

**Protocol for Tissue Sampling and
Testing for Vertebrate Pesticides in Animals**

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1. Introduction

When animals are found dead there may be a requirement to establish the cause of death, especially in situations where non-target species may have been exposed to vertebrate pesticides. Samples can be taken and analysed for a number of vertebrate pesticides to establish a diagnosis of pesticide poisoning.

Vertebrate pesticides are used throughout New Zealand for control of pests such as possums, rabbits, and rodents. There is potential for effects on non-target animals as well as the contamination of waterways, soil, leaf-litter (which is an important habitat for invertebrates), and plants (Spurr & Powlesland 1998).

Manaaki Whenua - Landcare Research maintains a national database of results of testing for vertebrate pesticide residues, based on samples submitted to the toxicology laboratory. The Vertebrate Pesticide Residue Database collates data from analyses of pesticide residues in plant, soil, target and non-target animal samples, for use by Department of Conservation (DOC) managers and researchers.

The database is supported by a vertebrate pesticide residue analytical service, and this protocol has been prepared to assist DOC staff, pest control contractors, and concerned members of the general public, in the process of collecting and handling samples for laboratory testing. It provides information on the appropriate sample types for each vertebrate pesticide, and on the preparation, storage, and transport of the samples to the testing laboratory.

2. Sampling Strategies

2.1 General

In order to collect appropriate samples, it is helpful to know what the suspected pesticide is. Animals should, where possible, be dissected by the client, and the appropriate tissues removed and placed separately in bags or specimen containers before freezing. If the specific pesticide of concern is not known, collect tissue samples appropriate to each pesticide (see Section 2.2) and pack each individually. Small animals up to the size of stoats may be frozen and sent whole, if preferred. Samples of stomach or intestinal tract contents are useful to examine for traces of bait material and for initial concentrations of pesticide. However, if time to death is prolonged, the active compound may not be detectable in stomach contents, and pesticide residues will only be found in appropriate organs and tissues (Wickstrom & Eason 1997).

2.2 Target tissue

2.2.1 Anticoagulants

The anticoagulant rodenticides are retained in the liver and to a lesser extent in muscle. For this reason, liver is the tissue of choice when looking for rodenticides such as pindone, brodifacoum, diphacinone, coumatetralyl, and warfarin.



2.2.2 Sodium monofluoroacetate (1080)

Sodium monofluoroacetate is a very water soluble compound and rapidly passes through the body. It is at its highest concentration in blood and stomach contents soon after poisoning. After death, muscle is the best tissue to take, along with stomach contents. The liver and kidneys do not normally retain large amounts of 1080, so are therefore not appropriate tissues to sample.

2.2.3 Cyanide

Cyanide is best measured in whole blood samples in living animals, or in the liver of dead animals.

2.2.4 Other pesticides

Less commonly tested are cholecalciferol (liver, kidney), and phosphorus (stomach contents). Please contact the laboratory manager for details if an analysis for these pesticides is required.

Contact:

Laboratory Manager

Phone 03 321 9617; Fax 03 321 9998

boothl@landcareresearch.co.nz

2.3 Sample types and quantities

Care must always be taken to ensure that samples are not contaminated by other tissues or by pesticide-contaminated surroundings during collection and storage. Always ensure that dissecting equipment is thoroughly cleaned before sampling each animal.

2.3.1 Blood samples

These can only be collected prior to, or immediately after, death. Blood samples should be taken if animals are found sick but still alive (NOTE: The person sampling has the responsibility for ensuring that blood sampling and/or the euthenasia of animals is carried out in an appropriate manner and with permits where required). Blood consists of red cells suspended in a straw-coloured liquid called plasma. If blood is taken in a tube and allowed to stand, the red cells settle, a plug or clot of fibrin forms, and a liquid layer called serum forms on top. By using a tube containing an anticoagulant to collect the sample, clotting is prevented and plasma separates. Blood cells make up approximately 45% of the total blood volume. Samples of blood for pesticide determination are normally submitted as serum samples, so blood samples should be placed in glass Vacutainer® plain serum tubes (red top). It is important to ensure that the syringe needle is removed before discharging the syringe into the **opened** tube to prevent rupturing the blood cells. It is not necessary to separate the serum from the coagulated and settled material before sending the sample. Each analysis normally requires 1 mL of serum or plasma, though 2–3 mL is preferred in case repeat analyses are required.

2.3.2 Stomach contents

These are taken by dissecting the abdominal cavity and removing the complete stomach intact. For small animals the whole stomach can be sent, but for large animals (sheep, cattle) a 100-g portion of the stomach contents is sufficient.

2.3.3 Liver

The liver should be removed whole, preferably before taking the stomach (to avoid contamination from seeping fluid). For larger animals, a 100-g portion of the liver is sufficient. It is also recommended that sampling should avoid contamination from the gall bladder and bile duct.

