



Annex IV: Procedural protocol for dealing with cases of poisoning in wildlife rescue centres and toxicology laboratories

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INTRODUCTION

The present document has been drawn up under the *Life* + *VENENO* (LIFE08 NAT/E/000062) coordinated by SEO/BirdLife with collaboration of all the key stakeholders in the fight against poison in Spain.

This protocol is the Annex IV of the **Action Plan to eradicate the illegal use of poison in the countryside**

This guide aims to serve as an aid for veterinarians in wildlife rescue centres (*centros de recuperación*) and the technicians of forensic and toxicology labs in cases of presumed poisoning. Availability of human and technical resources might vary between regions (*comunidades autónomas*) and laboratories, so this document should not be conceived as a strict protocol but rather a procedural model that is likely to give sound results. This procedural guideline starts with the arrival at the *Centro de Recuperación* of the animal or bait collected by the officers in the field and ends with the writing of the expert appraisal by the veterinarian of the *Centro de Recuperación*.

1. CARRYING OUT THE NECROPSY

The purpose of any necropsy is to establish as precisely as possible the causes and circumstances of the death of the bodies submitted for post mortem. The necropsy should therefore be complete, orderly and systematic. It must be conducted by a trained veterinarian with updated knowledge of the fundamental aspects of legal necropsy.

1.1 BACKGROUND INFORMATION

The first step is to read all the information recorded in the carcass removal report (the official report controlling removal of the carcass from the spot where it was found). If necessary the officer who wrote this report should be contacted before going ahead with the necropsy. It is vital to record as much information on the specimens as possible:

- Site where it was found, including the name of the spot, municipal district and, where possible UTM coordinates.
- Circumstances of the finding: time, day, position...
- Treatment received and handling of the animal since its collection, if it was originally found alive.

1.2 EXTERNAL EXAMINATION

The external examination has to be exhaustive and painstaking, with the aim of determining the age, recording biometric data and detecting external injuries.



First of all, each carcass should be photographed using a top-quality digital camera to make sure that the photos are useful for drawing up the expert appraisal. It is recommended that at least the following views should be photographed:

- Whole body, ventral view.
- Whole body, dorsal view.
- Lesions or signs deemed to be important.

The external examination of the animal will then be carried out to estimate the date of death (based on decomposition and forensic entomology) and look for any injuries, impacts and fractures. Special attention should be paid to any signs that might denote pre-death episodes of convulsions, diarrhoea or haemorrhages, since these will largely determine the analytical protocol pursued from then on. It is always recommendable to X-ray the animal beforehand to detect fractures and signs of being shot. The animal will then be weighed to assess its bodily condition (presence of body fat and state of pectoral muscles in birds). If there are any observable remains of vomit or food in or around the mouth, samples of these will be collected to see whether they contain high toxin levels.

The external examination will include:

- Ventral-dorsal X-ray and X-ray of any other part of the body deemed to be necessary.
- Examination and palpation of the whole skeleton, oesophagus and coelom.
- Complete examination of fur, plumage, mouth, beak, cere and claws.
- Examination of all bodily orifices and state of mucous membranes.
- Weight and size of the animal (readings to be taken to suit the particular species).
- Look for the presence of ectoparasites, type, identification, if possible, location and level of parasitism.

1.3 INTERNAL EXAMINATION

For the internal examination the animal will be placed in supine position and dissected in the manner to suit the particular animal species. In general the carcass dissection will commence in the abdominal area, drawing off the skin as possible to look for any signs of impact and haemorrhage. Consideration will be given to the possibility of an infectious process. Samples of the viscera will be taken with sterilised material and with a Bunsen burner or alcohol burner near the carcass; the necessary material will also be prepared for flaming the material if need be.

The viscera will be examined before and after being taken out of the body. It is recommended that they be taken out in the following order:

- Heart.
- Digestive tract from the oesophagus to the cloaca in birds, including liver, spleen and pancreas, closing off the opening of the digestive tract with mosquito forceps to avoid



contamination. In the case of mammals, depending on the size of the animal, the best procedure would be to take out the digestive tract in parts and the viscera separately.

- Dissection and extraction of tongue, trachea, bronchia and lungs.
- Adrenal glands, gonads and kidneys.
- Thyroid glands and thymus gland (if necessary).
- Encephalon.

These organs will be placed in a clean, regulated tray beside the Bunsen burner. The digestive tract will be placed apart in another tray to avoid any contamination from the rest of the organs.

