1. INTRODUCTION

Background and Objective:

The objective of this study was to independently validate an analytical method for the determination of d-phenothrin in surface water. The method was originally validated by Smithers Viscient using liquid/liquid clean-up with subsequent LC-MS/MS analysis to achieve a limit of quantitation (LOQ) of 0.005 µg/L.

2. EXPERIMENTAL

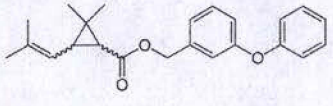
2.1 Test System

Surface water (River Brenz) was obtained locally. The surface water was characterized for its inorganic load (e.g. pH, conductivity, hardness) and its organic load (e.g. DOC/TOC), whereby these characterizations were performed (non-GLP) by Institut Alpha, Ulm, Germany, following appropriate DIN/EN procedures (see APPENDIX 3 for detailed characteristics).

2.2 Analytical Test and Reference Item(s)

One analytical standard of d-phenothrin obtained by Sumitomo Chemical Co., Ltd. (see Appendix 1 for Certificate of Analysis) was used as test / reference item:

d-phenothrin



Chemical Formula:	$C_{23}H_{26}O_3$	Molecular Mass: 350.458 g/moL	
CAS no:	26046-85-5	Expiry date:	27-Feb-2019
Batch/Lot no:	C170227	Purity:	99.8%

The analytical standard was stored in a refrigerator when not in use.

2.3 Analytical Method

2.3.1 Apparatus

2.3.1.1 Laboratory Equipment

Mettler-Toledo XS205DU analytical balance for analytical standard.
Rotary evaporators Büchi Rotavapor R 200 V800 and Büchi Rotavapor R 210 V850.
Ultrasonic bath Sonorex RK100, BANDELIN electronic.
Nitrogen evaporator, Thermo Scientific.
Vortex mixer REAX top, Heidolph.
Typical glassware and laboratory equipment.
All the glassware was cleaned in a laboratory dishwasher and air-dried before use.

2.3.1.2 LC-MS/MS System

Agilent 1290 infinity series LC system (vacuum solvent degasser, binary LC pump, column oven) and CTC Analytics HTC-Pal Autosampler.
Columns:
Agilent Poroshell C_8 column: Length: 50 mm, i.d.: 3.0 mm, particle size: 2.7 µm.
Pre-column: Phenomenex C_{18}, 4 mm length, 3 mm i.d.
Applied Biosystems API5500 Q-trap LC-MS/MS system with TurboIonspray (ESI) source.
Analyst 1.6.3 Instrument control and data acquisition software.

2.3.2 Solvents, Chemicals and Miscellaneous

Acetone, for Pesticide Residue Analysis, Promochem.
Acetonitrile, HPLC grade, Promochem.
n-Hexane, for Pesticide Residue Analysis, Promochem.
Water, LC-MS grade, Merck.
Methanol, LC-MS grade, Merck.
Millipore water, supply at EAG Laboratories GmbH.
Ammonium acetate, ≥ 98%, Sigma-Aldrich.
Nitric acid, 65%, Fluka.

2.3.3 Preparation of Standard Solutions

Two stock solutions of the analyte were prepared in acetone as exemplified:

Substance name	Weight* [mg]	Dissolve in [mL]	Obtain*[mg/mL]
d-phenothrin (purity 99.8%)	5.05	5.04	1.0
	50.38	5.03	10

(*): Purity was taken into account.

Fortification solutions with concentrations of 10 and 0.10 µg/mL were prepared in acetone diluting the 10 mg/mL d-phenothrin stock solution in acetone and a further subsequent dilution.

Calibration solutions were prepared from intermediate solutions in acetonitrile with concentrations of 10000, 100 and 10 ng/mL by volumetric dilution to obtain concentrations in a range from 0.005 ng/mL to 0.10 ng/mL in acetonitrile/water (8/2; v/v). For preparation of matrix-matched standards final extracts of residue-free control specimens (processed together with the validation specimens) were used. Aliquots of the final extracts were fortified with the analyte using an intermediate solution in acetonitrile with a concentration of 1.0 ng/mL. Matrix-matched standards with a matrix content of at least 90% were prepared in concentrations of 0.005, 0.010, 0.025, 0.050, 0.080 and 0.10 ng/mL.

