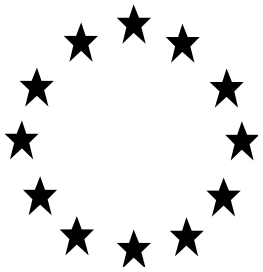
Competent Authority Report

Programme for Inclusion of Active Substances in Annex I to Council Directive 98/8/EC



Amines, N-C10–C16-alkyltrimethylenedi-, reaction products with chloroacetic acid; Ampholyt (PT 2, 3, 4)

CAS-No. 139734-65-9

DOCUMENT IIIA (A4)

Evaluation Report

Rapporteur: Ireland

April 2015

Ampholyt (PT2, 3, 4)

Document A4

CONTENTS

Section A4.1 Annex Point IIA4.1	Analytical method for determining the concentrations of the active substance(s) in the biocidal product	Official use only
	1 REFERENCE	
Reference	**Reference A4.1/01:** [redacted] Determination of the content of microbicidal amphotheric in TEGO 2000. NOTOX B.B. 's-Hertogenbosch, The Netherlands, unpublished report no. 285525, May 09, 2000.	
Data protection	Yes	
Data owner	Goldschmidt GmbH	
Companies with letter of access	No	
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	
Guideline study	No, determination of the content of microbicidal amphotheric in TEGO 2000 according to Shogenki method, AA HC 002 A, Goldschmidt.	
GLP	Yes	
Deviations	No	
	3 MATERIALS AND METHODS	
Preliminary treatment		
Enrichment	Not applicable	
Cleanup	Not applicable	

Section A4.1 Annex Point IIA4.1	Analytical method for determining the concentrations of the active substance(s) in the biocidal product	
Detection	The content of microbicidal amphoteric in TEGO 2000 was determined using a titration method. The results of the standardisation of the 0.1 mol/l sodiumthiosulphate solution are shown in Table 1 below. The results of the determination of the content microbicidal amphoteric in TEGO 2000 are shown in Table 2 below. Standardisation: About 150 mg (w g) of potassium iodate was weighed (to the nearest 0.1 mg) into a conical flask and dissolved in 40 ml of milli-Q water. The conical flask was securely closed and the solution was shook until all potassium iodate completely dissolved. 2 g of potassium iodide and 12 ml 1 mol/l hydrochloric acid solution was added. The liberated iodine was titrated with the sodium thiosulphate solution, with constant shaking. When the reaction was nearly complete, i.e. when the solution was pale yellow in colour, the solution was diluted to a volume of 200 ml with Milli-Q water. Starch indicator solution (2 ml) was added and the titration was continued until the solution became colourless (t ml). Determination of the content of microbicidal amphoteric: About 3.3 g TEGO 2000 was weighed (to the nearest 0.1 mg) into a conical flask and dissolved in 25 ml 1 mol/l hydrochloric acid and 25 ml sodium acetate. 50 ml of potassiumhexacyanoferrate was added, this solution was stirred and placed in the dark for 60 minutes. Thereafter the solution was filtered, the flask and the filter were washed with 2 x 50 ml water. To the filtrate 10 ml 1 mol/l hydrochloric acid, 10 ml 1 mol/l potassium iodide and 15 ml 10% zinc sulphate was added. Liberated iodine was titrated with the sodium thiosulphate solution, with constant shaking. When the reaction was nearly complete, i.e. when the solution was pale yellow in colour, the solution was diluted to a volume of 200 ml with Milli-Q water. Starch indicator solution (2 ml) was added and the titration was continued until the solution became colourless (t ml). Blank solution was prepared as described above, omitting the test substance.	
Separation method	Not applicable, titration method	
Detector	Not applicable. From the results of the colour titration method the content of the amphotheric matter in the amount of test substance was determined.	
Standard(s)	Not applicable	
Interfering substance(s)	none	
Linearity	Not applicable	
Calibration range		
Number of measurements		
Linearity		
Specificity: interfering substances		X
Recovery rates at different levels	Not stated	
Relative standard deviation	Not stated	
Limit of determination	Not applicable	

Section A4.1 Annex Point IIA4.1	**Analytical method for determining the concentrations of the active substance(s) in the biocidal product**	
Precision	The precision (repeatability) of the method was determined by fivefold analysis of one subset, the blanks were determined in duplicate.	
Repeatability	18.57 ± 0.02 % (w/w)	
Independent laboratory validation	Not stated	
	4 APPLICANT'S SUMMARY AND CONCLUSION	
Materials and methods	The content of microbicidal amphotheric in TEGO 2000 was determined using a titration method.	
Conclusion	The content of microbicidal amphotheric in TEGO 2000 is 18.57 ± 0.02 % (w/w).	X
Reliability	1	
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	17/01/13	
Materials and Methods	**X – 3.4 - Specificity: interfering substances** Titration methods of analysis are not considered to be specific methods of analysis. A confirmatory method of analysis should be presented in order to confirm identity. **X – 4.2 – Conclusion** The applicant has stated that "this method is merely designed to provide customers with an analytical method to establish a method to inspect the incoming AMPHOLYT 20 which they use to prepare their biocides. The detection extends into the 20% w/w area". However it should be noted that there is essentially no validation data accompanying this method of analysis (no linearity, recovery, %RSD available). The only validation data that has been presented is precision data. The applicant should fully validate the titration method if they intend to rely upon this method.	
Results and discussion		
Conclusion	Further validation required if the applicant intends to rely upon this method. Titration methods are not considered to be specific/highly specific methods of analysis. The applicant should use a specific method of analysis for determining the content of active substance in the technical material as manufactured. Methods of analysis cannot be considered reliable if they have not been fully validated. The applicant should validate their supporting methods of analysis in line with requirements outlined in guidance documents that are considered to be reliable under the EU evaluation process for Biocides.	
Reliability	3	
Acceptability	Not acceptable unless further validation data is supplied.	
Remarks	The applicant has stated that they are not relying upon this method for the purposes of the dossier. The applicant is relying upon another method of analysis (HPLC-CAD) for the purposes of the dossier. See Confidential Section A4.1/02 below.	
	COMMENTS FROM ...	
Date		
Materials and Methods		
Results and discussion		
Conclusion		
Reliability		
Acceptability		
Remarks		

Table 1: Standardisation of the 0.1 mol/l sodiumthiosulphate solution.

Test	Volume sodiumthiosulphate (ml)	Weight of the potassium iodate (g)	Concentration sodiumthiosulphate (mol/l)
Blank	0	–	–
Test 1	42.255	0.1500	9.95×10^{-2}
Test 2	42.098	0.1499	9.98×10^{-2}
Test 3	42.184	0.1501	9.98×10^{-2}
Mean concentration sodiumthiosulphate solution (mol/l)			9.97×10^{-2}
Standard deviation			1.37×10^{-4}
Coefficient of variation (%)			0.17

Table 2: Determination of the content of microbicidal amphoteric in TEGO 2000.