An exhaustive examination will then be made of each organ/system and the samples will be taken. Digital photos will also be taken of any observed lesions. Special attention should be paid to the presence of haemorrhages, congestion of viscera, exudates and oedemas and the state of gastrointestinal mucous membrane.

With all this information to hand, the veterinarian will write a preliminary expert appraisal, in which, on the basis of his or her experience, he or she will weigh up the possibility of intoxication as cause of death or the existence of possible bait toxins. A copy of this appraisal will be sent up to the toxicology laboratory together with a printout of the analysis request (ANNEX I) and the samples to be analysed. This preliminary appraisal report, pending analytical confirmation, could be used to extend the field investigation by the officers (so they should be diligently informed thereof) and for lodging the due injunctions.

2. SAMPLE TAKING FOR TOXICOLOGICAL ANALYSIS

2.1 TYPE OF SAMPLES

Different types of samples should be taken according to the observed lesions and the suspicion of products involved (Table 1). In general the following samples should always be taken:

- **Content of oesophagus and stomach:** Most of the toxins are quick-working and concentrate at high levels in the upper digestive tract. Once absorbed, toxins like organophosphates and carbamates break down quickly and may not be detectable in the liver. The makeup and nature of the digestive contents, if any, could give important information on the process. Its handling will depend on whether or not there are any visible signs of suspicious foreign bodies. If any foreign bodies like dust, paste or suspicious microgranules are observed, these should be placed in a small tube to avoid further mixture with the rest of the food; this will also facilitate the laboratory's analytical detection work. To weigh up the possibility of a lethal ingestion it is essential to work from information on the total weight of the stomach contents and/or the amount of dust, paste or microgranules of toxic products in this content. Conversely, when the toxins are evenly mixed in the stomach contents and the product is therefore invisible, the sample will be handled as a whole. Once the analysis results have been



obtained, then, on the basis of the detected concentration and the amount of stomach contents, a calculation can be made of the dose ingested. The estimated dose ingested, bearing in mind the weight of the animal involved, can then be gauged against the lethal doses described in the literature. In many cases, however, the toxin may have been largely absorbed or broken down, so the simple presence in the stomach contents, together with toxin-related lesions or symptoms observed and other analytical findings (e.g.: brain AChE inhibition in the case of phosphate pesticides and carbamates) are sufficient basis for the diagnosis.

- **Liver:** This organ receives everything absorbed by the digestive tract and should therefore ideally be sampled. Liver analysis might even sometimes be crucial for demonstrating the toxic cause of death, despite a positive result in the digestive tract. Moreover, when there is no digestive content or the analysis thereof comes out negative, then liver analysis is essential. Some slower-working toxins like anticoagulant rodenticides build up in the body and are mainly detected in the liver. Heavy metals like lead may also build up in the liver and kidney; the concentration levels in these tissues associated with lethal intoxication in animals have by now been well defined.
- **Encephalon** (not necessary to take it out intact): Most of the products used as poison today are cholinesterase-inhibiting pesticides, so it is recommendable to determine brain acetylcholinesterase activity, which will normally be very inhibited in the case of exposure to carbamates or organophosphates. This will serve as a useful guideline for any chemical analyses while also confirming animal death by toxic causes (inhibition >50% as compared to control values). Some carbamate-intoxicated animals, however, might still record a normal brain acetylcholinesterase activity, possibly due to post-mortem reactivation or because the symptoms were peracute and the effects on the peripheral nervous system were death-inducing even before the toxin got to the central nervous system. Mercury or some organochlorinated pesticides build up in the encephalon to reach lethal concentrations. For all these reasons the cholinesterase-inhibiting test should be taken as a useful complement in the laboratory process and diagnosis but not as definitive proof to rule out intoxication by cholinesterase-inhibitors.

Other revealing samples, depending on the circumstances in each case, might be:

- **Bait and vomit:** Suspicious material found close to the carcass. For obvious reasons concentration levels are usually higher than in the animals themselves and toxin detection more probable. This could be a sample of great value in the analysis laboratory, since the toxin is usually unaltered and both the extraction and analytical detection are much easier, with a significant saving of time and effort.
- **Blood:** Blood sampling is possible only for living or recently deceased animals (heart) but can be useful for determining exposure to lead and therefore quick application of treatment with chelating agents. For lead determination, blood should be sampled in tubes with lithium heparin. Blood could also be sampled in tubes with sodium citrate



as anticoagulant for carrying out coagulation tests in the case of intoxication by anticoagulant rodenticides antagonistic to vitamin K. It can also be used for carrying out a haemogram. Blood of a brown colour might indicate nitrate intoxication and in this case a determination could be made of the blood methaemoglobin percentage if it is analysed within a few hours or kept in liquid nitrogen.

