5. Summary

The final report of the study "*Residues determination of BRODIFACOUM, DIFENACOUM and BROMADIOLONE in soil*" summarizes the observation and the raw data collected during the study. According to the request of the sponsor (annex 1) has been prepared one final report for each active principle. This final report summarize the data collected during the *BROMADIOLONE* study. The aim of the study was to develop and validate an analytical method for the determination of *BROMADIOLONE* residues in soil in order to meet European Directive requirements. This active ingredient is an anticoagulant rodenticides. This method is applicable for the quantitative determination of residues of the test substances in soil. Residues of *BROMADIOLONE* are extracted from blank and spiking soil using chloroform : acetone 1:1 solution. The extract is concentrated by rotary evaporator and recovery with acetone and afterwards are purified with florisil-sodium sulphate column. The eluates are dried, resumed with methanol: water 1:1 and analyzed to HPLC UV-VIS. The method was validated with spiking solution over the concentration range of 0.017-0.165 µg/g in soil, with a calibration curve from 13.2 µg/mL to 0.264 µg/mL to detector and a validated limit of quantitation of 0.264 µg/g.

6. INTRODUCTION

6.1. Background

According to the Study Protocol, a literature search was performed in accordance with European Community guidelines on the development of an analytical method for the determination of *BRODIFACOUM, DIFENACOUM e BROMADIOLONE*, according to the directive 96/23/EC. The intention was to develop a single method that allowed the identification of all three actives. The result of the literature search is presented in Section 11. The analytical method developed is based on methods identified by the literature search.

6.2. Objective of the study

The aim of the study was to develop and validate an analytical method for the determination of *BRODIFACOUM, DIFENACOUM e BROMADIOLONE* residues in soil in order to meet European Directive requirements. These active ingredients are anticoagulant rodenticides.

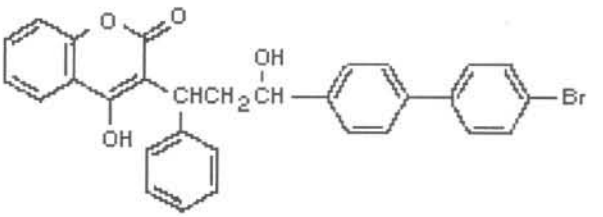
7. TEST PRODUCT

On June 24, 2005 the sponsor provided the test substances with the following characteristics:

7.1 Test items

Common name:	Bromadiolone
Chemical formula:	$C_{30}H_{23}BrO_4$
Chemical name (IUPAC):	3-[3-(4'-bromobiphenyl-4-yl)-3-hydroxy-1--phenylpropyl]-4-hydroxycoumarin

Structure:



CAS registry number:	[*56073-56-7*]
Molecular weight:	527.4
Appearance:	Bottle
Content:	White powder
Batch:	BROMADIOLONE Lot. L12478
Manufacturing date:	04/02/2003
Retest date:	02/2008
Produced by:	ACTIVA / Dr. Pezza

The delivery note, technical information and certificate of analysis of the test product and its label is presented in Annex 3.

This standard material was used to prepare standard solution for calibration and for samples spiking. This solutions were stored at +4°C in the dark when not in use.

8. MATERIALS AND METHODS

8.1. Housing and Management System

The trial was carried out in:

CERZOO
Centro Ricerche per la Zootecnia e l'Ambiente
S. Bonico, 29100 Piacenza (Italy)
Phone +39-0523-506102
Fax +39-0523-506345
E-mail: cerzoo_pc@unicatt.it

8.2. Outlines of test method

Test method for Bromadiolone determination in soil is based on a extraction from blank and spiking soil (40.0 g) using chloroform : acetone 1:1 solution. The extract is concentrated by rotary evaporator and recovery with acetone and afterwards are purified with florisil-sodium sulphate column. The eluates are dried, resumed with methanol: water 1:1 and analyzed to HPLC UV-VIS. The sorbent traps were extracted and then analysed immediately.

