INTRODUCTION

ChemService S.r.l. conducted a study to adjust and validate the analytical method for the determination of bromadiolone residues in drinking, ground and surface water samples.

The study was performed in accordance with Study Protocol CH - 290/2005 and the following guidelines:

EEC guideline SANCO/3030/99 rev. 4 dated 11/07/00:
"Working document "Technical Material and Preparation: Guidance for generating and reporting methods of analysis in support of pre and post registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414".

and the following data requirements:

Commission Directive 96/46/EC of 16 July, 1996 amending Council Directive 91/414/EEC.
Commission Directive 98/83/EC of 3 November, 1998 amending Council Directive 80/778/EEC.

The experimental phase of this study started on September 30, 2005 and it was completed on October 20, 2005.

MATERIALS

Test Article

Drinking, ground and surface water samples, on which the study was performed, were purchased on the market or sampled by ChemService and received on September 29, 2005.

Type of water	ChemService No.	Sampling
Drinking	AA15486	Natural mineral water Fiuggi in glass bottles
Ground	AA15487	Water sampled from Well SB1 I.Pi.Ci. in glass bottles
Surface	AA15488	Water from Italy's lake Garda sampled at Desenzano in glass bottles

The samples were stored in a refrigerator at 4°C in the dark.

Test Substance

A sample of bromadiolone technical product was received by ChemService on June 13, 2005 (ChemService number AA13821).
It was received as a 10 g sample in a polyethylene bottle with screw cap (see Appendix B).
Information on the test article is as follows:

Test article identification	:	Bromadiolone technical product
Chemical name (IUPAC)	:	3-[3-(4'-bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl]-4-hydroxycoumarin
Chemical formula	:	$C_{30}H_{23}BrO_4$
Chemical Class	:	rodenticide (coumarin anticoagulant)
C.A.S. number	:	28772-56-7
Molecular weight	:	527.4
Batch number	:	L12478
Declared purity	:	99.5%
Preparation date	:	February 04, 2003
Expiry date	:	February, 2008

The test article was stored at room temperature in the dark.

Reference Material

Common name	:	Bromadiolone, analytical standard (supplied by the Sponsor, see Appendix B)
C.A.S. number	:	28772-56-7
Purity	:	99.5%
Batch number	:	L02478
ChemService code	:	STU 31.2

Because of the low concentration of the spikes in water samples, for the calculation the analytical standard purity can be considered 100 %.
The reference material was stored in a freezer at -20°C.

EQUIPMENT

- HPLC/MS/DAD, Thermo Finnigan Surveyor, equipped with quaternary pump, autosampler, and coupled with a DAD detector and with a LCQ Advantage ionic trap mass detector by both ESI and APCI interfaces, software for data processing, ChemService code No. 229
- Analytical balance, Mettler AG204, ChemService code No. 102
- Rotary evaporator Büchi– ChemService code No. 197
- Volumetric glassware: pipettes, flasks, measuring cylinders
- Usual laboratory glassware

REAGENTS

- Dichloromethane, for residues analysis (Merck)
- Acetone, for residues analysis (Merck)
- Methanol, HPLC grade (Merck)
- Water, HPLC grade (Merck)
- Triethylamine, reagent grade (Merck)

EXPERIMENTAL

Specificity

The analytical conditions were suitably adapted to obtain the best results on the bromadiolone residues in the three types of water.
The method was demonstrated to be specific for the determination of bromadiolone residues in water samples by virtue of the HPLC/MS/MS technique with SIM mode (Selected ion Monitoring) and SRM mode (Selected Reaction Monitoring).
The SIM mode is ideally suited to trace analysis in complex matrices because it permits the configuration of the MS detector to monitor a m/z value characteristic of the targeted compound, thus improving sensitivity and increasing definition of the chromatogram peak profile.
With the SRM mode a parent ion and product ion pairs are monitored. It is a very specific technique and false positives can be avoided because if there is a potential interference of a parent ion of the same m/z value of the target compound would also need to fragment to form a product ion of the same m/z ratio as the target compound in order not to be detected.
The presence of bromadiolone in the water samples was identified by comparison of the peak retention time, the SIM and SRM data with those obtained for the known standards.