- **Plasma:** Plasma sampling can be used for determining some toxins but above all for ascertaining plasma cholinesterase activity for the same reason as already given for encephalon sampling. It can also be used for making a routine biochemical profile.
- **Kidney:** In the case of intoxication by bipyridyl herbicides (paraquat) the toxin builds up in the kidney. In intoxications of this type, significant lung lesions are observed (congestion, oedema). The kidney could also be a useful sample if the carcass has been devoured by predators and there are no remains of the digestive tract or liver. Cadmium builds up throughout the animal's life in the kidney, sometimes to very high levels.
- **Body fat:** This tissue is not apt for ascertaining acute exposures but is useful for monitoring exposure to persistent lipophilic compounds such as organochlorine compounds. The pesticides of this family most likely to build up in the body are no longer in use but are still detectable in animals. Halogenated compounds like polychlorinated biphenyls, polybromated diphenyl ethers and others are highly persistent and also need to be monitored.
- **Bone:** Lead builds up in the bones throughout an animal's life so it is a useful sample for monitoring studies. On certain occasions, when only the skeleton is available, bone could be used for extracting bone-marrow remains for carrying out an analysis, though the chances of demonstrating death by intoxication are very slight.
- **Fur, feathers and claws:** Some elements like mercury and arsenic (and even lead) might build up in these structures, indicating chronic exposure or even lethal acute exposure.
- **Earth beneath the carcass:** In cases of completely decomposed carcasses, the first 5 cm of soil below the dead body can be taken, always taking into consideration the size of the body in each case (small bodies, small earth samples) and also the possibility of depredation and removal from the initial decomposition site.

2.2 SAMPLE CONSERVATION AND SENDING METHOD

The following premises should always be borne in mind when conserving samples for dispatch:

- Samples should be free of any external chemical contamination (dust, hair, earth, etc), unless, obviously, this is precisely part or all of the sample to be sent.



- Samples should be frozen at -20 °C immediately after collection and kept under the same conditions until arrival at the laboratory. The only exception to this rule is blood sampling; blood is kept at refrigeration temperature (c. +4°C) to be able to conduct coagulation tests and the haemogram.
- Each sample should be kept in an independent container (of the urine-sample type or plastic bag with Ziploc closure), duly labelled with the case reference and nature of the sample. Bags have the advantage of taking up less space and they are also easily sealable with a self-sticking label, including sample information (case number and type of sample). The set of samples of a particular case should be kept and sent in a numbered flange seal bag.
- All containers, whether bags or plastic vases, must be hermetically sealed. In the case of samples in which trace levels of organic compounds type PCBs or pesticides need to be detected, the sample could be wrapped in aluminium foil before being put in the container.
- Never use preservatives unless expressly told to do so by the laboratory. If any preservative is in fact added to the sample, the type and amount should be specified in the accompanying report and a sample thereof shall be sent to the laboratory. This shall be sent in a separate container and duly labelled so that there is no possibility of confusion with a sample.
- In the case of volatile compounds, such as ammonium or cyanide intoxication, the rumen, blood and serum content should be frozen immediately to prevent volatilisation loss.
- All containers with each sample taken from the case should be placed in suitable packaging, which will then be sealed in such a way as to betray unequivocally any tampering with the sample thereafter. This packaging shall be labelled in the same way as the containers and always bear reference to the case and accompanying reports.
- Samples will be sent to the laboratory in EPS boxes or the like, suitably packed with refrigerating elements to ensure that the sample does not arrive totally thawed out. The inside should also be padded against impact and accidental opening of the containers during transport. Samples should ideally be sent at the start of the week to prevent them from thawing out during the weekend.
- It is also recommendable to include fixed samples for histopathology to confirm the diagnosis in the case of any doubt.