2.3.4 Skeletal muscle

Muscle is best taken from the hindquarters of mammals or from the breast of birds. When collecting muscle samples avoid tough sinew and other materials that will be difficult to homogenise in the laboratory. A minimum of 10 g of sample should be provided although 50 g is preferred.

2.3.5 Invertebrates

Large invertebrates such as beetles, wētā, and cockroaches may be analysed individually. Smaller invertebrates (<0.1 g) are better tested as a pooled sample in groups that represent a certain time-point, location, or species. Whole invertebrates are homogenised and tested. At least 0.5 g of sample is required.

3. Storage and transport

3.1 Sample packaging and storage

Snap-top plastic bags are the most appropriate packaging for liver, muscle, small stomach contents, and invertebrate samples, but take care to seal the bags properly to prevent leaking. Screw-top specimen pots are also effective for small tissue samples, and larger screw-top containers for large-animal samples, such as stomach contents. **Each sample must be placed in its own container and not mixed with other samples.** Serum samples can be submitted in Vacutainer®-type collection tubes or screw-capped vials, suitably packed with padding to prevent breakage.

Samples (except blood samples) should be frozen to below -10°C as soon as possible after collection, and certainly within 8 hours. Serum tubes with whole blood should be stored in a refrigerator at 4°C . A chillybin packed with ice is suitable for temporary storage in the field until the samples can be placed in a fridge or freezer.

Each sample should be labelled **externally** with as much information as necessary to fully identify the sample on the test report. Clear labelling with a waterproof medium is essential as the freezing/thawing process tends to obliterate markings. It is better to use an attached label, either tied or adhesive, than rely on marking the plastic bag directly. Pencil, though waterproof, is not easy to read in some circumstances and should be avoided. A tissue sample form is available to assist in supplying the details necessary for the Vertebrate Pesticide Residue Database.

Note that the processing of samples submitted without appropriate information may be delayed until the details are supplied.

3.2 Transport

Individual tissue samples or serum tubes should be transported to the testing laboratory in an insulated pack such as a chilly bin. Alternatively, samples well wrapped in insulating material or layers of newspaper and packed in a cardboard carton will suffice. Samples (e.g., of invertebrates) may be quite small and will thaw out rapidly in transit. To prevent this they should be placed in contact with freezer packs or plastic soft-drink bottles filled with water and frozen, before wrapping.



Samples are to be sent to:

Laboratory Manager
Toxicology Laboratory
Landcare Research
54 Gerald St
Lincoln, Canterbury 7608
Phone 03 321 9617; Fax 03 321 9998

Samples should be sent door-to-door early in the week to avoid the possibility of thawing over the weekend. They should be marked **“Urgent tissue samples - please keep frozen”**. No special declaration is required for samples sent from within New Zealand unless the samples are likely to be infected (e.g., with Tb). Permits may be required for samples sent from other countries depending on the type of sample.

On receipt at the laboratory, the sample details will be entered in the laboratory sample register and the sample placed in a freezer at -20°C to await analysis. Blood samples will be separated by centrifuging and the serum decanted and repacked before freezing.

4. Testing procedures

The analysis is carried out in a laboratory accredited by International Accreditation New Zealand (IANZ). Most of the methods used for low concentration pesticide analyses are IANZ registered. If samples are to be retained as evidence in a potential legal case, the toxicology laboratory is to be given specific instructions. Samples are normally discarded after 3 months unless directed otherwise.

4.1 1080

Testing is carried out using a gas chromatography method TLM 005, "Assay of 1080 in water, soil, and biological materials by GLC". This method was developed by Landcare Research, based on the work of Ozawa & Tsukioka (1989).

Aqueous extracts are obtained from tissue, plant, and blood serum or plasma samples by dispersing in an alcohol/water mixture, centrifuging, filtering, and passing through an ion exchange column to extract the sodium monofluoroacetate (1080). The 1080 is eluted with sodium chloride solution, acidified with hydrochloric acid, and converted to the dichloroaniline derivative by using N,N'-dicyclohexyl carbodiimide (DCC) and 2,4-dichloroaniline (DCA). The derivative is cleaned on a silica cartridge, eluted with toluene, and quantified by gas chromatography with electron-capture detection. The limit of detection is 0.001 mg/kg in a 5-g tissue sample.

4.2 Brodifacoum

This method was adopted by Landcare Research, based on the work of Hunter (1983).

A sample of tissue (normally liver) is chopped finely and a 2-g sub-sample placed in a centrifuge tube. Anhydrous sodium sulphate is added, followed by 20 mL chloroform/acetone (1:1). The contents of the tube are homogenised with a tissue disperser, shaken, and centrifuged. The supernatant is decanted, and the extraction process repeated twice more. The combined extracts are evaporated and taken up in hexane/chloroform/acetone for application to a gel permeation column for clean-up. The eluent from the column is again evaporated and taken up in the mobile phase for HPLC analysis using a C18 column with methanol/water/acetic acid as the solvent. A post-column pH switching technique is used with a fluorescence detector. The limit of detection in liver tissue is 0.001 mg/kg. The rodenticide difenacoum is used as an internal standard.