All solutions containing the analyte were stored refrigerated, when not in use.

2.3.4 Residue Analysis

Preparation of non-disposable glassware:

1. Rinse all non-disposable glassware either three times with equivalent amounts of 20% nitric acid or by soaking for 15 minutes.
2. Rinse the glassware three times with millipore water until no nitric acid is left.
3. Rinse the glassware two additional times with millipore water, followed by three equivalent volumes of acetone.
4. Finally rinse the glassware with the final dilution solvent.

d-phenothrin

1. 500 mL surface water were transferred into a 1 L separation funnel.
2. Specimens were fortified, if necessary.
3. 200 mL of n-hexane were added and shaken for about 1 minute.
4. The n-hexane phase was transferred into a 500 mL round-bottom flask.
5. Step 3 and 4 were repeated.
6. The water was discarded after the second extraction.
7. The separation funnel was rinsed with 50 mL n-hexane which were added to the appropriate round-bottom flask.
8. The volume was concentrated to about 2 mL using a rotary evaporator at < 35°C.
9. 100 mL of acetone were added to the round-bottom flask.
10. The volume was concentrated to about 5 mL using a rotary evaporator at < 35°C.
11. The remaining solution was transferred into a 15 mL glass centrifuge tube.

12. The round-bottom flask was rinsed with 5 mL of n-hexane followed by 5 mL of acetone.
13. The sample was concentrated to incipient dryness using a gentle stream of nitrogen at room temperature.
14. 1.6 mL of acetonitrile were added, vortexed for 30 seconds and sonicated for 5 minutes.
15. 0.4 mL of millipore water were added, vortexed for 30 seconds and sonicated for 5 minutes.
16. 250 µL of the extract (or 500 µL for blanks) were transferred into a vial containing 4.75 mL (or 9.5 mL for blanks) acetonitrile/water (8/2; v/v). High concentration level samples were additionally diluted into the standard curve range with acetonitrile/water (8/2; v/v).

2.4 LC-MS/MS Analysis

Specimen extracts and calibration solutions were analyzed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) with the following methods:

Method for d-phenothrin:

LC System	Agilent 1290 infinity series LC system (vacuum solvent degasser, binary LC pump, column oven) and CTC Analytics HTC-Pal Autosampler.
LC Column	Agilent Poroshell C_8 column: Length: 50 mm, i.d.: 3.0 mm, particle size: 2.7 µm. Pre-column: Phenomenex C_{18}, 4 mm length, 3 mm i.d.
LC Injection Volume	100 µL
Temperature	40 °C
LC Method	Solvent A: Water containing 10mM ammonium acetate Solvent B: Methanol Mobile Phase Composition: Time (min) / Flow rate (mL/min) / % A / % B 0.00 / 0.400 / 70 / 30 5.00 / 0.400 / 10 / 90 7.00 / 0.400 / 0 / 100 9.00 / 0.400 / 0 / 100 9.10 / 0.400 / 70 / 30 10.00 / 0.400 / 70 / 30
Retention time	≈ 6.3 min for d-phenothrin
MS/MS System	Applied Biosystems API5500 Q-trap LC-MS/MS system with TurboIonspray (ESI) source.
Ion Source Conditions ESI Positive Polarity	Source temperature: 550°C GS 1: 50 (arbitrary units) GS 2: 60 (arbitrary units) Curtain gas: 20 (arbitrary units) CAD gas: Medium Entrance potential: 10 V IonSpray voltage: 5500 V Resolution: Q1: Unit, Q3: Low