2.3 INFORMATION ENCLOSED WITH THE SAMPLES

Samples will be accompanied by an envelope with reports on each case, giving all the following details:



- Species.
- Number of animals involved in the case.
- Case number of the *centro de recuperación* (unique reference).
- Date of the finding/death.
- Locality: Municipality and province.
- Field details: Habitat, type of crops, livestock, hunting grounds, power line pylons, death of other animals, timeline of appearance of the cases, radio-monitoring data, treatment with phytosanitary or zoosanitary products ...
- Necropsy findings. The necropsy report should ideally also be enclosed.
- Hard-copy or digital photos.

Table 1. This table shows the samples for determining the toxins involved or some of the toxic effects. Nonetheless, in order to conduct a differentiating diagnosis, if there are no clear grounds for suspicion and also to rule out/identify the various toxins, it might be necessary to take all possible samples.

	Priority general			or Additional or specific						
	Stomach contents	Liver	Encephalon	Bait, vomit	Blood	Plasma	Kidney	Body fat	Bone	Fur, feathers, claws
Organophosphates and carbamates	F	F	F	F	F	F				
Organochlorines	F	F	F	F	F	F		F		
Strychnine	F	F								
Anticoagulant rodenticides		F			R					
Bipyridyl herbicides	F	F					F			
Alphachloralose	F	F								
Methaldehyde	F									
Lead	F	F			F		F		F	
Other pesticides	F	F								
Mercury		F	F		F		F			F
Arsenic	F	F			F		F			F
Cadmium		F			F		F			
Other metals and metalloids		F								
Nitrates		F			F+					
Cyanide		F								

F: frozen at -20°C; F+: frozen in liquid nitrogen, R: refrigerated and analysed in a few hours.



- Type of samples sent. We recommend assigning different letters to the various samples of the same case. For example, if two vultures have died and a sample has been taken of the stomach contents and liver we will assign the number CRFS001A/07 to the stomach contents and CRFS001B/07 to the liver of the first vulture, CRFS001C/07 and CRFS001D/07 to the samples of the second.
- Suspicions of potential toxic products or compounds that might be involved in the case, based on any available information: former cases, local customs, rumours, etc. In any case it is best to quote the degree of certainty about the suspicion.
- Date of dispatch.
- Person responsible for the dispatch (write full name, identity document number [DNI in Spanish initials], signature and seal of the centre).
- Person carrying out the transport. If this is a company, name it and give contact details (telephone, fax or email).
- Date of reception.
- Person responsible for the reception (write full name, DNI, signature and seal of the centre).

A signed and sealed copy of this report will be returned to the sender. A note will be made of any incidences related to the dispatch and sample (codification errors, poor conservation ...). Attached hereto (Annex I) is a form that can be used as reference.

3. TOXICOLOGICAL ANALYSES

The protocol under this heading could depend on the equipment and characteristics of each toxicology laboratory. The one detailed here is a compilation and pooling of the procedures used in three laboratories signing this protocol.

The laboratory shall keep a register of the entry of all cases, noting down at least the following details: date of entry of the case, sender of the sample, sender's case reference, laboratory's case reference, samples sent and state, considerations or observations about the seal (if necessary).

3.1 USE OF BIOMARKERS

Bearing in mind the clinical history and findings of the necropsy, a determination can be made of brain acetylcholinesterase enzyme (AChE) activity using the Ellman method (Hill and Fleming 1982). This biomarker can be useful in many of the anticholinesterase poisoning cases, which account for the majority nowadays. The values obtained can be compared with those obtained from *in vitro* enzyme reactivation by dilution of the sample and addition of 2-PAM. The usefulness of this determination in the brain has been commented on in the previous section. This test can be conducted at the beginning of the study of each case as a guide to the analysis thereafter, or afterwards to confirm death by exposure to an anticholinesterase (inhibition >50%) rounding out the other laboratory analyses for the final diagnosis.



3.2 DETECTION OF THE TOXIN

First and foremost, in the case of bait and stomach contents, a visual examination of the sample will be made to check for any granular formulations or other sign of the presence of phytosanitary products. A general method of extraction, purification and determination allows us to identify most toxins but if there is evidence of a particular toxin or when several possibilities have been ruled out, it might then be necessary to carry out more specific analytical methods. These analysis protocols are based on methods published by the toxicology laboratories that have drawn up this text and in fact are largely based on the procedure used by the Wildlife Incident Investigation Scheme of the UK's Central Science Laboratory (Brown et al., 2005). Depending on the technical resources available in each laboratory, there are various alternative methods that might also be acceptable. In general, and to ensure correct identification of the toxin, the organic compounds should be analysed using a mass detector coupled up with a gas or liquid chromatograph. If this analytical technique is unavailable, attempts will be made, if possible, to confirm the result by means of two different analytical techniques (e.g.: colorimetry and thin layer chromatography for strychnine).