Linearity and System Precision

Linear regression analysis was performed using the least squares method.
The correlation coefficient was calculated using regression analysis.
The linearity test documented in this report was performed with solutions containing 0.1, 0.2, 0.3, 0.4 and 0.5 μg/mL (ppm) of the bromadiolone analytical standard.
The bromadiolone stock solution was prepared in acetone and the diluted solutions, as well as the relevant working standard solutions, were prepared in methanol, using 10 mL volumetric flasks and suitable pipettes (details are reported in ChemService Analytical Method No. 290/2005, Appendix A).
From the lowest to the highest concentration, four series of injections were performed and a solvent wash was injected after each highest standard concentration solution, in order to verify that no significant memory peaks were detected.
Means and standard deviations for each concentration were calculated using the data from four replicate injections.

Repeatability (Precision) and Recovery (Accuracy)

Both repeatability and recovery tests were performed using freshly fortified control samples of all three types of water (drinking, ground and surface, ChemService No. AA15486, AA15487 and AA15488).
Each water sample was intended to be fortified at the limit of quantification (0.05 μg/L) and at L.O.Q. x 10 (0.5 μg/L), L.O.Q. x 100 (5.0 μg/L) and L.O.Q. x 1000 (50 μg/L).
For each of the four fortification levels, five fortified samples were processed.
Precision (repeatability) and accuracy (recovery) of the analytical method were assessed with the data obtained.

Specificity

A comparison of the chromatograms (see Figures 1 to 5) of the bromadiolone analytical standard, solvent wash and control sample extract of each of the three water types, did not show any interference.
Therefore, by using the conditions stated in the method, interferences can be avoided and the bromadiolone residue can be determined reliably in each of the three types of water samples.

Linearity and System Precision

Stock standard solution at 1000 µg/mL, the diluted solutions at 10.0 and 1.0 µg/mL and five working standard solutions were prepared, using volumetric glassware, as described in ChemService Analytical Method No. 290/2005, (Appendix A).

From the lowest to the highest concentration, four series of injections were performed and a solvent wash was injected after each highest standard concentration, in order to verify that no significant memory peaks were detected.
The data obtained are reported in Tables 1 and 2.

No significant memory peak was detected in the washing solvent injected after each standard solution at 0.5 µg/mL.
The range tested was from 0.1 to 0.5 µg/mL, corresponding to concentrations from 0.05 to 0.25 µg/L in the water samples, and was found to be linear (correlation coefficient > 0.99).

The SANCO guideline requires any interference from the untreated control sample to be lower than 30 % at the L.O.Q. In all sequences of analysis of fortified samples at the four fortification levels (0.05 µg/L, 0.50 µg/L, 5.0 µg/L and 50 µg/L), the analysis of the control samples gave no interferences.

The limit of quantification (L.O.Q.) of this method is defined as the lowest validated level, i.e. 0.1 µg/mL (ppm) corresponding to 0.05 µg/L in the water matrix samples.

The limit of detection (L.O.D.) of this method is defined as 50% of the lowest validated level, i.e. 0.05 µg/mL corresponding to 0.025 µg/L in the water matrix samples.

Residue results calculated as values < 0.025 µg/L are classified as not detected (n.d.).

Residue results calculated as greater than the limit of detection but less than limit of quantification, are designated as < 0.05 µg/L.

If the residue amount is greater than 0.25 µg/L, the organic extract must be suitably diluted in volumetric flasks.