MS/MS Conditions for d-phenothrin	MS/MS transition for quantification:	351 m/z → 183 m/z
	Collision energy (CE):	27 V
	Cell exit potential (CXP):	12 V
	Dwell time:	300 ms
	Declustering potential (DP):	126 V
	MS/MS transition for confirmation:	351 m/z → 249 m/z
	Collision energy (CE):	21 V
	Cell exit potential (CXP):	20 V
	Dwell time:	300 ms
	Declustering potential (DP):	126 V
	MS/MS transition for confirmation :	351 m/z → 305 m/z
	Collision energy (CE):	15 V
	Cell exit potential (CXP):	24 V
	Dwell time:	300 ms
	Declustering potential (DP):	126 V

The quantitative determination was carried out by external standardization using matrix-matched standards. Calibration functions (≥ 5 levels) for d-phenothrin with three ion mass transitions ranging from 0.005 ng/mL to 0.10 ng/mL were used to evaluate the extracts (Figure 2) except for the transitions 351 m/z -> 249 m/z and 351 m/z -> 305 m/z where the lowest calibration of 0.005 ng/mL gave no detectable response (Figure 3). The lowest calibration standard used for these MRMs was 0.010 ng/mL.

Representative LC-MS/MS ion chromatograms of matrix-matched standard solutions, reagent blanks and for extracts of fortified and control specimens are presented in Figure 4 to Figure 8. The product ion spectrum of d-phenothrin is presented in Figure 1.

2.5 Calculations

Recovery results for d-phenothrin with full validation for surface water derived from LC-MS/MS and calculations are shown in detail in Table 1.

The following equation was used to calculate the individual residues R in µg/L:

$$R = C_{End} \times DF \times (V_1 \times V_{End}) / (V_{Sample} \times V_{Aliquot})$$

$$= C_{End} \times DF \times \text{Multiplier M}$$

R: Analyte residue in µg/L.

C_{End}: Final concentration of analyte in specimen extract, in ng/mL.

DF: Dilution factor.

V_1: Solvent volume for reconstitution after evaporation to dryness: 2.0 mL.

V_{End}: End volume: 5.0 mL (10 mL for blanks).

V_{Sample}: Sample volume: 500 mL.

$V_{Aliquot}$: Aliquot volume of V_1: 0.25 mL (0.50 mL for blanks).

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = $(R / R_{fort}) \times 100\%$

Example for Calculation:

The calculation is exemplified with the surface water specimen P4686-59 fortified at 0.0050 µg/L (LOQ). The undiluted final extract was examined by LC-MS/MS in run file P4686API5#061 (Figure 7) to give a peak area of 1.74e+005 counts for d-phenothrin at the transition 351 m/z to 183 m/z. Using the respective calibration curve a final concentration of 0.051 ng/mL is calculated (see Table 1).

Thus:

R = $C_{End} \times DF \times (V_1 \times V_{End}) / (V_{Sample} \times V_{Aliquot})$

= $C_{End} \times DF \times$ Multiplier M

= 0.051 ng/mL x 1 x (2.0 mL x 5.0 ml) / (500 mL x 0.25 mL)

= 0.0041 µg/L

Rec. = $(R / R_{fort}) \times 100\%$

= (0.0041 µg/L / 0.0050 µg/L) x 100% = 82%.

Calculations were performed with full precision. Thus minor insignificant discrepancies may arise when recalculated.