- **Determination of different types of neurotoxic substances (organophosphates, carbamates, organochlorines, alphachloralose, barbiturates):** Working from baits or stomach contents, an extraction with dichloromethane will be made. Depending on the type of sample, the analysis can be conducted without more purification after evaporation by means of a rotary evaporator or nitrogen flow and resuspension in 0.5 ml of ethyl acetate. In the case of the liver or other parenchymatous tissue, the tissue will first be homogenised with anhydrous sodium sulphate and is then extracted in the same way as stomach contents with dichloromethane. When extracts need previous purification this can be done by means of gel permeation chromatography through a phase of Bio-Beads S-X3 (Bio- Rad) with ethyl acetate and cyclohexane (1:1) as mobile phase. Alternatively, if there is any indication of the type of toxin sought, solid phase extraction (SPE) columns can be used. In some cases, the extract obtained by GPC can also be additionally purified by means of alternative techniques (e.g.: SPE, Quechers, etc) before being analysed. Purified extracts of each sample can be analysed by means of liquid and gas chromatography but results should always be confirmed by means of mass spectrometry, either by comparison with standard spectra or with commercial databases. Some less specific and sensitive techniques, such as thin layer chromatography, might come in useful for a first study of the samples, especially for the rapid analysis of microgranules and samples with a high toxin concentration.
- **Determination of strychnine:** Although this might be detectable with the same procedure described above, it is recommendable to carry out a more specific extraction with dichloromethane and subsequent purification by liquid-liquid extraction. Extracts obtained will be analysed as in the above section.
- **Anticoagulant rodenticides:** The liver or bait is homogenised with anhydrous sodium sulphate; the extraction is made with dichloromethane or other solvent mixtures and purification by SPE (this can be varied according to the type of



rodenticides to be analysed, indandione or cumarin) and finally analysed with liquid chromatography coupled with mass spectrometry.

- **Metals and metalloids:** A freeze-dried sample of the liver or bait is digested with nitric acid and hydrogen peroxide (or other acid mixtures) in microwaves or open glass (or quartz) tubes and analysed by specific techniques (atomic absorption spectroscopy, ICP, voltammetry).

4. INTERPRETATION OF ANALYTICAL RESULTS AND DRAWING UP THE TOXICOLOGY REPORT

The toxicology report shall include at least the following information in the stated order:

4.1 DESCRIPTION OF CASE DATA AND DOCUMENTS

The report must be complete, including the case information furnished by the veterinarian of the *centro de recuperación* in his or her necropsy report, especially the species, type of samples and case references (number of seals, number of reports or case number in the *centro de recuperación*). Reference must also be made to any additional documentation (necropsy reports, reports by other professionals, graphical documents, etc.) which has been sent to the laboratory together with the samples, and which has been used to carry out the analyses and interpretation of results.

4.2 ANALYTICAL METHODS

Details will be given of the analytical methods used and also the samples that have been analysed. In some cases it is not necessary to analyse all the samples sent, so thoroughgoing justification shall be given of the selection of samples to be analysed. Detailed information will also be given on validation of the methods used and applied quality control (blanks, standards used, recovery rate, detection limits...).

4.3 RESULTS

This section will give information on the type of toxin detected and the concentration found when this information is necessary for drawing definitive conclusions. An indication will also be given of any toxins that have been ruled out on the basis of the analytical methods used.

Cross-checked information of maximum scientific rigour (including references) will then be given on the toxin(s) detected, ideally with at least the following data:

- **Mean lethal dose** in similar species to those studied to be able to weigh up the risk of its being a case of lethal intoxication on the basis of information on weight of stomach contents, bait or number of microgranules.
- **Commercial applications** of these products (agrochemical, zoosanitary...) so that the officers studying the case can work with more information about the possible perpetrators of the poisoning case.



- **Action mechanism and associated symptoms**, so that the veterinarian of the *centro de recuperación* can write up the definitive report.
- **Final interpretation** weighing up whether the animal might have been lethally intoxicated or the chances of the bait proving lethal to the animal once ingested. Information will also be given on the chances of its being a case of secondary intoxication or whether it might be acute or chronic intoxication. A correct joint interpretation of the set of case data (clinical reports, analyses, etc.) is crucial in support of the final diagnosis. A specimen toxicology report is attached hereto in ANNEX II.
- **Complementary information:** Chromatograms and mass spectra to support the weighing up of results.

