relative standard deviation	N/A
3.6	**Limit of determination**	Sensitivity limt: 5ng/ml for serum/plasma/blood. 10ng/g for tissue
3.7	**Precision**	
3.7.1	Repeatability	CV % of intrarun and interrun data for serum and tissues at different concentrations range from 5% and 18%.
3.7.2	Independent laboratory validation	N/A

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	NaOH n (0.05 ml) is added to 1 -2 ml of serum/plasma/blood containing 100 ng of brodifacoum as internal standard. The sample is extracted with 4ml of ethyl acetate vortexing for 3 min. The mixture is centrifuged and the organic layer taken to dryness in a gentle stream of nitrogen. The residue is then reconstituted with 0.1 ml of the mixture methanol:water (1:1) and injected into the HPLC system. Tissues (liver, spleen, lung, kidney, etc) : 10 grams of tissue are homogenized with 10ml of water with a homogenizer: 2ml of sample homogenized containing 100ng/ml of internal standard are extracted.
4.2	**Conclusion**	Each represented matrix has all the relevent fields of information reoprted., including limits of determination and recovery rates. The limits of detection allow determination of the active substance at the no adverse effect concentration.
4.2.1	Reliability	2
4.2.2	Deficiencies	No

Section A4.2 (d) Analytical Methods for Detection and Identification

Annex Point IIA4.2 & IIIA-IV.1

Methods of analysis in human and animal body fluids and tissues.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE FINLAND
Date	4 August 2006
Materials and methods	NaOH n (0.05 ml) is added to 1 -2 ml of serum/plasma/blood containing 100 ng of brodifacoum as internal standard. The sample is extracted with 4ml of ethyl acetate vortexing for 3 min. The mixture is centrifuged and the organic layer taken to dryness in a gentle stream of nitrogen. The residue is then reconstituted with 0.1 ml of the mixture methanol:water (1:1) and injected into the HPLC system. Tissues (liver, spleen, lung, kidney, etc) : 10 grams of tissue are homogenized with 10ml of water with a homogenizer: 2ml of sample homogenized containing 100ng/ml of internal standard are extracted.
Conclusion	The analytical technique is considered to be commonly available. In 3.3 Linearity the equation of the calibration line and correlation coefficient have been submitted, but the typical calibration plot is missing. In 3.5 Recovery the recovery rate for for serum and plasma (over 65%) and for tissue (over 50%) has been reported. In 3.6 Limit of determination the sensitivity limit has been reported to be 5 ng/ml for serum/plasma/blood and 10 ng/g for tissue. However, due to several major deficiencies the method is not sufficiently validated and does not cover all requirements for analysis of body fluids and tissues. The study is not done in compliance with the GLP.
Reliability	3
Acceptability	not acceptable
Remarks	In 3.3 Linearity either duplicate determinations at three or more concentrations or single determinations at 5 or more concentrations must be made. The calibration range has been mentionned, but the number of determinations and concentration levels are missing. In 3.5 Recovery the recovery rates have been reported, but the levels for the determinations are missing. The mean recoveries for each level should be in the range 70-110% and the control samples should be analysed concurrently. The precision of the method must be reported as repeatability of recovery at each fortification level and the overall RSD must also be reported. Five determinations should be made at each fortification level. The blank values should not exceed 30% of the LOQ. The study is not done in compliance with the GLP.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Results and discussion	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant´s summary and conclusion.* *Discuss if deviating from view of rapporteur member state*

Section A4.2 (d) Analytical Methods for Detection and Identification

Annex Point IIA4.2 & IIIA-IV.1

Methods of analysis in human and animal body fluids and tissues.

Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A4.3 Analytical Methods for Detection and Identification

Annex Point IIA4.1/4.2 & IIIA-IV.1

Validation of Analytical Methodology to Determine Rodenticides in Food Matrices

Official use only

1 REFERENCE

1.1	**Reference**	Turnbull, G (2005) Validation of Analytical Methodology to Determine Rodenticides in Food Matrices. Central Science Laboratory: PGD-180.
1.2	**Data protection**	Yes
1.2.1	Data owner	The CEFIC Rodenticide Group
1.2.2	Companies with access to data	PelGar International Ltd and Activa srl The Rodenticide Group and those wishing to comply with FIFRA Section 10.
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of it's entry into Annex I.

2 GUIDELINES

2.1	**Guideline**	SANCO/825/00 rev. 6
2.2	**GLP**	Yes
2.3	**Deviations**	

3 MATERIALS AND METHODS

3.1 Preliminary treatment

3.1.1 Enrichment

Analytical method for determination of Difenacoum in the cucumber

From the stock solutions prepare fortification solutions in methanol.

Control samples (30g) are fortified using a microsyringe or glass pipette by adding a volume of fortification solution as described below.

Fortification level (mg/kg)	Concentration of fortification sol. (µg/ml)	Volume of fortification sol. (µg)
0.01	1	300
0.1	10	300

presence of solid carbon dioxide.

Weigh 30 g of sample into 250 ml Schott bottle. Any control sample requiring fortification should be fortified at this point. Add 60 ml of ethyl acetate and 30g (+/-5g) of sodium sulphate. Homogenise using the Ultra Turrax for 1 minute on the red setting, pour the extract through a funnel with a non-absorbent cotton wool plug and a layer of sodium sulphate into one or more 37 ml amber vials.