8.3. Experimental condition

8.3.1.Equipment

8.3.1.1 *Glassware and materials*

- Glass pipettes, and flask Class A
- Sovirel 500 ml
- Glass columns for purification

8.3.1.2 *Laboratory equipment*

- Balance analytical, model Mettler AE260
- Balance technical, model Sartorius BL6
- Rotary evaporator model IKA-WERK
- HPLC UV-VIS 1100 Agilent: with: Degasser, Binary pump, Autosampler, Chiller for column and DAD UV-VIS detector.
- Column for HPLC Synergy 4u Fusion RP80A Phenomenes 150 x 4,60 mm S/N 224016-2

8.3.1.3 *Reagents*

- Acetone ACS-ISO for Analysis code 400974 Carlo Erba
- Chloroform ISO for Analysis code 438603 Carlo Erba
- Methanol for HPLC RS code 412532 Carlo Erba
- Acetonitrile Gradient Grade for liquid chromatography L129430-343 Merck
- Formic acid 99% RPE-ACS code 405792 Carlo Erba
- Water bidistillate
- Florisil 60-100 RS code 452273 Carlo Erba
- Sodium sulphate anhydrous ACS-ISO for analysis code 483007 Carlo Erba

8.3.1.4 *Standard*

- BROMADIOLONE technical grade Lot. L12478

8.4. Soil property

In the table below there are the chemical-physical property of soil test

pH	Cationic Exchange Capacity (meq/100g)	Organic matter (%)	Cd (mg/Kg)	Hg (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Cu (mg/Kg)	Zn (mg/Kg)	B & J Test Cr (IV) µM/g
5,95	36,42	5,04	< 0,015	0,024	161,75	12,65	393,65	215,30	0,023

Silt (%)	Clay (%)	Sand (%)	Assimilable P (mg/Kg)	Total N Kjeldhall (%)	Assimilable K (mg/Kg)
25,84	26,52	47,64	134,54	0,25	2227,40

8.5. Extraction and analysis

8.5.1 Preparation of standard

0.0126 g BROMADIOLONE technical grade Lot. N. L12478 was weighted and quantitatively transfer to a 100-mL volumetric flask, and then dilute to volume with acetone to obtain a 132-µg/mL stock solution

8.5.2 Preparation of Spiking Solutions

Table 1

Name of mix	Concentration of Stock Sol. (Approx. µg/mL)	Aliquot of Stock Sol. mL	Final Sol. Volume mL	Spiking Sol. Final Conc. µg/mL	Equivalent Sample Conc.[a] µg/g
A	132	1	10	13,2	0,33
B	132	0,5	10	6,6	0,165
C	132	1	25	5,28	0,132
D	132	0,5	25	2,64	0,066
E	13,2	1	10	1,32	0,033
F	6,6	1	10	0,66	0,017
G	5,28	1	10	0,528	0,013
H	2,64	1	10	0,264	0,007

[a]The equivalent sample concentration is based on fortifying a 40.0 g soil sample with 1.0 mL of spiking solution.

8.5.3 Instrument conditions

The analysis was performed with a HPLC UV-Vis equipped with a DAD (Diode Array Detector). The solvents utilized were water with 0.1 % of formic acid and acetonitrile with the following solvent gradient program:

Table 2

Time (min)	Flow (ml/min)	Water 0.1% formic ac. (%)	Acetonitrile (%)
0.00	1.000	50.0	50.0
5.00	1.000	50.0	50.0
10.00	1.000	10.0	90.0
15.00	1.000	10.0	90.0
18.00	1.000	50.0	50.0
23.00	1.000	50.0	50.0

Characteristic of instrument:

Instrument	Agilent™ HPLC 1100 binary pump with DAD detector, autosampler, degaser and chilller
Column for HPLC	Synergy 4u Fusion RP80A Phenomenes 150 x 4,60 mm S/N 224016-2
Volume and type of injection	20 µL with autosampler
Temperature of chiller	25°C
λ of detection	264 nm with a window of 4 nm and a reference to 360 with a window of 100 nm
Type of spectrum recovered	All in analysis
Software	Agielent ChemStation A 7.0

8.5.4 Sample preparation

-Extraction

40.0 g of soil has been weighted into a series of 500 ml sovirel. The fortified samples, has been prepared adding 1.0 ml aliquots of the appropriate spiking solutions, mix B, D and F approximately from 0.66 to 6.6 µg/g. 100 ml of the 50% acetone/50% chloroform extraction solution has been added to the sovirel. The sovirel has been closed and shaken for a minimum of 30 minutes on a shaker at approximately 180 movements/minute. The solvent has been collected in a 500 ml rotavapor balloon after filtration on glass fiber. Another quantity of 100 ml of the extraction solution has been added and the process repeated for a further 30 min. The solvent has been filtered and the extraction has been repeated with a 50 ml of extraction solution. The three filtered solutions has been combined and evaporated with the rotavapor to 200 mm of Hg.