5. DEFINITIVE REPORT BY THE VETERINARIAN OF THE RESCUE CENTRE

Once the veterinarian has received the toxicology report, he or she shall then draw up the **definitive expert appraisal** of the case, which, on the basis of the analytical results (toxicological or others like histopathology, microbiology ...) will assess whether the animal has been intoxicated or whether the bait in question is capable of causing intoxication to protected fauna. Furthermore, on the basis of the information culled by the officers and with the analytical results to hand, the veterinarian will determine whether it is a case of deliberate, accidental or secondary intoxication, etc. The report will also weigh up the possibility of endangered protected species being intoxicated in the area where the bait or dead animals have been found. A specimen of this type of final report is attached hereto as ANNEX III.

References

Brown, P.M., Turnbull, G., Charman, S., Charlton, A.J., Jones, A. 2005. Analytical methods used in the United Kingdom Wildlife Incident Investigation Scheme for the detection of animal poisoning by pesticides. *J AOAC Int.* 88:204-20.

Hill, E.F., Fleming, W. J., 1982. Anticholinesterase poisoning of bird: field monitoring and diagnosis of acute poisoning. *Environ Toxicol Chem* 1:27-38.

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ANNEX I: REQUEST FOR A TOXICOLOGICAL ANALYSIS

(This form has been furnished by IREC Toxicology laboratory)

Address of the sample analysing laboratory

Tel , Fax , @

Case number of the *centro de recuperación*:

Species: Number of animals involved in the case:

Date of finding /death:

Municipality: Provincial:

Findings in the field:

Necropsy findings:

Suspected toxins:

Type of samples sent and references:

Seal number:

Email/mobile phone of the Officer:

Email/mobile phone of the Veterinarian of the *centro de recuperación*:

Date of dispatch: Person responsible:

Transported by:

Reception date: Person responsible:

Incidents:



ANNEX II: TOXICOLOGY REPORT

(This form has been furnished by IREC Toxicology laboratory)

Laboratory Logos

Mr./Ms.

Organisation

C/

00000

TOXICOLOGY REPORT. Laboratory case reference; *centro de recuperación* ref:

Applicant:

Entry in the laboratory:

Clinical history: Suspicion of the placement of poisoned bait.

Samples received: We received sample A (eggshell N) and B (black liquid N) with blue seal number:

Analyses asked for: Determination of carbamates, cyanide, strychnine and organophosphate compounds.

Analytical methods:

An extraction was made with solvents of samples A and B, separately, after homogenisation with anhydrous sodium sulphate; one part of the extract was purified selectively for alkaloids; another was purified by gel permeation chromatography for pesticides, followed in both cases by gas chromatography coupled to mass spectrometry (Brown et al., 2005; J AOAC Int. 88:204-20).

Results:

Toxin detected: **Dimethoate** in sample A and **dimethoate, diazinon (dimpylate) and chlorpyrifos** in sample B.

Concentration: **0.15 µg/g** of dimethoate in sample A, **88.66 ng/µl** of dimethoate, **4.04 ng/µl** of diazinon and **1.69 ng/µl** of chlorpyrifos in sample B.

Acute oral median lethal dose in living weight: 60 mg/kg in rats and 42 mg/kg in ducks for dimethoate, 66 mg/kg in rats and 3.5 mg/kg in ducks for diazinon and 82 mg/kg in rats and 76 mg/kg in ducks for chlorpyrifos (Toxnet).

Formulations:

a) Dimethoate: Marketed in Spain as a foliar application insecticide in powder, liquid or



emulsifiable concentrate with concentrations ranging from 3 to 50 % and accompanied by chlorpyrifos.

b) Diazinon: Currently marketed in Spain as non-farming biocide in liquid spray anti-parasite formulations (Zooveca) and in flea collars (Prevender), although up to 2007 (Directive 2007/25/EC (LCEur 2007,664)) it was marketed as a foliar application farming insecticide in powder, liquid or granules with concentrations ranging from 2.5 to 60 %.

c) Chlorpyrifos: Marketed in Spain as foliar application insecticide in powder, liquid or granules with concentrations of between 1 and 75 % and accompanied by cypermethrin, phosmet and dimethoate.