Transfer 20 ml of extract into a 25ml graduated tube and evaporate to dryness. Re-dissolve the residue in 5ml of acetone. Using a glass microsyringe add 200 µl of 2-butlyamine.

3.1.2 Cleanup

The extract from above is loaded onto a SPE column which is eluted with 2 solvents and 2 different fractions are collected. One fraction is

evaporated to dryness and a derivative formed which is determined by GC-MS.

3.2 Detection

3.2.1 Separation method: Liquid chromatography

3.2.2 Detector:

Mass spectrometer:	Sciex API 2000 (PE/Applied Biosystems)
Column:	Phenomenex Luna 150 mm x 2 mm i.d. packed with 5 µm Phenyl-Hexyl, no guard column.
Mobile phase:	
A:	10 mM ammonium acetate
B:	methanol
Flow rate:	0.2 ml/min
Ionisation mode:	Turboionspray negative ion
Injection Volumn:	5 µL

3.2.3 Standard(s): internal standard: coumatetralyl

3.2.4 Interfering substance(s): None stated. The specificity of the methods were tested using control (untreated) matrices.

3.3 Linearity: Non-entry field

3.3.1 Calibration range: Not applicable.

3.3.2 Number of measurements: 5 measurements made at each of the two fortification levels.

3.3.3 Linearity: Calibration curve values (R^2) ranged from 0.9162 to 0.9969

3.4 Specifity: interfering substances: None stated.

3.5 Recovery rates at different levels

Validation study	Difenacoum (LC-MS-MS)			
	Fortification level	Recovery (%)	Mean recovery (%)	RSD(%)
Cucumber	0.01	94-109	100	7
	0.1	91-102	98	5
Wheat	0.01	102-124	117	8
	0.1	64-101	86	13
Meat	0.01	65-78	71	7
	0.1	41-82	58	29
Oil-seed	0.01	101-123	111	9

3.5.1 Relative standard deviation

3.6 Limit of determination: 0.01 mg/kg stated.

3.7 Precision

Section A4.3 Analytical Methods for Detection and Identification

Annex Point IIA4.1/4.2 & IIIA-IV.1

Validation of Analytical Methodology to Determine Rodenticides in Food Matrices

3.7.1	Repeatability	Validation of procedure at LOQ and at 0.1 mg/kg.
3.7.2	Independent laboratory validation	None.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	SANCO/825/00 rev. 6 The specificity of the methods were tested using control (untreated) matrices. The determination for difenacoum was performed by liquid chromatography followed by mass spectrometry for identification.
4.2	**Conclusion**	Validation data have been provided by the analysis of fortified samples and by comparison with unfortified samples. The methods validated in this study are multi-residue in nature in that they allow determination of all 8 analytes in the same sample extract. It was possible to detect all analytes in all matrices studied. For most of the analytes/matrix combinations studied, mean recoveries are >70% with RSD values of <20% and the methods are also suitable for quantitative determination. For combinations in which mean recoveries are <70% and/or RSD values >20% the methods in this study may be used to determine whether an analyte is present in a sample but for quantitative measurement a separate procedure would be required.
4.2.1	Reliability	2
4.2.2	Deficiencies	Statistical analysis to support the limit of quantitation was not presented. Recoveries from meat were poor, and the relative standard deviations for all the crops were quite large.

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE FINLAND

Date	12 September 2006, 13 November 2006
Materials and methods	The validation has been made at fortification levels of 0.01 mg/kg and 0.1 mg/kg for five matrix (cucumber, wheat, meat, oil-seed rape, and lemon). The determination for difenacoum was performed by liquid chromatography followed by mass spectrometry for identification.

Section A4.3 Analytical Methods for Detection and Identification

Annex Point IIA4.1/4.2 & IIIA-IV.1

Validation of Analytical Methodology to Determine Rodenticides in Food Matrices

Conclusion	The validation study and the method seem to be acceptable. In 3.3 Linearity the measurements were done at four concentrations (0.03, 0.1, 0.4, and 1,2 µg/ml) for all five matrices. A typical calibration plot has been submitted for one matrix (lemon). The equation of the calibration line and the correlation coefficient for that line has been reported. In 3.5 Recovery the validation has been made at fortification levels of 0.01 mg/kg and at 0.1 mg/kg for five matrix (cucumber, wheat, meat, oil-seed rape, and lemon). Five determinations have been made at both fortification level for each matrices. The mean recoveries are within accepted limits (70-110%) in both fortification level for cucumber, wheat, and lemon. For meat the mean recovery in higher fortification level was too low (58%) and for oil-seed-rape it was too high (118%). The relative standard deviations have been reported to all matrices in both fortification levels. Only for meat in higher fortification level (0.1 mg/kg) the relative standard deviation was higher than 20%. For each fortification level and matrix, a control sample has been reported to analyse and the values was less than 30% of the lowest fortification level. For the reasons listed above, it can be concluded that the analytical method is in compliance with the validation and other criteria required from such method in the SANCO/3029/99 Guidance Document.
Reliability	2, except for meat and oil-seed-rape –validation criteria 3
Acceptability	acceptable
Remarks	

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Results and discussion	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant´s summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	