Test	Volume sodium thiosulphate (ml)	Weight of the potassium iodate (g)	Concentration sodium thiosulphate (mol/l)
Blank1	24.669	–	–
Blank2	24.698	–	–
Blank mean	24.684		
Test 1	14.276	3.3204	18.58
Test 2	14.192	3.3494	18.57
Test 3	14.186	3.3478	18.59
Test 4	14.316	3.3126	18.55
Test 5	14.248	3.3296	18.58
Mean content microbicidal amphoteric, % (w/w)			18.57
Standard deviation			1.52×10^{-2}

Evaluation by Competent Authorities	
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	29/07/13
Materials and Methods	**Other methods of analysis** The applicant also provided a method of analysis for the individual components of Ampholyt 20. The HPLC –CAD method is evaluated as part of the Confidential Section. The HPLC-CAD evaluation is included in the Confidential Section of the CAR. The following analytical reports are included in the confidential section of the CAR: (1) Analytical procedure – short description, analytical method for the analysis of Ampholyt 20 by HPLC – CAD & (2) Characterisation of Ampholyt 20 by HPLC – CAD. Validation Report & (3) Determination of acetic acid in Ampholyt 20 by HPLC-UV The exact references for the three study reports are provided in the reference part of this Section.
Results and discussion	APCP WG III 2014 decided that only the overall min. purity of 100% w/w (TC) will appear in the Inclusion Regulation. The exact identity and concentration ranges of the individual components in Ampholyt 20 will not be disclosed in the Inclusion Regulation or the non-confidential sections of the CAR.
Conclusion	
Reliability	Not applicable – just a comment from the RMS.
Acceptability	Not applicable – just a comment from the RMS.
Remarks	APCP WG III 2014 decided that the min. purity of 100% w/w (TC) will appear in the Inclusion Regulation. The exact identity of the individual components in Ampholyt 20 will not be disclosed in the Inclusion Regulation or the non-confidential sections of the CAR.
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (a) soil	Official use only

REFERENCE

Reference **A4.2/01:**

[redacted] Validation for the substance-specific analysis of Ampholyt 20/100 in soil. Report no. EBR-013/7-24, Fraunhofer-Institute for Molecular Biology and Applied Ecology (IME), Schmallenberg, Germany, November 14, 2007 (unpublished).

Data protection Yes

Data owner: Goldschmidt GmbH

Companies with letter of access: No

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.

GUIDELINES AND QUALITY ASSURANCE

Guideline study Yes

EC Commission Directive 91/414/EEC, SANCO/825/00 rev.7, 17/03/04

EC Commission Directive 91/414/EEC, SANCO/3029/99 rev.4, 11/07/00

GLP Yes

Deviations No

Further remarks The test material is a multi-component substance as specified in Section A2. In this validation study the determination of Ampholyt 20/100 was restricted to four representative "lead components" of Ampholyt 20/100. The substance consists of various components (see Section A2) most of which are not commercially available as analytical standards. Therefore, and also due to the low proportion of most components in Ampholyt 20/100 it was not possible to validate the analytical method for all components of the active substance.

However, the selected "lead components" all have the C_{12}-alkyl group as moiety and they represent all functional groups by which the active substance is characterised. In summary, the C_{12} group makes up approximately 75% (w/w) of the chain length distribution of Ampholyt 20/100. All other components in the Ampholyt 20/100 mixture occur at much lower amounts, and only differ in their chain length (varying from C_{10} to C_{16}). The C_{12}-compounds are thus considered to be representative for the whole complex active substance which may safely be assumed to behave in a similar way as the chosen "lead components":

[redacted]

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (a) soil	
	MATERIALS AND METHODS	
Preliminary treatment		
Enrichment	To enrich the four components of Ampholyt 20/100, the residues were extracted from spiked Lufa 2.2 soil four times with: • 25 ml acetone (extract 1) • 25 ml methanol/dichlormethane/25% ammonia 50:50:1 (v/v/v) (extract 2) • 25 ml methanol/dichlormethane/98% formic acid 50:50:5 (v/v/v) (extract 3) • 25 ml methanol/dichlormethane/98% formic acid 50:50:5 (v/v/v) (extract 4). The four extracts were combined and an aliquot was reduced to dryness, re-dissolved in 0.6 ml methanol/acetonitrile/98% formic acid (50:50:0.5, v/v/v), diluted with water and spiked with triflouoroacetic acid and internal standard (2-Aminononanoic acid).	
Cleanup	Not applicable.	
Detection		
Separation method	High-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) HPLC-equipment: Waters 2795, Alliance HT Column: Luna C8(2), 100x 2.0 mm, 3 µm 100 A° (Phenomenex) MS/MS-equipment: Micromass, Quattro Ultima Pt	
Detector	Multiple reaction monitoring (MRM) for peak identification; software: MassLynx Vers. 3.5	
Standard(s)	Internal standard: 2-Aminononanoic acid	
Interfering substance(s)	None	
Linearity		
Calibration range	Calibration curves for each of the four “lead components” for a lower and a higher concentration range: 0.5 ng/ml to 10 ng/ml 10 ng/ml to 500 ng/ml	
Number of measurements	Two calibration curves (low and high concentration range) for each “lead component” were plotted based on seven different concentrations, respectively, each of them injected twice.	
Linearity	Linear relationships between the peak area the peak area, i.e. the response and the nominal concentration were obtained. The determined coefficients of determination ranged between R^2 = 0.889 and 0.997.	
Specificity: interfering substances	No interferences or interfering peaks were recorded in the total ion chromatogram (TIC of 5 mass channels). The highly selective detection of electrospray ionisation mass spectrometry coupled to multiple reaction monitoring (ESI_MS/MRM) allows unequivocal identification and quantification without significant interferences of interfering peaks.	X

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (a) soil	

Recovery rates and relative standard deviation at different levels

Fortification level	Recovery	RSD	n
"Lead component" 1			
0.05 mg/kg	105 %	11 %	5
0.5 mg/kg	108 %	9 %	5
"Lead component" 2			
0.05 mg/kg	111 %	5 %	5
0.5 mg/kg	101 %	13 %	5
"Lead component" 3			
0.05 mg/kg	108 %	10 %	5
0.5 mg/kg	111 %	11 %	5
"Lead component" 4			
0.05 mg/kg	91 %	9 %	5
0.5 mg/kg	73 %	7 %	5

Relative standard deviation: See above.

Limit of determination

Limit of quantification:

"Lead component"1, 2, 3, and 4: LOQ = 0.05 mg/kg

Limit of detection (LOD):

"Lead component" 1	LOD = 0.004 mg/kg
"Lead component" 2	LOD = 0.005 mg/kg
"Lead component" 3	LOD = 0.005 mg/kg
"Lead component" 4	LOD = 0.007 mg/kg

X

Precision

Repeatability: The reproducibility of the analytical method is given by the relative standard deviation (RSD) of the determined recovery rates at a certain fortification level. In no case the RSD exceeded 20 %.

Independent laboratory validation: Not applicable.

APPLICANT'S SUMMARY AND CONCLUSION

Materials and methods

A LC-MS/MS method for the determination of four representative "lead components" of Ampholyt 20/100 in Lufa 2.2 soil was developed and validated. The soil samples were extracted with acetone, methanol/dichlormethane/25% ammonia, methanol/dichlormethane/98% formic acid, and methanol/dichlormethane/98% formic acid, sequentially. Determination was performed by LC-MS/MS.

X

Conclusion

Average recoveries were in the range between 73 and 110 % (except two cases of 111 %) and with relative standard deviations below 20%. Interfering blanks were not observed. Therefore the method fulfils the requirements of SANCO/825/00 rev. 7 and can be used as an enforcement method for the determination of residues of Ampholyt 20/100 in soil.

Reliability: 1

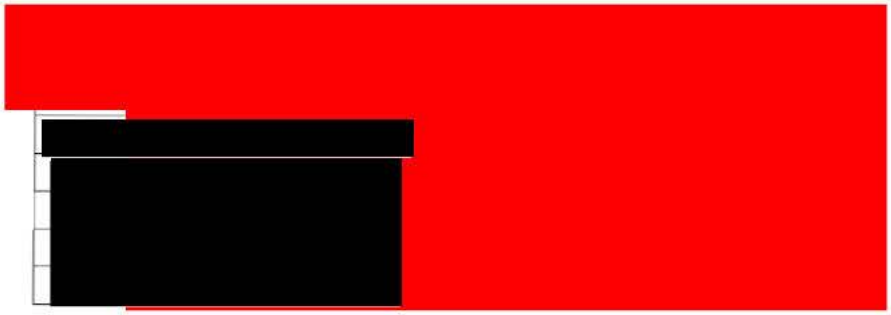
Deficiencies: Yes

Mean recovery rates of > 110 % for the lower fortification level (0.05 mg/kg) of "lead component" (2) and the higher fortification level

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (a) soil	
	(0.5 mg/kg) of "lead component" (3) were caused by narrow contamination effects during the intricate sample preparation. Moreover, memory effects in the MS/MS-equipment influenced the measurement. This is, however, related to the properties of the substance, rendering it difficult to analyse and does therefore, not compromise the general validity of the method.	