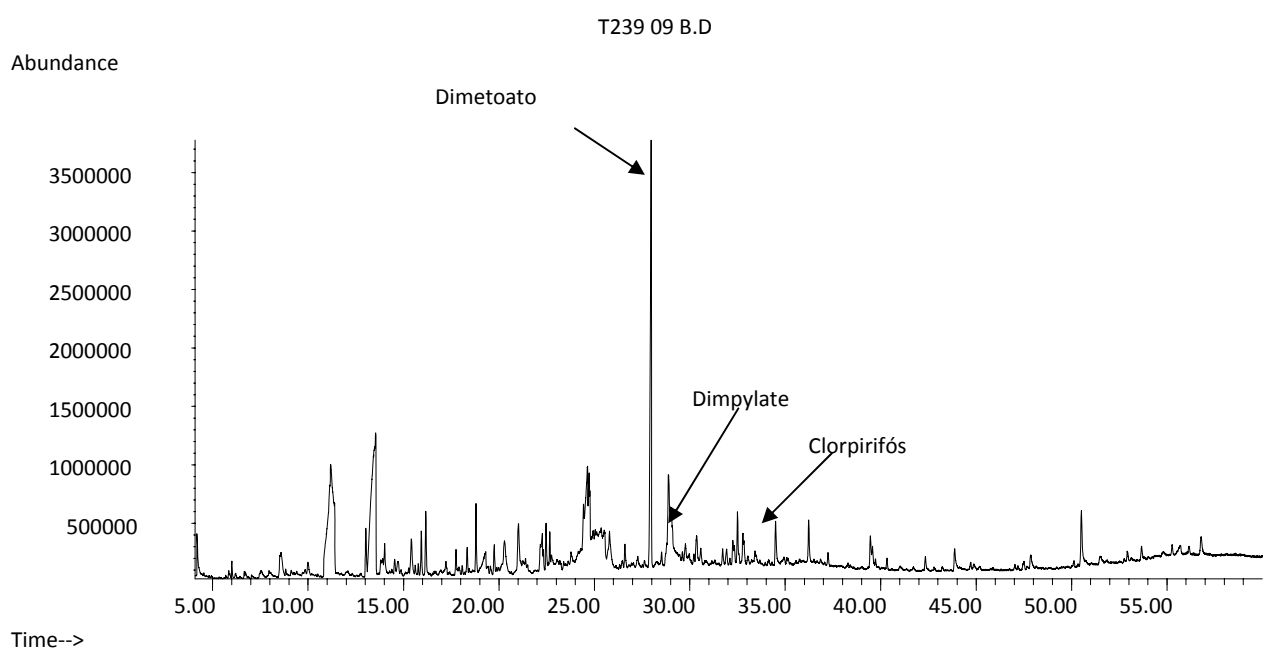
Interpretation: Dimethoate, diazinon and chlorpyrifos are low persistence, cholinesterase-inhibiting pesticides. Cholinesterases are enzymes involved in the correct transmission of nerve impulses and their inhibition upsets the nervous system, causing death by respiratory arrest. This action mechanism makes these compounds very quick-acting neurotoxins capable of producing death in 10 to 30 minutes with doses higher than LD50 and between 30 minutes and six hours at lower doses, although this may be prolonged up to 12 or even 24 hours for some latent cholinesterase inhibitors (Hill 2004, in Hoffman et al., Handbook of Ecotoxicology).

Exposure to organophosphates in birds and mammals produces survival-threatening muscle paralysis, reducing their capacity of moving about, depending on the doses and toxin they have been exposed to (Hill 2004).

On the basis of the high toxicity of the compounds and the concentrations detected in the samples, we confirm the intentionality of the use of the analysed bait to poison animals.