Appendix A

ChemService Analytical Method No. 290/2005

Determination of bromadiolone residues in drinking, ground and surface waters by liquid extraction followed to HPLC/MS detection

AUTHORS

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Scope

This method is applicable to the quantitative determination of bromadiolone residues in drinking, ground and surface waters to a lowest validated level of 0.05 µg/L The method has been validated by the analysis of untreated and fortified samples for residues of bromadiolone in drinking, ground and surface waters over the range 0.05 – 50 µg/L.

Principle of the method

For each type of water, 1 L is extracted with 3 x 50 mL of dichloromethane, then the organic extract is evaporated to dryness at 40°C by a rotary evaporator. The residue is re-dissolved with 0.5 mL of methanol and injected into an HPLC coupled with an ion trap mass detector.

Safety precaution

Each analyst should be acquainted with potential hazards of the reagents, products and solvents before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, LITERATURE AND OTHER INTERNALLY-GENERATED DATA. Safety information on products should be requested from the supplier. Disposal of reagents, reactants and solvents must be in compliance with the appropriate government regulations.

Equipment

- HPLC/MS equipped with quaternary pump, auto sample, ionic trap mass detector ESI positive interfaces and software for data processing
- Analytical balance, 0.1 mg precision
- Volumetric glassware: pipettes, flasks, measuring cylinders
- Rotary evaporator
- Usual laboratory glassware

Reagents

- Water, HPLC grade
- Methanol, HPLC grade
- Acetone, for residues analysis
- Dichloromethane, for residues analysis
- Triethylamine, reagent grade

Water samples

Type of water	**Sampling**
Drinking	Natural mineral water Fiuggi in glass bottles
Ground	Water sampled from Well SB1 I.Pi.Ci. in glass bottles
Surface	Water from Italy's lake Garda sampled at Desenzano in glass bottles

Water samples storage

All three types of water must be taken from the refrigerator at 4°C and allowed to stand one hour at room temperature before the extraction.

Test Substance

- Bromadiolone, technical grade active ingredient

Reference Material

- Bromadiolone, analytical standard

Preparation of the stock and diluted standard solutions

Weigh 50 mg of bromadiolone analytical standard into a 50 mL volumetric flask, using the analytical balance, then dissolve to volume with acetone (1000 µg/mL).
Transfer 1.00 mL of this stock solution, taken by a volumetric pipette, into a 100 mL volumetric flask and make to volume with methanol (10.0 µg/mL).
Transfer 5.00 mL of this stock solution, taken by a volumetric pipette, into a 50 mL volumetric flask and make to volume with methanol (1.0 µg/mL).

Preparation of the working standard solutions

Prepare five working standard solutions for linear calibration using volumetric flasks and pipettes, as follows:

WSS1. Transfer 1.0 mL of the 1.0 µg/mL diluted standard solution into a 10 mL volumetric flask and make to volume with methanol (working standard solution at 0.1 µg/mL)
WSS2. Transfer 2.0 mL of the 1.0 µg/mL diluted standard solution into a 10 mL volumetric flask and make to volume with methanol (working standard solution at 0.2 µg/mL)
WSS3. Transfer 3.0 mL of the 1.0 µg/mL diluted standard solution into a 10 mL volumetric flask and make to volume with methanol (working standard solution at 0.3 µg/mL)
WSS4. Transfer 4.0 mL of the 1.0 µg/mL diluted standard solution into a 10 mL volumetric flask and make to volume with methanol (working standard solution at 0.4 µg/mL)
WSS5. Transfer 5.0 mL of the 1.0 µg/mL diluted standard solution into a 10 mL volumetric flask and make to volume with methanol (working standard solution at 0.5 µg/mL)

The range of linearity tested is from 0.1 µg/mL to 0.5 µg/mL corresponding to a range from 0.05 µg/L to 0.25 µg/L for bromadiolone in the water samples.