-Purification and concentration

The recovery has been made with 10 ml of acetone and purify in a glass column with 6 g of florisil and 1 g of anhydrous sodium sulphate. The solution has been washed with 40 ml of acetone and recovery of all solvent in a flask. The acetone has been evaporated with nitrogen. One ml of methanol:water (1:1) has been added and then centrifuged for 5 minutes at 2000 rpm. The final solution has been transferred in a 2 ml vial and cap for inject to HPLC or storage in a freezer at –20°C if didn't inject immediately.

8.8. Calculation

Using the slope (a) and the intercept (b) from the standard calibration curve (y = ax + b) and, if the samples area are in the calibration curve, to calculate the concentration from the samples area.

The calculated concentration was been divided for 40 (from 40 g of soil to 1 ml of methanol:water 1:1)

The residue level in the sample is determined with the external standard method; the residue of brodifacoum is calculated with the following equation:

Analitical Residue = (area residue – b)/a

Where:

a = slope of calibration curve
b = intercept of calibration curve

The percentage of recovery was calculated according to the formula:

$$R\ \% = \frac{A\ (\mu g)}{B\ (\mu g)} \times 100$$

where

A = residue determined in fortified sample
B = effective fortification amount

9. LAYOUNT OF EXPERIMENT

9.1. Check of untreated control samples

Prior to recovery determination the control samples of soil were analysed according to the considered analytical method. The control samples were used after verifying that their signal did not exceed the 50% of the signal obtained at the limit of detection.

9.2. Validation tests

Validation of the analytical method for Bromadiolone determination in soil (table 5) was be performed at 3 levels with 4 replicates for each levels. Moreover control samples was be composed with unspiked soil, were analysed 4 times.

The efficiency of analysis of Bromadiolone from soil was evaluated by determining the recovery rates of test item after applying the spiking solution

11 Archives

All data and documents generated in connection with the analytical phase of this study, together with the study logbook and a copy of the final report, are kept in the archives (fireproof cabinet) of CERZOO for at least 10 years after completing the study. All data are available for inspection by competent authorities.

12 References

12.1 Law references

- D.L. n. 120/92 Attuazione delle direttive n. 88/320/CEE e n. 90/18/CEE in materia di ispezione e verifica della Buona Pratica di Laboratorio (BPL) come modificata dal D.M. 02/08/1999 Disposizioni relative all'ispezione e verifica della buona prassi di laboratorio in recepimento delle direttive 1999/11/CE e 1999/12/CE
- Direttiva 2004/10/CE del Parlamento EUROPEO E DEL Consiglio dell'11 febbraio 2004 concernente il riavvicinamento delle disposizioni legislative, regolamentari ed amministrative relative all'applicazione dei principi di Buona Pratica di Laboratorio ed al controllo della loro applicazione per le prove sulle sostanze chimiche.
- EMEA/CVMP/590/98 Final. Guideline on validation of analytical procedures: definition and terminology.
- EMEA/CVMP/591/98 Final. Guideline on validation of analytical procedures: methodology.
- Decisione 2002/657/CE della Commissione del 12 agosto 2002 che attua la Direttiva 96/23/CE del Consiglio relativa al rendimento dei metodi analitici e all'interpretazione dei risultati.

12.2 Methodology references

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- High-performance liquid-chromatographic determination of seven anticoagulant rodenticides in plasma. Guan,-FY; Liu,-L; Luo,-Y *Fenxi-Huaxue.* Feb 1995; 23(2): 159-162
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- Liquid-chromatographic determination of bromadiolone in rodent baits. Koeppen,-B *J-Assoc-Off-Anal-Chem*. May-Jun 1990; 73(3): 429-430
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- Modification to procedures for the determination of chlorophacinone and for multi-residue analysis of rodenticides in animal tissues. Hunter,-K; Sharp,-EA *J-Chromatogr*. 11 Mar 1988; 437(1): 301-305
- Determination of diastereoisomers of bromadiolone, an anticoagulant rodenticide, in animal tissues by high-performance liquid chromatography. Hunter,-K; Sharp,-EA; Newton,-A *J-Chromatogr*. 1 Jan 1988; 435(1): 83-95
- Simple, rapid determination of coumarin rodenticides in swine tissues by high-performance liquid chromatography. Nojiri,-T; Hashidzume,-N; Satoh,-T; Mori,-G; Yamauchi,-S; Hosoi,-Y *Journal-of-the-Japan-Veterinary-Medical-Association*. 2001; 54(3): 213-216
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