Section A4.2(e)

**Annex Point IIA,
IV.4.2(e)/01**

Analytical Methods for Detection and Identification

Difethialone residues in treated food and feedingstuffs

			Official use only

1   REFERENCE

1.1	**Reference**	Wolf, X. (2006). Development and validation of a residue analytical method for difethialone in meat (muscle), oil seed rape (seed) and lemon (whole fruit). XXX Xxx., unpublished report number XXXXXX, 22 June 2006.
1.2	**Data protection**	Yes.
1.2.1	Data owner	LiphaTech SAS.
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. 7.
2.2	**GLP**	Yes.
2.3	**Deviations**	No.

3   MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Extraction	Samples are extracted by blending and then shaking with methanol (meat and lemon) or methanol/water 4+1 v/v (oil-seed rape). After centrifugation the samples are diluted with methanol/water.
3.1.2	Cleanup	None.
3.2	**Detection**	
3.2.1	Separation method	HPLC, Thermo Hypersil-Keystone, Fluophase PFP column with mobile phase A: 95:5 v/v water/acetonitrile + 5 mM ammonium formate + 0.1% formic acid and B 95:5 v/v acetonitrile/water + 5 mM ammonium formate + 0.1% formic acid.

The "x" marks appear in the Official use only column for rows 3.1.1 and 3.3.

Time [minutes]	0	2.0	3.5	3.6	5.0
A [%]	60	0	0	60	60
B [%]	40	100	100	40	40
Flow [µL/minutes]	400	400	400	400	400

3.2.2	Detector	MS-MS primary method (m/z: 81.0). Confirmation ion (m/z: 79.3).
3.2.3	Standard(s)	External standard.
3.2.4	Interfering substance(s)	Analysis of control samples demonstrated that there were no known substances which interfered with the detection of difethialone.
3.3	**Linearity**	
3.3.1	Calibration range	0.05 to 5.0 ng/mL.
3.3.2	Number of	Seven.

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	measurements	
3.3.3	Linearity	R^2 = >0.9995.
3.4	**Specifity: interfering substances**	Analysis of control samples showed that there were no substances which interfered with the detection of difethialone. An HPLC/MS-MS method with a different MS-transition (m/z: 79.3) was used for confirmation. The use of LC/MS-MS is considered to be highly specific.
3.5	**Recovery rates at different levels**	Recoveries from fortified oil seed rape, meat and lemon were as follows:

Matrix	Fortification level (mg/kg)	Recovery (%)		
		range	mean	n
Oil seed rape	0.01	70 – 95	85	5
(seeds)	0.10	90 – 99	95	5
	overall	70 – 99	90	10
Meat (muscle)	0.01	78 – 87	81	5
	0.10	78 – 97	87	5
	overall	78 – 97	84	10
Lemon (whole	0.01	87 – 99	92	5
fruit)	0.10	94 – 102	98	5
	overall	87 – 102	95	10

3.5.1	Relative standard deviation	RSD values were as follows:

Matrix	Fortification level (mg/kg)	RSD (%)	Overall RSD (%)
Oil seed rape	0.01	12.8	10.3
(seeds)	0.10	3.8	
Meat (muscle)	0.01	4.5	7.8
	0.10	9.4	
Lemon (whole	0.01	5.0	5.2
fruit)	0.10	3.8	

3.6	**Limit of determination**	The limit of determination is 0.01 mg/kg (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.

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	4	**APPLICANT'S SUMMARY AND CONCLUSION**
4.1	**Materials and methods**	Samples are extracted by blending and then shaking with methanol (meat and lemon) or methanol/water (oil-seed rape). After centrifugation the samples are diluted with methanol/water. Determination is by HPLC/MS-MS with Thermo Hypersil-Keystone, Fluophase PFP column with mobile phase: 95:5 v/v water/acetonitrile + 5 mM ammonium formate + 0.1% formic acid and 95:5 v/v acetonitrile/water + 5 mM ammonium formate + 0.1% formic acid (ion monitored m/z: 250.3).
4.2	**Conclusion**	The method for determination of residues of difethialone in oil seed rape, meat and lemon has been adequately validated. The method was successfully evaluated and meets the EU criteria with respect to specificity, linearity and accuracy according to the guidance given in SANCO/825/00. The method requires equipment and instrumentation which is commonly available in most well-equipped laboratories. Therefore, the method is suitable for enforcement purposes.
4.2.1	Reliability	1
4.2.2	Deficiencies	No