Water samples extraction

Measure 1 L of the water sample by a glass graduated cylinder, transfer into a 2 L glass separatory funnel and extract three times with 50 mL of dichloromethane. Collect together the organic extracts into a 250 mL round bottom flask and evaporate to dryness by a rotary evaporator at 40°C.
Re-dissolve the residue with 5 mL of dichloromethane, transfer into a 7 mL glass test tube and make again to dryness with a gentle nitrogen flow.
Re-dissolve the residue with 0.5 mL of methanol, transfer into an autosampler vial and inject 10 µL into the HPLC/MS system.

Preparation of eluent C

In a 250 mL conical flask, place 200 mL of HPLC water and add 280 µL of triethylamine (TEA).

Mass scan parameters

The mass scan parameters are reported in the following table.

Compound	Ion mode	Collision Energy (%)	m/z Molecular Ion (SIM)	m/z Daughter (SRM)
Bromadiolone	ESI –	60	527	509

Chromatographic conditions

HPLC/MS instrument	:	Thermo Finnigan LCQ Advantage
HPLC Column	:	ChemService code No. 131
Teknokroma or equivalent		Tracer Excel 120 ODSB 5 µm, 50 x 2.1 mm i.d.
HPLC pre-column	:	C18 (ODS) 4.0 x 3.0 mm
Interface	:	Electron spray ionization (ESI), negative ions
Detector	:	Mass (Scan in SIM and SRM mode)
Column temperature	:	room temperature
Eluent A	:	water
Eluent B	:	methanol
Eluent C	:	water with 10 mM of TEA
Gradient	:	A:B:C 65:30:5 hold 1 minute from A:B:C 65:30:5 to 0:95:5 in 5 minutes, hold 10 minutes from A:B:C 0:95:5 to 65:30:5 in 2 minutes, hold 10 minutes
Eluent flow	:	0.2 mL/min
Volume of injection	:	10 µL
Bromadiolone ret. Time	:	ca. 11 minutes
Total Analysis Time	:	30 minutes

Calculations

The bromadiolone residue content in the water samples is calculated by the following formula.

$$\text{Bromadiolone (µg/L)} = \frac{C_S \times V_S}{V_W}$$

where:

C_S = Bromadiolone concentration in the water sample extract (µg/mL)

V_S = Volume of the water sample extract (0.5 mL for unknown water sample and for the lowest fortification level, suitably higher for the other fortification levels)

V_W = Volume of extracted water sample (1 L)

This C_S value is calculated from the A_S data using the linear calibration curve obtained with C_{std} and A_{std} data from injections of the working standard solutions.

where:

A_S = Bromadiolone peak area in the water sample extract

C_{std} = Concentration of the working standard solution (µg/mL)

A_{std} = Bromadiolone peak area in the working standard solution

The percentage recovery is calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{µg/L found - µg/L control}}{\text{µg/L added}} \times 100$$

Method validation

The analytical conditions were suitably adapted to obtain the best results on the bromadiolone residues in the three types of water. The validation of the analytical procedure was performed following the SANCO/3029/99 rev. 4 guideline.

Specificity

The method was demonstrated to be specific for the determination of bromadiolone residues in water samples by virtue of the HPLC/MS/MS technique with SIM mode (Selected ion Monitoring) and SRM mode (Selected Reaction Monitoring).

The presence of bromadiolone in the water samples was identified by comparison of the peak retention time, the SIM and SRM data with those obtained for the known standards.

Representative chromatograms are presented in Figures 1 to 5.

Linearity

Stock standard solution at 1000 µg/mL, the diluted solutions at 10.0 µg/mL and 1.0 µg/mL and the five working standard solutions were prepared, using volumetric glassware, as described above.
Means and standard deviations for each level were calculated using the data from replicate injections.

No significant memory peak was detected in the washing solvent injected after each standard solution at 0.5 µg/mL.
The range tested was from 0.1 to 0.5 µg/mL, corresponding to concentrations from 0.05 to 0.25 µg/L in the water samples, and was found to be linear (correlation coefficient > 0.99).

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