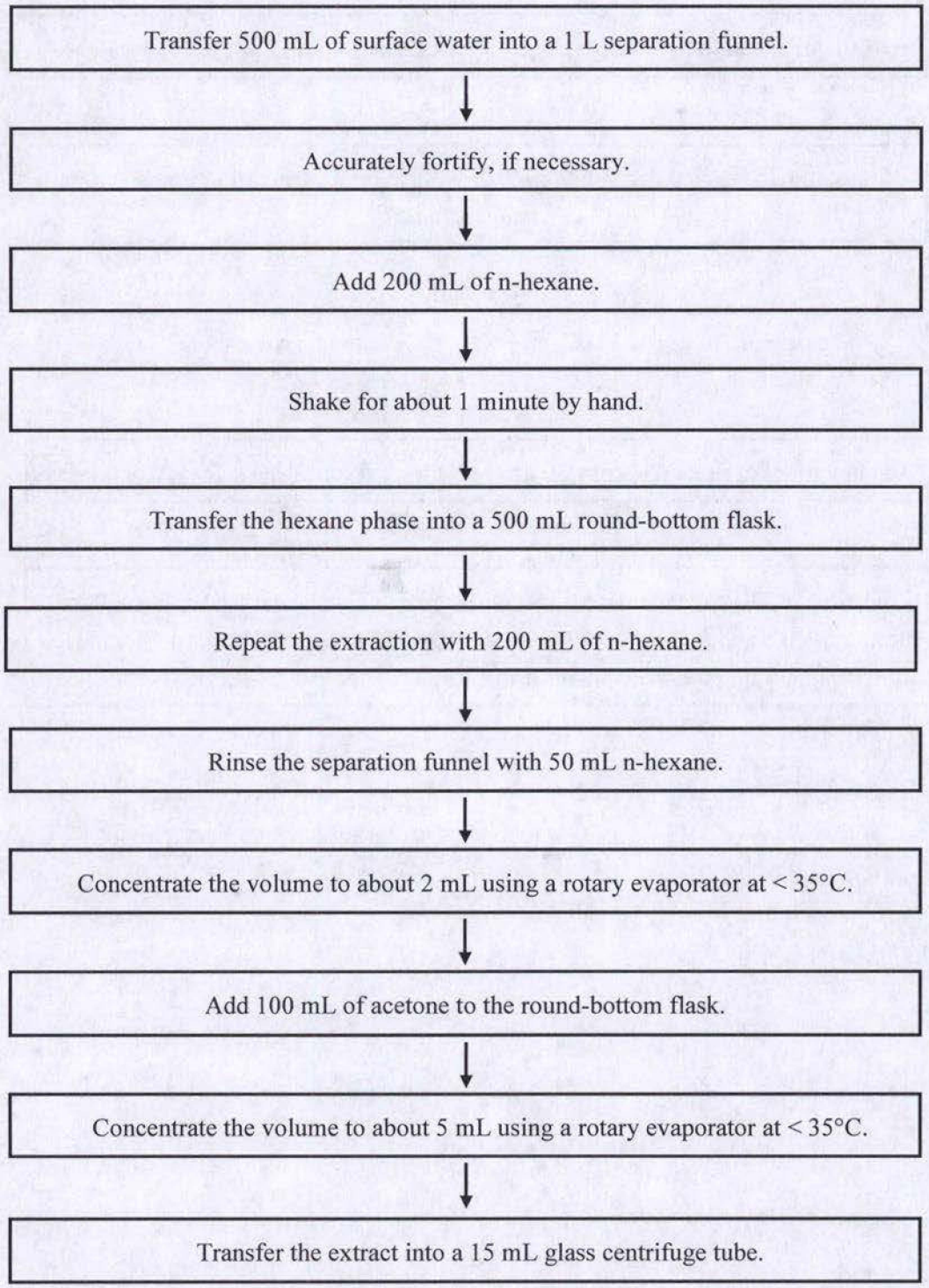
3.6 Deviation to Method

Minor deviation from the original method:
Due to different technical equipment samples were kept at room temperature and not cooled to 5°C while standing in the autosampler. This minor deviation has no impact on the outcome of the study.

3.7 Deviation to Study Plan

Matrix-matched standards were used for the evaluation of the results as a strong suppression on instrument response was observed for surface water. For the same reason the lowest calibration of 0.005 µg/L in surface water gave no detectable response for both confirmatory transitions. Furthermore, this also implicated that calibration functions obtained from injections of matrix-matched standards consisted of ≥ 5 different concentrations and not ≥ 6 concentration levels as required by the Study Plan. Nevertheless, the existing calibrations range cover the range from no more than 30% of the LOQ and at least +20% of the highest analyte concentration level detected in a fortified sample as described in the protocol. There is no impact on the outcome of the study.

Appendix 4 Method Flow Chart



Appendix 4 Method Flow Chart (continued)