4.3 Pindone

This method is based on Hunter (1984).

A sample of tissue (normally liver) is chopped finely and a 2-g sub-sample placed in a centrifuge tube. Anhydrous sodium sulphate is added, followed by 15 mL chloroform/acetone (1:1). The contents of the tube are homogenised with a tissue disperser, and then centrifuged. The supernatant is decanted, and the extraction process repeated twice more. The combined extracts are evaporated and taken up in hexane/chloroform/acetone for application to a gel permeation column fitted with a silica solid-phase extraction cartridge for clean-up. The cartridge is eluted with dichloromethane, evaporated, and taken up in mobile phase for HPLC analysis using a C18 column and ion-paired chromatography with tetrabutyl ammonium phosphate and a UV/vis detector at 280 nm. The limit of detection in liver is 0.2 mg/kg.

4.4 Diphacinone

This is tested similarly to pindone. Chlorophacinone is used as an internal standard. The limit of detection is 0.02 mg/kg.

4.5 Multiple rodenticides

Liver tissue may be analysed for multiple rodenticides. The coumarin rodenticides warfarin, coumatetralyl, bromadiolone, difenacoum, flocoumafen, and brodifacoum may be analysed using the same chromatography as given above for brodifacoum, with slightly modified gel permeation chromatography clean-up procedures. Pindone and diphacinone are similarly both analysed from liver samples using the chromatographic techniques for pindone.

4.6 Cyanide

This method is based on the work of Krynitsky et al. 1986, Wiemeyer et al. 1986, and Valentour et al. 1974. Whole blood is homogenised and a 1-mL sub-sample placed in a diffusion cell. Samples are digested by the addition of 10% H₂SO₄. A polythene cup containing 0.3 M NaOH is placed in the cell and the cell closed. Absorption takes place overnight. The collected free cyanide in the alkaline absorbant is converted to cyanogen chloride by the addition of hexane, 1 M sodium dihydrogen phosphate and 0.25% chloramine T. The mixture is cooled and a portion of the hexane layer removed for GLC analysis. Tissue samples, usually liver, are chopped and homogenised with an equal amount



of water. A 2 g aliquot of the homogenate is placed directly in the bottom of the diffusion cell and treated as for whole blood.

5. Interpretation

A number of variables will affect the concentration of pesticide residue present in animal tissue after death. Ambient temperature will play a part, but the main factors are biological considerations such as the amount of pesticide ingested, the age and condition of the animal, time-to-death, age of the carcass and the processes of absorption, distribution, metabolism and excretion of the pesticide prior to death and degradation characteristics in the carcass. The concentration found in tissue or blood is not related to the lethal dose value of the pesticide for particular animal species.

The presence of any measurable pesticide in a dead animal is an indication of exposure, but not necessarily proof of cause of death.

The limit of detection of the method is influenced by the size and quality of the sample.

6. Prices

The cost of the analysis of samples for vertebrate pesticides relevant to this protocol varies with pesticide and sample type. Any queries should be addressed to the Laboratory Manager (ph. 03 321 9617, boothl@landcareresearch.co.nz).

7. References

Hunter, K. 1983: Determination of coumarin anticoagulant rodenticide residues in animal tissue by high-performance liquid chromatography: I. Fluorescence detection using post-column techniques. *Journal of Chromatography* 270: 267–276.

Hunter, K. 1984: Reversed phase ion-pair liquid chromatographic determination of chlorophacinone residues in animal tissues. *Journal of Chromatography* 299: 405–414.

Ozawa, H. T.; Tsukioka, T. 1989: Determination of monofluoroacetate in soil and biological samples as the dichloroanilide derivative. *Journal of Chromatography* 473: 251–259.

Spurr, E.B.; Powlesland, R.G. 1998: Manual for monitoring the impacts of vertebrate pest control operations on non-target wildlife species. Landcare Research Contract Report LC9899/35 (unpublished). 46p.

Wickstrom, M.; Eason, C. 1997: Sample collection for the diagnosis of 1080 poisoning in livestock. *Vetscript* 10 (7): 30.

Krynitsky, A.J.; Wiemeyer, S.N.; Hill, E.F.; Carpenter, J.W. 1986: Analysis of cyanide in whole blood of dosed cathartids. *Environmental Toxicological Chemistry*.5: 787-789.

Valentour, J.C.; Aggarwal, V.; Sunshine, I. 1974: Sensitive gas chromatographic determination of cyanide. *Analytical Chemistry* 46: 924 – 925.

Wiemeyer, S.N.; Hill, E.F.; Carpenter, J.W.; Krynitsky, A.J. 1986: Acute oral toxicity of sodium cyanide in birds. *Journal of Wildlife Diseases* 22: 538 – 546.

8. Landcare Research Website

Copies of this protocol and the pesticide analysis sample details form are available on the Manaaki Whenua - Landcare Research website at:

<http://www.landcareresearch.co.nz/services/laboratories/toxlab/index.asp>



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