Evaluation by Competent Authorities	
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	

	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	26/03/2013
Materials and Methods	**X - Further remarks:** The "sum of lead compounds" is proposed by the applicant for the residue definition in soil. The applicant had included four lead components of Ampholyt 20 for method validation in soil. The four lead components make up approximately 55 – 65% w/w of technical Ampholyt 20/100. It should also be noted that it would be unreasonable to request applicants and Member State Monitoring Laboratories to monitor for 20+ components in order to monitor the use of one active substance. The applicant has provided member states with the possibility of monitoring for four lead components of Ampholyt 20. The residue definition for monitoring was discussed during the EU peer-review commenting stage. The final residue definition for monitoring includes three lead components: It was considered that it was unnecessary to monitor for all four components and that the above lead components were sufficiently representative of Ampholyt 20. The applicant has been able to synthesise reference standards (characterised by NMR and MS) for the four lead components. The reference standards used for method validation of the soil method have been reported to have the following purity - It is noted by the RMS that the % purity of two of the reference standards is low, however taking into consideration that the standards are not commercially available and that the applicant has had considerable difficulty in synthesising the standards, the % purity of the reference standards should be considered acceptable. The applicant has correctly stated that "an experimental determination of the degradation half-life (DT_{50}) of Ampholyt 20 in soil is not considered to be required in view of the ready biodegradability and overall limited direct release to soil…… ...the selected "lead components" all have the C_{12}-alkyl group as moiety and in summary, the C_{12} group makes up approximately 75 % (w/w) of the chain length distribution of Ampholyt 20/100. All other components in the Ampholyt 20/100 mixture occur at much lower amounts, and only differ in their chain length (varying from C_{10} to C_{16}). The C_{12}-compounds are thus considered to be representative for the whole complex active substance which may safely be assumed to behave in a similar way as the selected "lead components". It should be noted that the RMS does not have any specific information in relation to the stability and degradation of the four lead components in soil. **X – Specificity** The applicant validated the method of analysis for the four lead components using a single ion transition for each lead component.

	
Results and discussion	It should be noted that it has previously been agreed agreed by Member States that in order for MS/MS to be considered to be highly specific, 2 ion transitions need to be monitored. The applicant should provide validation data for a second ion transition for the four components if they are going to be included in the residue definition for monitoring. **X-LOQ:** The given LOQ of 0.05 mg/kg is considered acceptable in terms of the appropriate NO(A)EC. The lowest EC_{50} for soil organisms was observed in Lactuca sativa with 363 mg a.s/kg dry weight soil. The NO(A)EC is < 363 mg a.s/kg dry soil, and therefore the LOQ is acceptable. **X – Materials and methods** The use of hazardous substances should be avoided if possible. It should be noted that dichloromethane is classified as a carcinogen. It would have been preferable to have used an alternative solvent. The validation data as presented by the applicant is acceptable for the "three lead components" with the exception of the number of ion transitions used for method validation. The applicant should provide two validated ion transitions for each target species considered to be part of the residue definition for monitoring.
Conclusion	Further validation is required with respect to a second ion transition.
Reliability	3
Acceptability	Not acceptable.
Remarks	Further validation and discussion required.

	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Section A4.2 Annex Point IIA4.2	Analytical Methods for Detection and Identification in (b) air	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ] Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	A method for the detection of residues in air is not submitted, since Ampholyt 20 is neither volatile nor intended to be applied by high-pressure spraying or in any other way resulting in occurrence of Ampholyt 20 in air. This is supported by study B6.6/04, where the MMAD of droplets resulting from spraying of application solutions of TEGO 51 (a product equivalent to TEGO 2000, thus equivalent to Ampholyt 20, see sections A2 and B2) was ≥ 185 µm. In view of the current cut-off criterion of an MMAD of 50 µm for inhalation exposure, the submission of an analytical method in air is not considered to be required.	
Undertaking of intended data submission [ ]		

Evaluation by Competent Authorities	
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	26/03/2013
Materials and Methods	
Results and discussion	
Conclusion	The requirement for a method of analysis for air was discussed at APCP WG III. It was decided that tox. input post WG would be required with regards to possible inhalation exposure exposure before a final decision could be made. The RMS tox. experts decided that Ampholyt 20 exposure in air is not considered to be significant with regards to the method of application and intended use pattern (foam application – not a spray application). However, if other methods of application and intended uses are requested in the future, the requirement for a method of analysis for air will have to be revisited.
Reliability	1
Acceptability	Acceptable.
Remarks	No further data required.
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (c) water	
	REFERENCE	Official use only
Reference	**A4.2/02:** [redacted] Validation for the substance-specific analysis of Ampholyt 20/100 in water. Report no. EBR-013/7-26, Fraunhofer-Institute for Molecular Biology and Applied Ecology (IME), Schmallenberg, Germany, November 14, 2007 (unpublished).	X
Data protection	Yes	
Data owner	Goldschmidt GmbH	
Companies with letter of access	No	
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.	
	GUIDELINES AND QUALITY ASSURANCE	
Guideline study	Yes, EC Commission Directive 91/414/EEC, Sanco/825/00 rev.7, 17/03/04 EC Commission Directive 91/414/EEC, Sanco/3029/99 rev.4, 11/07/00	
GLP	Yes	
Deviations	No	
Further remarks	The test material is a multi-component substance as specified in Section A2. In this validation study the determination of Ampholyt 20/100 was restricted to four representative "lead components" of Ampholyt 20/100. The substance consists of various components (see Section A2) most of which are not commercially available as analytical standards. Therefore, and also due to the low proportion of most components in Ampholyt 20/100 it was not possible to validate the analytical method for all components of the active substance. However, the selected "lead components" all have the C12-alkyl group as moiety and they represent all functional groups by which the active substance is characterised. In summary, the C12 group makes up approximately 75% (w/w) of the chain length distribution of Ampholyt 20/100. All other components in the Ampholyt 20/100 mixture occur at much lower amounts, and only differ in their chain length (varying from C10 to C16). The C12-compounds are thus considered to be representative for the whole complex active substance which may safely be assumed to behave in a similar way as the chosen "lead components": [redacted] MATERIALS AND METHODS	X