Evaluation by Competent Authorities
EVALUATION BY RAPPORTEUR MEMBER STATE

Date	31 December 2006
Materials and methods	Agree with applicant's summary and conclusions.
	Comments (3.1.1): Type of meat has not been specified.
	Comments (3.3): A non-linear regression has been applied in order to achieve the best fit to the calibration data. The non-linear (exponential) equation is given as $y = a*x^b$.
Conclusion	Agree with applicant's version.
Reliability	1
Acceptability	Acceptable
Remarks	-

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<table>
<tr><td></td><td>**5   REFERENCE**</td><td>Official use only</td></tr>
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5.1	**Reference**	Xxxxxxxx, X. (2005). Validation of Analytical Methodology to Determine Rodenticides in Food Matrices. Xxxxxxx Xxxxxxxxxx unpublished report number XXXXX, 16 June 2005.
5.2	**Data protection**	Yes.
5.2.1	Data owner	LiphaTech SAS.
5.2.2	Companies with letter of access	None.
5.2.3	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.

6   GUIDELINES AND QUALITY ASSURANCE

6.1	**Guideline study**	SANCO/825/00 rev. 6.
6.2	**GLP**	Yes.
6.3	**Deviations**	No.

7   MATERIALS AND METHODS

7.1	**Preliminary treatment**	
7.1.1	Extraction	<u>Cucumber</u> Difethialone 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> Difethialone 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.
7.1.2	Cleanup	Gel permeation chromatography or SPE catridge.
7.2	**Detection**	
7.2.1	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.
7.2.2	Detector	MS-MS (primary ion m/z: 79-81).
7.2.3	Standard(s)	External standard.
7.2.4	Interfering substance(s)	Analysis of control samples demonstrated that there were no substances which interfered with the detection of difethialone. There were no chromatographic peaks above 30% of the LOQ at the retention time of difethialone.
7.3	**Linearity**	
7.3.1	Calibration range	0.03 to 1.2 µg/mL.

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7.3.2	Number of measurements	Eight.

7.3.3	Linearity	R^2 (cucumber) = 0.951 and 0.955
		R^2 (wheat) = 0.972 and 0.996

7.4   Specifity: interfering substances

Analysis of control samples showed that there were no substances which interfered with the detection of difethialone. 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 difethialone.

7.5   Recovery rates at different levels

Recoveries from fortified cucumber and wheat were as follows:

Matrix	Fortification level (mg/kg)	Recovery (%)		
		range	mean	n
Cucumber	0.01	72 – 94	88	5
	0.10*	88 – 100	94	5
	overall	72 – 100	91	10
Wheat	0.01	81 – 117	101	5
	0.10	75 – 92	84	5
	overall	75 – 117	93	10

* Values at this validation level were determined without the use of an internal standard

7.5.1	Relative standard deviation	RSD values were as follows:

Matrix	Fortification level (mg/kg)	RSD (%)	Overall RSD (%)
Cucumber	0.01	10.2	8.5
	0.10*	5.8	
Wheat	0.01	13.3	14.5
	0.10	9.3	

* Values at this validation level were determined without the use of an internal standard

7.6   Limit of determination

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

7.7   Precision

7.7.1	Repeatability	RSD values are presented above under 3.5.1.

7.7.2	Independent laboratory validation	Not applicable.

8   APPLICANT'S SUMMARY AND CONCLUSION

8.1   Materials and   Cucumber

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	methods	Difethialone 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	
		Difethialone 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.	
8.2	**Conclusion**	The methods for determination of residues of difethialone 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.The method requires equipment and instrumentation which is commonly available in most well-equipped laboratories. Therefore, the methods are suitable for enforcement purposes.	
8.2.1	Reliability	1	
8.2.2	Deficiencies	None	

Evaluation by Competent Authorities
EVALUATION BY RAPPORTEUR MEMBER STATE

Date	31 December 2006
Materials and methods	Agree with applicant's version.
Conclusion	Agree with applicant's version.
Reliability	1
Acceptability	Acceptable
Remarks	-