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CERZOO
CENTRO DI RICERCHE PER LA ZOOTECNIA E L'AMBIENTE

Study: **CZ/05/002/ACTIVA/SOIL**
Document: **CZ/05/002/ACTIVA/SOIL/RF_DIFENACOUM**

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RESIDUES DETERMINATION OF BRODIFACOUM, DIFENACOUM AND BROMADIOLONE IN SOIL

Final Report for

DIFENACOUM RESIDUE DETERMINATION

Editor of the final report:
dr. Mauro Morlacchini
Centro Ricerche per la Zootecnia e l'Ambiente
S. Bonico, 29100 Piacenza - I

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CZ/05/002/ACTIVA/SOIL/RF_DIFENACOUM

INTEGRATION

1.- Extension for method: "*Residues determination of brodifacoum, difenacoum and bromadiolone in soil. Difenacoum*" for determination in sediment

Analysis of sediment could be carried out with the same method developed for soil analysis. Both matrices are similar and the only difference is the moisture. For this reason it isn't necessary to develop a specific method for Difenacoum analysis in the sediment, but it can be adopted the same method developed for soil analysis. The result of analysis should be corrected for the moisture percent.

2.- Some point of the final report needs an explanation.

- The measure unit, in column 4 of table 4, "µg/g" is a press error. The correct unit is "µg/ml".
- Column 6 indicates which concentration would correspond 1 ml of solution standard distributed on 40 g of soil, as indicated in the method.
- The column "difference %" indicates the difference in the percentage between column 4, where the value indicates the prepared concentration, and column 7, where the concentration is recalculate from the calibration chart
- The negative value in column "difference %" depends on the result of the relationship between the column "Conc." and the column "Conc. Calculated" Expressed in %. The used formula is: "100- (Conc. /Conc. Calculated*100)"

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 *DIFENACOUM* study.
The aim of the study was to develop and validate an analytical method for the determination of *DIFENACOUM* 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 *DIFENACOUM* 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 0.016-0.158 µg/g in soil, with a calibration curve from 12.6 µg/mL to 0.252 µg/mL to detector and a validated limit of quantitation of 0.252 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.

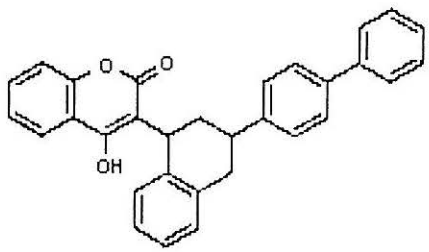
6.2. Objective of the study

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:	difenacoum
Chemical formula:	$C_{31}H_{24}O_3$
Chemical name (IUPAC:	3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)--4-hydroxycoumarin
Structure:	

CAS registry number:	[*56073-07-5*]
Molecular weight:	444.5
Appearance:	Bottle
Content:	White powder
Batch:	DIFENACOUM Lot. L13653
Manufacturing date:	12/02/2003
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 Difenacoum 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*

- DIFENACOUM technical grade Lot. N. L13653

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 DIFENACOUM technical grade Lot. N. L13653 was weighted and quantitatively transfer to a 100-mL volumetric flask, and then dilute to volume with acetone to obtain a 126-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	126	1	10	12,6	0,315
B	126	0,5	10	6,3	0,158
C	126	1	25	5,04	0,126
D	126	0,5	25	2,52	0,063
E	12,6	1	10	1,26	0,032
F	6,3	1	10	0,63	0,016
G	5,04	1	10	0,504	0,013
H	2,52	1	10	0,252	0,006

[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 0.63 to 6.3 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.

8.6 HPLC UV-Vis final analyses

After extraction the analysis has been performed as detailed in section 8.3.2. Each sample was been determinate with a calibration curve. The entire sample was been analysed in a unique series. The list of analysis has been tabulated below:

Table 3

File	Date	Sample name	Conc. µg/g Difenacoum
10190000	18/10/2005	solvent	0
10190001	18/10/2005	H	0.252
10190002	18/10/2005	G	0.504
10190003	18/10/2005	F	0.63
10190004	18/10/2005	blank	0.00
10190005	18/10/2005	Rec 1	0.63
10190006	18/10/2005	Rec 2	2.52
10190007	18/10/2005	Rec 3	6.30
10190008	18/10/2005	E	1.26
10190009	18/10/2005	D	2.52
10190010	19/10/2005	C	5.04
10190011	19/10/2005	B	6.3
10190012	19/10/2005	A	12.6
10190013	19/10/2005	blank	0.00
10190014	19/10/2005	Rec 4	0.63
10190015	19/10/2005	Rec 5	2.52
10190016	19/10/2005	Rec 6	6.30

10190017	19/10/2005	blank	0.00
10190018	19/10/2005	Rec 7	0.63
10190019	19/10/2005	Rec 8	2.52
10190020	19/10/2005	Rec 9	6.30
10190021	19/10/2005	solvent	0
10190022	19/10/2005	H	0.252
10190023	19/10/2005	G	0.504
10190024	19/10/2005	F	0.63
10190025	19/10/2005	blank	0.00
10190026	19/10/2005	Rec 10	0.63
10190027	19/10/2005	Rec 11	2.52
10190028	19/10/2005	Rec 12	6.30
10190029	19/10/2005	E	1.26
10190030	19/10/2005	D	2.52
10190031	19/10/2005	C	5.04
10190032	19/10/2005	B	6.3
10190033	19/10/2005	A	12.6

The file name is the progressive number of analysis.

The sample name is a brief description of the sample: from A to H is a standard, "Rec" from 1 to 12 are spiked samples with the relative concentration.