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (c) water	
Preliminary treatment		
Enrichment	To enrich the four components of Ampholyt 20/100, the residues were extracted from spiked and acidified (formic acid) water by solid phase extraction (SPE): The water sample was loaded onto conditioned SPE-columns with BondElut C2 material (VARIAN). The "lead components" were eluted sequentially with • 3 ml acetone/98 % formic acid 100:1 (v/v) (extract 1) • 5 ml methanol/acetonitrile/dichlormethane/98 % formic acid 50:25:25/2 (v/v/v/v) (extract 2) • 3 ml methanol/acetonitrile/dichlormethane/25% ammonia 50:25:25:1 (v/v/v/v) (extract 3) The eluate was concentrated to dryness under a stream of nitrogen at 40 °C, re-dissolved in 0.6 ml methanol/acetonitrile/98% formic acid 50:50:0.5 (v/v/v), diluted with 0.6 ml water and spiked with trifluoroacetic acid and internal standard (2-Aminononanoic acid).	
Cleanup	Not applicable.	
Detection		
Separation method	The "lead components" in the worked-up water samples were quantified by means of high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS). HPLC-equipment: Waters 2795, Alliance HT Column: Luna C8(2), 100x 2.0 mm, 3 µm 100 A° (Phenomenex) MS/MS-equipment: Micromass, Quattro Ultima Pt	
Detector	Multiple reaction monitoring (MRM) for peak identification, software: MassLynx Vers. 3.5	
Standard(s)	Internal standard: 2-Aminononanoic acid	
Interfering substance(s)	None	
Linearity		
Calibration range	Calibration curves for each of the four "lead components" for a lower and higher concentration range: 0.5 ng/ml to 10 ng/ml 10 ng/ml to 500 ng/ml	
Number of measurements	Two calibration curves (low and high concentration range) for each "lead component" were plotted based on seven different concentrations, respectively, each of them injected twice.	
Linearity	Linear relationship between the peak area the peak area, i.e. the response and the nominal concentration were obtained. The determined coefficients of determination ranged between r^2 = 0.9647 and 0.9914.	
Specificity: interfering substances	No interferences or interfering peaks were recorded in the total ion chromatogram (TIC of 5 mass channels). The highly selective detection of electrospray ionisation mass spectrometry coupled to multiple reaction monitoring (ESI_MS/MRM) allows unequivocal identification and quantification without significant interferences of interfering peaks.	X

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (c) water	

Recovery rates at different levels

Fortification level	Recovery	RSD	n
"Lead component" 1			
0.1 µg/l	101 %	10 %	5
1.0 µg/l	93 %	15 %	5
"Lead component" 2			
0.1 µg/l	111 %	6 %	5
1.0 µg/l	89 %	12 %	5
"Lead component" 3			
0.1 µg/l	103 %	8 %	5
1.0 µg/l	95 %	12 %	5
"Lead component" 4			
0.1 µg/l	115 %	5 %	5
1.0 µg/l	91 %	14 %	5

Relative standard deviation: See above.

Limit of determination

Limit of quantification:

"Lead component"1, 2, 3, and 4: LOQ = 0.1 µg/l

Limit of detection (LOD):

"Lead component" 1	LOD = 0.005 µg/l
"Lead component" 2	LOD = 0.015 µg/l
"Lead component" 3	LOD = 0.004 µg/l
"Lead component" 4	LOD = 0.01 µg/l

X

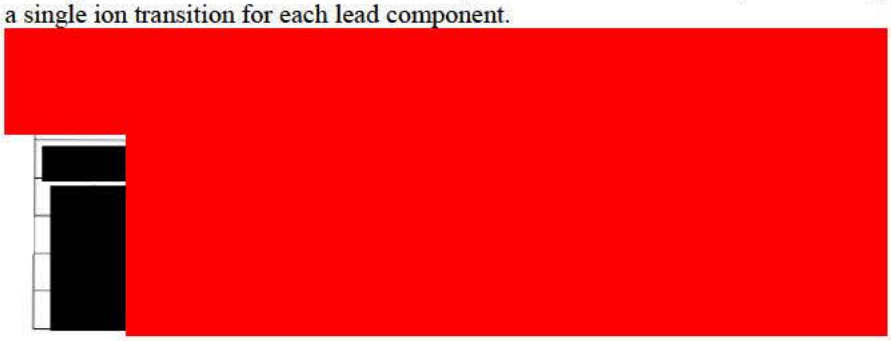
Precision

Repeatability: The method was successfully validated with five values at both fortification levels for the four "lead components", with recoveries in the range from 89 % to 115% and relative standard deviations of the single measuring series between 5 and 15 %.

Independent laboratory validation: Not applicable.

Section A4.2 Annex Point IIA4.2	Analytical methods for detection and identification in (c) water	
	APPLICANT'S SUMMARY AND CONCLUSION	
Materials and methods	A LC-MS/MS method for the determination of 4 selected "lead components" of Ampholyt 20/100 in water was developed and validated. The water samples were loaded onto SPE-columns with C2 material. The "lead components" were eluted sequentially with acetone/98 % formic acid, methanol/acetonitrile/dichlormethane/98 % formic acid, and methanol/acetonitrile/dichlormethane/25% ammonia. Determination of the four lead components (and the internal standard) was performed by LC-MS/MS.	X
Conclusion	Average recoveries were in the range between 70 and 110 % (except two cases of >110 %) and with relative standard deviations not exceeding 20%. The values of the blanks did not exceed 30% of the values of the lowest fortification level. Therefore, the method can be used as an enforcement method for the determination of residues of Ampholyt 20/100 in water.	
Reliability	1	
Deficiencies	Yes Mean recovery rates of > 110 % for the lower fortification level (0.1 µg/l) of "lead component" (2) and "lead component" (4) were caused by narrow contamination effects during the intricate sample preparation. Moreover, memory effects in the MS/MS-equipment influenced the measurement. This is, however, related to the properties of the substance, rendering it difficult to analyse and does, therefore, not compromise the general validity of the method.	

	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
Date	EVALUATION BY RAPPORTEUR MEMBER STATE (*) 26/03/2013	
Materials and Methods	**X – Reference** The study title refers to "water". The study matrix used for method validation was tap water. The applicant also needs to validate their method of analysis for surface water and sediment. **X - Further remarks:** The "sum of lead compounds" is proposed by the applicant for the residue definition in drinking and surface water. The applicant has included four lead components of Ampholyt 20 for method validation. The four lead components make up approximately 55 – 65% w/w of technical Ampholyt 20/100. It should also be noted that it would be unreasonable to request applicants and Member State Monitoring Laboratories to monitor for 20+ components in order to monitor the use of one active substance. The applicant has provided member states with the possibility of monitoring for four lead components of Ampholyt 20. The residue definition for monitoring was discussed during the EU peer-review commenting stage. The final residue definition for monitoring includes three lead components: It was considered that it was unnecessary to monitor for all four components and that the above lead components were sufficiently representative of Ampholyt 20. The applicant has been able to synthesise reference standards (characterised by NMR and MS) for the four lead components. The reference standards used for method validation of the tap water method have been reported to have the following purity - It is noted by the RMS that the % purity of two of the reference standards is low, however taking into consideration that the standards are not commercially available and that the applicant has had considerable difficulty in synthesising the standards, the % purity of the reference standards should be considered acceptable. The applicant has correctly stated that "Ampholyt 20 is hydrolytically and photolytically stable, therefore it will be detectable in drinking and surface water. In view of its intended indoor use as a disinfectant (PT 2, 3 and 4), any release will be through sewage treatment plants. Ampholyt 20 is aerobically readily biodegradable, leading to an efficient elimination of the active substance in sewage treatment plants, which has also been demonstrated by two simulation tests in sewage treatment plants with removal rates of 81 ± 5 % (A7.1.2.1.1/01) and 92–99 % (A7.1.2.1.1/02), respectively, and a DT_{50} of 0.693 hours". It should be noted that the RMS does not have any specific information in relation to the stability of the four lead components in water.	

Results and discussion	**X – Specificity** The applicant validated the method of analysis for the four lead components using a single ion transition for each lead component.  It should be noted that it has previously been agreed that in order for MS/MS to be considered to be highly specific, 2 ion transitions need to be monitored. The applicant should provide validation data for a second ion transition for the four components. **X – LOQ** The LOQ of the method for drinking water matrices is 0.1µg/L. The LOQ is acceptable. The applicant needs to validate the method of analysis for surface water. The LOQ for the method in surface water should be below the relevant NOEC by a factor of 10 or more. The relevant NOEC is 2.3µg/L (Daphnia magna). **X – Materials and methods** The use of hazardous substances should be avoided if possible. It should be noted that dichloromethane is classified as a carcinogen. It would have been preferable to have used an alternative solvent. The validation data as presented by the applicant is acceptable for the "three lead components" with the exception of the number of ion transitions used for method validation. The applicant should provide two validated ion transitions for each target species considered to be part of the residue definition for monitoring. The applicant needs to validate the method for surface water and sediment matrices.
Conclusion	Further validation and clarification required.
Reliability	3
Acceptability	Not acceptable.
Remarks	Further validation and discussion required. The results of the second ion transition validation for drinking water, and the validated surface water method of analysis should be provided to the RMS at least 6 months before the date of entry into force.

	COMMENTS FROM ...
Date	
Materials and Methods	

Section A4.2 Annex Point IIA4.2	Analytical Methods for Detection and Identification in (d) animal and human body fluids and tissues	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	Submission of such a method is not considered to be required in view of the toxicological properties of Ampholyt 20: Since this compound is not classified as toxic or highly toxic, there is no need to establish a method for determination of the a.s. in body fluids and tissues. For further information, please refer to toxicological data in section 6 of this dossier.	
Undertaking of intended data submission [ ]		

	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)	
Date	14/08/2013	
Evaluation of applicant's justification	The active substance is classified as Toxic (R48) under Dir. 67/548/EEC and Danger (H372) under Reg. 1272/2008.	
Conclusion	The applicant will need to provide a validated method of analysis for monitoring in body fluids and tissues with an LOQ which allows for determination well below the critical NOAEL (21.012 mg/kg)..	
Remarks	Further data required.	
	COMMENTS FROM ...	
Date		
Evaluation of applicant's justification		
Conclusion		
Remarks		

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs	Official use only
	REFERENCE	
Reference	**A4.3/01:** [redacted] Über den Stellenwert amphoterer Desinfektionsmittelspuren in Speisegelatine – Ergebnisse entsprechender rückstandsanalytischer, mikrobiologischer und toxikologischer Untersuchungen. Archiv für Lebensmittelhygiene 29: 62-65.	
Data protection	No	
Data owner	Public domain	
Companies with letter of access	Not applicable	
Criteria for data protection	None	
	GUIDELINES AND QUALITY ASSURANCE	
Guideline study	No	
GLP	No	
Deviations	Not applicable	
	MATERIALS AND METHODS	
Preliminary treatment	The method described here aims at determination of TEGO 51 (for similarity with Ampholyt 20 see Appendix 1 to Doc. III-A, confidential data) in gelatine. However, the method is claimed to be generally applicable to "amphotensides".	
Enrichment	Digestion with HCl (5 ml HCl conc. per 1 g gelatine) under reflux for min. 8 h. Transfer to adequately sized flask, filled up with HCl, take aliquot. Adjusted to alkaline pH with 100 ml 1 n NaOH per 5 ml original sample volume. Extract three times by shaking with 5 ml $HCCl_3$, respectively; the organic extracts should not be removed before the solution is clear.	
Clean-up	The pooled organic extracts are evaporated to dryness and the solid residue dissolved in 5 mL $HCCl_3$. To this solution, Orange II-solution and HCL (0.1 mol/L) are added and shaken on a mechanical shaker for 15 min. The organic phases are subjected to photometric measurement.	
Detection		
Separation method	None	
Detector	UV-Spectrophotometer (485 nm)	
Standard(s)	None (method description only)	
Interfering substance(s)	Not applicable	

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs
Linearity	
Calibration range	Not stated; method description only.
Number of measurements	Not stated; method description only.
Linearity	To be determined for the respective analysis.
Specificity: interfering substances	Specificity is claimed for “amphotensides”.
Recovery rates at different levels	100 ± 5%
Relative standard deviation	Not stated
Limit of determination	1.6 ppm (calculated for 100 % of a.s.)
Precision	
Repeatability	Not stated
Independent laboratory validation	Not applicable
	APPLICANT’S SUMMARY AND CONCLUSION
Materials and methods	Quantitative determination of TEGO 51 in gelatine (analytic residue test method) by digestion under reflux with HCL conc. The measurement was performed by photometric determination (485 nm) of the Orange II organic salt.
Conclusion	The method was found to be suitable for the determination of TEGO 51 in gelatine. Acceptable recovery rates were obtained (100 ± 5%). A control determination of blank values was carried out by TLC. **Comment:** The second method described in the publication (TLC) is only a semi quantitative determination method and therefore not described in this summary.
Reliability	2
Deficiencies	Only a method description, without validation data, is provided.

Evaluation by Competent Authorities	
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	09/08/2013
Materials and Methods	The method has little or no validation data available. The method reported in this section can only be viewed as supplementary data. There is a more suitable method reported in Section A4.3/04: [redacted] Ampholyt 20 Components – Analytical Method for the Determination of Residues in Food of Animal Origin, Wine, and Beer. Study No. CRA13322, Dr. U. Noack-Laboratorien, Sarstedt, Germany. June 21, 2010 (unpublished). The study report in Section A4.3/04 contains validation data for an LC-MS/MS method of analysis.
Results and discussion	The method of analysis has not been validated in accordance with current requirements.
Conclusion	The method of analysis is not acceptable. There is a more suitable method of analysis available in Section A4.3/04.
Reliability	3
Acceptability	Not acceptable.
Remarks	See Section A4.3/04 of the CAR.
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs	Official use only
	REFERENCE	
Reference	**A4.3/02:** [redacted] Adsorptionsvermögen von mikrobiziden Amphotensiden auf harten Oberflächen. TH. Goldschmidt AG, Germany, Analysenvorschrift THG-AL/022-02-B, 15. Oktober 1993 (unpublished). (In German; English translation included)	
Data protection	Yes	
Data owner	Goldschmidt GmbH	
Companies with letter of access	No	
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.	
	GUIDELINES AND QUALITY ASSURANCE	
Guideline study	No	
GLP	No	
Deviations	Not applicable	
	MATERIALS AND METHODS	
Preliminary treatment	The method described here is designed to determine residues of microbicidal amphotensides on hard surfaces. Therefore, hollow bodies of defined materials and surface area (inorganic and organic materials) were exposed to an application solution of microbicidal amphotensides for a given period. After removal of the application solution, the hollow bodies were rinsed several times with a defined quantity of water.	
Enrichment	**For inorganic hollow bodies (i.e. high-grade steel, aluminium, glass):** The hollow bodies were sealed on one side with a PE-foil coated rubber plug and filled with a defined quantity of water (dest.), HCl (1 mol/L), Orange-II-solution and $CHCl_3$. They were then sealed with a second plug and placed on a mechanical shaker for 20 min. The contents of the hollow bodies was transferred to centrifuge vials, respectively. **For organic hollow bodies (i.e. PVC and PE):** The hollow bodies were sealed on one side with a PE-foil coated rubber plug and rinsed with a defined amount of ethanol. The ethanolic extracts were transferred into round flasks and evaporated to dryness. The round flask was filled with a defined quantity of water (dest.), HCl (1 mol/L), Orange-II-solution and $CHCl_3$. The contents of the round flasks was transferred to centrifuge vials.	

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs
Clean-up	By centrifugation, the ion pair complex is completely separated into the organic phase. The absolutely clear organic phases are subjected to photometric measurement. Blanks were extracted from untreated pipes and examined in parallel.
Detection	
Separation method	Not applicable
Detector	UV-spectrophotometer (485 nm)
Standard(s)	None
Interfering substance(s)	None
Linearity	
Calibration range	Not stated
Number of measurements	8 concentration levels
Linearity	To be determined for the respective analysis
Specificity: interfering substances	Specificity is claimed for “microbicidal amphotensides”.
Recovery rates at different levels	Not stated
Relative standard deviation	Not stated
Limit of determination	0.17 mg/m^2 a.s.
Precision	
Repeatability	Not stated
Independent laboratory validation	Not applicable
	APPLICANT’S SUMMARY AND CONCLUSION
Materials and methods	Quantitative determination of microbicidal amphotensides on hard surfaces by photometric determination (485 nm) of the Orange II organic salt. Each measurement was carried out in six fold.
Conclusion	The method was found to be suitable for the determination of microbicidal amphotensides on hard surfaces. Acceptable reproducibility and specificity was given. The estimated standard deviation for the analysis procedure in any analysis series represents the total standard deviation, amounting to ± 0.18 mg/m².
Reliability	2
Deficiencies	No detail information of recovery rates was given.

Evaluation by Competent Authorities	
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	09/08/2013
Materials and Methods	The method has little or no validation data available. The method reported in this section can only be viewed as supplementary data. There is a more suitable method reported in Section A4.3/04: [redacted] Ampholyt 20 Components – Analytical Method for the Determination of Residues in Food of Animal Origin, Wine, and Beer. Study No. CRA13322, Dr. U. Noack-Laboratorien, Sarstedt, Germany. June 21, 2010 (unpublished). The study report in Section A4.3/04 contains validation data for an LC-MS/MS method of analysis.
Results and discussion	The method of analysis has not been validated in accordance with current requirements.
Conclusion	The method of analysis is not acceptable. There is a more suitable method of analysis available in Section A4.3/04.
Reliability	3
Acceptability	Not acceptable.
Remarks	See Section A4.3/04 of the CAR.
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Section A4.3 Annex Point IIIA 4.1	**Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs**	
	REFERENCE	Official use only
Reference	**A4.3/03:** Anonymous (1991): Photometric micro-method for determination of TEGOL 2000 residues in food substrates. Th. Goldschmidt AG, Essen, Germany, June 1991 (unpublished).	
Data protection	Yes	
Data owner	Goldschmidt GmbH	
Companies with letter of access	No	
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.	
	GUIDELINES AND QUALITY ASSURANCE	
Guideline study	No	
GLP	No	
Deviations	Not applicable	
	MATERIALS AND METHODS	
Preliminary treatment	The method described here is designed to determine residues of TEGOL 2000 (which a trade name for Ampholyt 20) in food substrates like white and red wine, aqueous palatinose, beer and milk.	

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs

Enrichment	**For white and red wine, aqueous palatinose and beer:** In a measuring cylinder sodium carbonate and sodium hydroxide are dissolved in water (dest.), a defined amount of the relevant matrix is added to this solution and filled up to the mark. The resulting solution is quantitatively transferred into a separation apparatus (see Figure A4.3-1) and carefully covered with a layer of acetic ester. The washing bottle in the gas stream inlet (N_2) is filled with acetic ester to 2/3 of its capacity. A N_2 stream is led through the apparatus for a defined time. Subsequently the acetic ester phase is separated and the aqueous phase is newly covered with a layer of acetic ester. After further 15 minutes of expellation of the active substance with inert gas both organic phases are combined and transferred into a round flask evaporated to dryness and filled with water (dest.), HCl (1 mol/L), Orange-II-solution and $CHCl_3$. **For milk:** Milk is evaporated to dryness in a round flask, then HCl (conc.) is added and the resulting solution is digested under reflux for approx. 10 hours until the disturbing components are completely degraded. After cooling, the resulting hydrolysis products are filtered off via a black band filter and the filtrate is transferred into a round flask. The residue is washed with a small volume of HCl (conc.) and the washing liquid is united with the filtrate evaporated to dryness and filled up with HCl (conc.) again. NaOH (1 mol/L) is added and the resulting solution is transferred quantitatively into a separation funnel and extracted tree times with $CHCl_3$. The organic phases are combined, evaporated to dryness and filled with $CHCl_3$, HCl (1 mol/L) and Orange-II-solution.
Clean-up	The flasks are closed, placed for 20 min on a shaking machine. The contents of the round flasks are transferred into centrifuge vials. By centrifugation, the ion pair complex is completely separated into the organic phase. After the phases are separated, the aqueous phase is removed by a pipette and the CHCl3 phase is centrifuged. The absolutely clear organic phases are subjected to photometric measurement. Blanks are determined analogously.
Detection	
Separation method	Not applicable
Detector	UV-spectrophotometer (485 nm)
Standard(s)	None
Interfering substance(s)	None
Linearity	
Calibration range	0.5 ng/L – 1 µg/L
Number of measurements	8 different concentration s, single determination
Linearity	To be determined for each matrix in the course of the respective analysis.
Specificity: interfering substances	Specificity is claimed for “microbicidal amphotensides”.
Recovery rates at different levels	Not stated

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs
Relative standard deviation	Not stated
Limit of determination	0.05 ppm a.s. (white and red wine, aqueous palatinose and beer) 1 ppm (milk)
Precision	
Repeatability	Not stated
Independent laboratory validation	Not applicable
	APPLICANT'S SUMMARY AND CONCLUSION
Materials and methods	Quantitative determination of TEGOL 2000 by photometric determination (485 nm) based on the formation of an ion pair complex. By quantitative protonation the active ingredients present in TEGOL 2000 are transferred completely into their cationic-active form. By reaction with the anionic dye Orange II an ion- pair complex is formed which is soluble in organic solvents. The colour intensity is proportional to the concentration of the microbicidal active substance. Each sample should be measured in quadruplicate.
Conclusion	The method is considered to be suitable for the determination of TEGOL 2000 in food substrates like white and red wine, aqueous palatinose, beer and milk.
Reliability	2
Deficiencies	No detail information of recovery rates was given.

Evaluation by Competent Authorities	
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	09/08/2013
Materials and Methods	The method has little or no validation data available. The method reported in this section can only be viewed as supplementary data. There is a more suitable method reported in Section A4.3/04: [redacted] Ampholyt 20 Components – Analytical Method for the Determination of Residues in Food of Animal Origin, Wine, and Beer. Study No. CRA13322, Dr. U. Noack-Laboratorien, Sarstedt, Germany. June 21, 2010 (unpublished). The study report in Section A4.3/04 contains validation data for an LC-MS/MS method of analysis.
Results and discussion	The method of analysis has not been validated in accordance with current requirements.
Conclusion	The method of analysis is not acceptable. There is a more suitable method of analysis available in Section A4.3/04.
Reliability	3
Acceptability	Not acceptable.
Remarks	See Section A4.3/04 of the CAR.
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Figure A4.3- 1: Illustration of the separation apparatus.

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs	

		Official use only
	REFERENCE	
Reference	**A4.3/04:** [redacted] Ampholyt 20 Components – Analytical Method for the Determination of Residues in Food of Animal Origin, Wine, and Beer. Study No. CRA13322, Dr. U. Noack-Laboratorien, Sarstedt, Germany. June 21, 2010 (unpublished).	
Data protection	Yes	
Data owner	Evonik Industries AG (former Evonik Goldschmidt GmbH)	
Companies with letter of access	No	
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.	
	GUIDELINES AND QUALITY ASSURANCE	
Guideline study	Yes. SANCO/825/00 rev. 7 (17/03/04), Guidance document on residue analytical methods	
GLP	Yes	
Deviations	No	
Further remarks	The test material is a multi-component substance as specified in section A2. In this study the determination of Ampholyt 20 was restricted to four representative "lead components" of Ampholyt 20 (Ampholyt 20/100 likewise). The substance consists of various components (see section A2) most of which are not commercially available as analytical standards. Therefore, and also due to the low proportion of most components in Ampholyt 20/100 it was not possible to determine all components of the active substance analytically. However, the selected "lead components" all have the C_{12}-alkyl group as moiety and represent all functional groups by which the active substance is characterised. In summary, the C_{12} moiety makes up approximately 75 % (w/w) of the chain length distribution of Ampholyt 20/100. All other components in the Ampholyt 20/100 mixture occur at much lower amounts, and only differ in their chain length (varying from C_{10} to C_{16}). The C_{12}-compounds are thus considered to be representative for the whole complex active substance; the remaining components may safely be assumed to behave in a similar way as the representative "lead components": [redacted]	

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs

MATERIALS AND METHODS

Preliminary treatment	The method aims at determination of the representative lead components of Ampholyt 20 (C12 PDA, N-C12-Gly, N'-C12-Gly, N'-C12-diGly) in food of animal origin (meat, animal fat, milk), red and white wine, and beer. These matrices were fortified with the lead components to levels of 1× and 10× LOQ (0.01 mg/kg) and (0.1 mg/kg), each. Two stock solutions of the lead components in methanol were prepared to spike the food matrices (for 1 × or 10 × LOQ) (as given in section 3.1.1).
Enrichment	Meat: For each replicate, 2 g of minced meat were defrosted and spiked with spiking solutions and left for 20 min. The samples were mixed with 15.51 mL methanol + 2 % formic acid with the ultraturrax for 2 min (10 rpm) and then treated with ultrasound for 15 min at 30 °C. The samples were then shaken on the rotary shaker for 30 min (10 rpm) and centrifuged for 5 min (3000 rpm). 1 mL of the supernatant was diluted with 1 mL HPLC water and filtered before analysis by LC-MS/MS. Fat: For each replicate, 2 g of fat (warmed-up to 50 °C, molten) were spiked with spiking solutions (for 1 × or 10 × LOQ) and kept in a water bath at 50 °C during extraction. The fat was vigorously mixed with 5 mL of methanol + 1 % trifluoroacetic acid for 2 min thrice. After the fat had settled down, 0.2 mL of the supernatant was diluted with 1.4 mL methanol:HPLC water (50:50) + 0.05 % trifluoroacetic acid and homogenised. Samples were filtered before analysis by LC-MS/MS. Milk: For each replicate, 4 mL of milk (corresponding to 4 g) were spiked with spiking solutions (for 1× or 10 × LOQ) and diluted with 4 mL HPLC water. Each replicate was given on Oasis HLB cartridges (conditioned with 5 mL methanol and 5 mL HPLC water). After washing of the cartridges with 5 mL HPLC water the samples were eluted with 10 mL methanol + 2 % formic acid. Samples were then diluted with HPLC water (factor 2) before analysis by LC-MS/MS. Red wine, white wine, beer: For each replicate, 4 mL (corresponding to 4 g) of each test system were spiked with spiking solution. Each replicate was diluted by factor 10 with methanol:HPLC water + 0.05 % trifluoracetic acid (for 1 × LOQ) before analysis by LC-MS/MS. For determination of C12 PDA, the samples were diluted by factor 100 instead of factor 10.
Clean-up	See above (3.1.1 Enrichment)
Detection	Detection was carried out by electrospray ionisation in positive mode using an external standard giving a linear response.
Separation method	Ultra performance liquid chromatography
Detector	Mass spectrometric detection (MS/MS detector, Xevo, WATERS)
Standard(s)	Certified lead components (C12 PDA, N-C12-Gly, N'-C12-Gly, N'-C12-diGly) were used as external standards.
Interfering substance(s)	Not applicable

Section A4.3 Annex Point IIIA 4.1	Analytical methods including recovery rates and the limits of determination for the active substance, and for residues thereof, in/on food or feedstuffs	
Linearity		
Calibration range	Meat, fat, milk: The analytical system gave a linear response in the nominal range of 0.25–10 µg/L (n=7) for each lead test item component [redacted] White wine, red wine, beer: The analytical system gave a linear response between nominal 0.025 and 3.2 µg/L (n = 8) for C12 PDA and 0.25 and 32 µg/L (n = 8) for the three other components [redacted]	
Number of measurements	n = 7 (meat, fat, milk) n = 8 (white wine, red wine, beer)	
Linearity	$r^2 \geq 0.992$	
Specificity: interfering substances	Specificity is given by the mass spectrometric method, due to the formation of specific mother and daughter ions of the test item. The response of the blank values of the two control samples was lower than 30 % of LOQ for each test system.	
Recovery rates at different levels	The mean recovery rates in foodstuff of animal origin are given in Table A4.3- 3 to Table A4.3- 8.	
Relative standard deviation	The relative standard deviations are given in Table A4.3- 3 to Table A4.3- 8.	
Limit of determination	Limits of detection (LODs) and limits of quantification (LOQs) for Ampholyt 20 components are given in Table A4.3- 1.	X
Precision	No detectable chromatographic interferences >30 % LOQ were determined in the control samples.	
Repeatability	For the repeatability of injections please refer to Table A4.3- 2 (6 sub-samples of a higher and the lowest calibration concentration of each lead substance)	
Independent laboratory validation	Not applicable	
	APPLICANT'S SUMMARY AND CONCLUSION	
Materials and methods	The method aims at determination of the lead components of Ampholyt 20 [redacted] in food of animal origin, wine, and beer. The determination was carried out via ultra performance LC-MS/MS on a reversed-phase column. Analysis was performed in gradient mode. Detection was carried out by electrospray ionisation in positive mode, using the lead test item components as external standards.	
Conclusion	The results confirm that the described method is suitable for the determination of residues of Ampholyt 20 components in meat, fat, milk, white wine, red wine, and beer at the limit of quantification (1 × LOQ) and the tenfold value of the limit of quantification (10 × LOQ).	X
Reliability	1	
Deficiencies	None	

	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)	
Date	26/03/2013	
Materials and methods	**X - Further remarks:** Analytical methods for residues are required, presuming that the biocidal product may come in contact with food, foodstuffs and feeding stuffs. This is always the situation for product- types 3, 4 and 5. The "sum of lead compounds" is proposed by the applicant for the residue definition in/on food or feedstuffs. The applicant has included four lead components of Ampholyt 20 for method validation. The four lead components make up approximately 55 – 65% w/w of technical Ampholyt 20/100. It should also be noted that it would be unreasonable to request applicants and Member State Monitoring Laboratories to monitor for 20+ components in order to monitor the use of one active substance. The applicant has provided member states with the possibility of monitoring for four lead components of Ampholyt 20. The applicant has been able to synthesise reference standards (characterised by NMR and MS) for the four lead components. The reference standards used for method validation of the food and feeds method have been reported to have the following purity - [redacted] It is noted by the RMS that the % purity of two of the reference standards is low, however taking into consideration that the standards are not commercially available and that the applicant has had considerable difficulty in synthesising the standards, the % purity of the reference standards should be considered acceptable. The applicant has stated that "Ampholyt 20 is hydrolytically as well as photolytically stable, it is easily detectable in food and feedstuffs. Furthermore, the lead components could also be detected in a mammalian metabolism study in which it was abundant in faeces, urine and the only component in plasma. Whereas the initial absorption lies between 20 to 34% and the vast majority was excreted rapidly within 24 hours. The selected "lead components" all have the C_{12}-alkyl group as moiety. In summary, the C_{12} group makes up approximately 75 % (w/w) of the chain length distribution of Ampholyt 20/100. All other components in the Ampholyt 20/100 mixture occur at much lower amounts, and only differ in their chain length (varying from C_{10} to C_{16}). The C_{12}-compounds are thus considered to be representative for the whole complex active substance which may safely be assumed to behave in a similar way as the selected lead components". The RMS notes that there are no metabolism studies available for Ampholyt 20 in food and feed, therefore the RMS does not have any specific information in relation to the stability of the four lead components in these matrices. It is very difficult to propose a residue definition for monitoring if the RMS has no information regarding the stability of these four components in food and feed. The applicant needs to elaborate further regarding the suitability of these four components for monitoring in food of plant and animal origin. The applicant's decision to include four lead in the residue definition proposal is not ideal - ideally monitoring methods of analysis should have one simple target molecule if possible. The applicant has proposed that all four components should be included in the	

	residue definition for monitoring. The inclusion of four lead components in the residue definition for monitoring adds a greater degree of certainty regarding analysis but will also introduce significant practical implications for monitoring laboratories at Member State level. It needs to be considered if it is possible to reduce the number of target analytes (lead components) in the residue definition so that enforcement laboratories have a more practical residue definition for enforcement purposes. **X – Specificity** The applicant validated the method of analysis for the four lead components using a single ion transition for each lead component. It should be noted that it has previously been agreed that in order for MS/SM to be considered to be highly specific, 2 ion transitions need to be monitored. The applicant should provide validation data for a second ion transition for the four components. **X-LOQ** An LOQ of 0.01 mg/kg was achieved for the four lead components in each matrices. The LOQ is acceptable. **X-Conclusion** ILV studies are necessary to perform when compliance with an MRL is required in order to demonstrate the reproducibility of the analytical method. ILV studies are generally needed for the determination of residues in plant materials and additionally for methods for the determination of residues in food of animal origin, if such methods are required. Usually, an ILV should be conducted with samples of the representative commodities and tissues. The sample set (number of samples and fortification levels) of the primary validation has to be applied for the ILV also. The laboratory chosen to conduct the ILV trials must not have been involved in the method development and in its subsequent use. Provided this criterion is met, the laboratory chosen to conduct the ILV trials may be in the applicant's organisation, but must not be at the same location. If the chosen laboratory requires communication with the developers of the method to carry out the analysis, this should be reported. Also any subsequent additions or modifications to the original method should be reported. APCP WG III discussed the requiremenmt for an I.L.V. method of analysis for food of plant and animal origin. The meeting agreed that the request for an ILV will be reconsidered at product authorisation when additional guidance on how to derive MRLs for biocides will be possibly in place.
Results and discussion	Residue transfer into food and feed of plant and animal origin is unlikely due to risk mitigation measures and the intrinsic properties of the substance, however, the accuracy of this statement cannot be concluded due to the absence of relevant guidance or data to quantitatively confirm this assertion. Please refer to Section 2.2.1 of Doc I. The validation data as presented by the applicant is acceptable for the "four lead components" with the exception of the number of ion transitions used for method validation. The applicant should provide two validated ion transitions for each target species considered to be part of the residue definition for monitoring. The applicant has demonstrated that they have at least two ion transitions available for method validation for each target analyte:

	 The comprehensive validation results reported in the actual study report and the summary document only refer to the validation results for the quantifier transition. It also needs to be considered if it is possible to reduce the number of lead components in the residue definition for monitoring taking into consideration the practical implications for monitoring laboratories at Member State level. The applicant should comment regarding the specificity of the four lead components and whether they are common to other active substances. The issue of requiring an ILV study will be revisited at the product authorisation stage..
Conclusion	Further validation and clarification required.
Reliability	3
Acceptability	Not acceptable.
Remarks	Further validation and clarification required. Outstanding validation data and clarifications with respect to the primary method of analysis should be provided 6 months before entry into force. The issue of requiring an ILV study will be revisited at the product authorisation stage.
	COMMENTS FROM ...
Date	
Materials and methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Table A4.3- 1: Limit of quantification (LOQs) for representative lead components of Ampholyt 20 in food matrices

Lead component	LOQ [mg/kg]	LOQ [mg/kg]	LOQ [mg/kg]	LOQ [mg/kg]	LOQ [mg/kg]	LOQ [mg/kg]
	Meat	Animal Fat	Milk	White wine	Red wine	Beer
[redacted]	0.01	0.01	0.01	0.01	0.01	0.01
[redacted]	0.01	0.01	0.01	0.01	0.01	0.01
[redacted]	0.01	0.01	0.01	0.01	0.01	0.01
[redacted]	0.01	0.01	0.01	0.01	0.01	0.01

Table A4.3- 2: Repeatability of injections of the lead components of Ampholyt 20

Test item		Repeatability of injections, peak area [counts]	
		Mean ± SD	RSD [%]
[redacted]	0.25 µg/L	113 ± 22.0	19.5
	10 µg/L	65737 ± 585	0.890
[redacted]	0.25 µg/L	88.8 ± 11.9	13.4
	10 µg/L	4804 ± 89.5	1.86
[redacted]	0.25 µg/L	139 ± 21.7	15.6
	10 µg/L	6534 ± 201	3.08
[redacted]	0.25 µg/L	147 ± 5.45	3.71
	10 µg/L	6704 ± 136	2.03

Table A4.3- 3: Mean recovery rates (%) in meat

Meat	1 × LOQ			10 × LOQ		
Lead components	Mean	SD	RSD [%]	Mean	SD	RSD [%]
[redacted]	81	3.44	4.25	108	2.83	2.62
[redacted]	78	7.97	10.2	86	3.27	3.80
[redacted]	73	5.89	8.07	82	4.28	5.22
[redacted]	86	16.9	19.7	78	4.58	5.87

Table A4.3- 4: Mean recovery rates (%) in animal fat

Animal fat	1 × LOQ			10 × LOQ		
Lead Components	Mean	SD	RSD [%]	Mean	SD	RSD [%]
[redacted]	82	9.37	11.4	96	2.35	2.45
[redacted]	84	7.47	8.89	89	4.56	5.12
[redacted]	90	10.4	11.6	95	5.39	5.67
[redacted]	108	14.9	13.8	100	6.15	6.15

Table A4.3- 5: Mean recovery rates (%) in milk

Milk	1 × LOQ			10 × LOQ		
Lead Components	**Mean**	**SD**	**RSD [%]**	**Mean**	**SD**	**RSD [%]**
[redacted]	87	4.64	5.33	100	6.07	6.07
[redacted]	84	6.66	7.93	85	2.83	3.33
[redacted]	90	6.57	7.30	95	4.66	4.91
[redacted]	108	8.44	7.81	102	5.77	5.66

Table A4.3- 6: Mean recovery rates (%) in white wine

White wine	1 × LOQ			10 × LOQ		
Lead Components	**Mean**	**SD**	**RSD [%]**	**Mean**	**SD**	**RSD [%]**
[redacted]	80	4.83	6.04	93	5.32	5.72
[redacted]	104	10.9	10.5	104	5.52	5.31
[redacted]	101	5.89	5.83	107	6.30	5.89
[redacted]	94	5.72	6.09	97	6.28	6.47

Table A4.3- 7: Mean recovery rates (%) in red wine

Red wine	1 × LOQ			10 × LOQ		
Lead Components	**Mean**	**SD**	**RSD [%]**	**Mean**	**SD**	**RSD [%]**
[redacted]	78	4.28	5.49	105	2.30	2.19
[redacted]	89	5.81	6.53	97	1.14	1.18
[redacted]	90	1.79	1.99	101	1.41	1.40
[redacted]	96	4.83	5.03	99	4.47	4.52

Table A4.3- 8: Mean recovery rates (%) in beer

Beer	1 × LOQ			10 × LOQ		
Lead Components	**Mean**	**SD**	**RSD [%]**	**Mean**	**SD**	**RSD [%]**
[redacted]	85	8.47	9.96	108	1.34	1.24
[redacted]	77	2.92	3.79	77	2.77	3.60
[redacted]	80	3.54	4.43	77	2.49	3.23
[redacted]	104	7.70	7.40	100	2.39	2.39