Chromatogram:

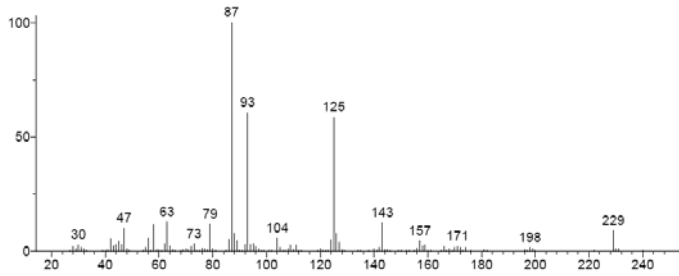
Chromatogram obtained after gas chromatography coupled with mass spectrometry:



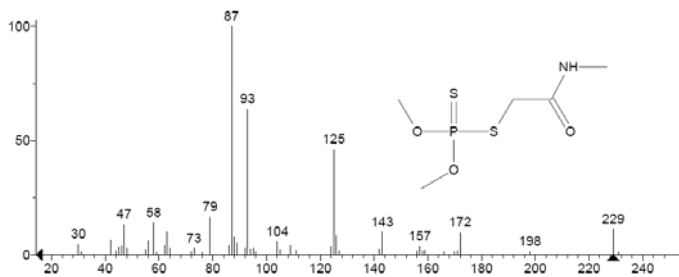


Mass spectrum in the peak at 27.976 minutes and identification of the peak by comparison with the mass spectra recorded in the NIST Mass Spectral Library:

Pico a 27.976 min: T239 09 B.D

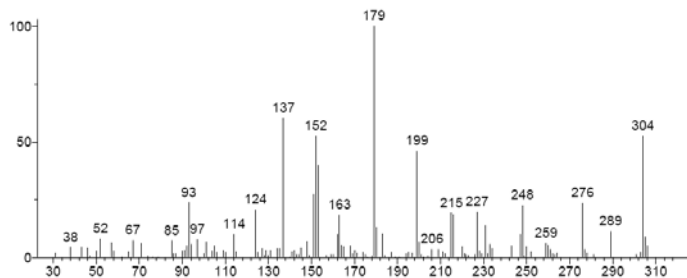


Hit 1 : Dimethoate
C5H12NO3PS2; MF: 880; RMF: 927; CAS: 60-51-5; Lib: replib; ID: 11026.

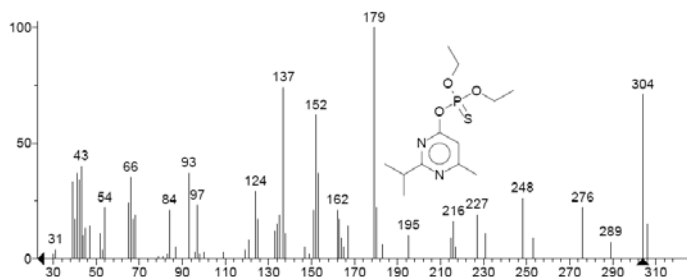


Mass spectrum in the peak at 29.093 minutes and identification of the peak by comparison with the mass spectra recorded in the NIST Mass Spectral Library:

Pico a 29.093 min: T239 09 B.D



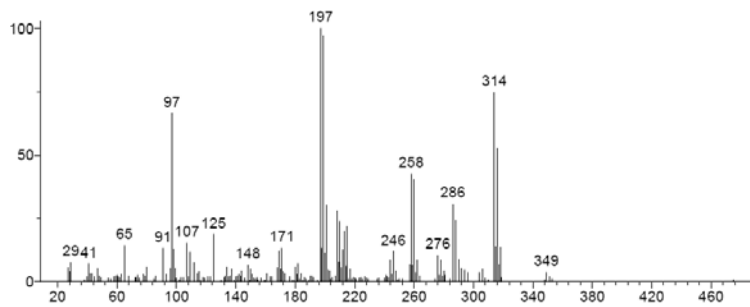
Hit 1 : Dimpylate
C12H21N2O3PS; MF: 587; RMF: 778; CAS: 333-41-5; Lib: replib; ID: 22500.



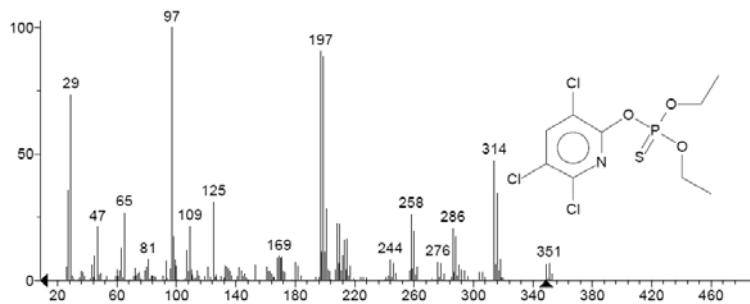


Mass spectrum in the peak at 32.779 minutes and identification of the peak by comparison with the mass spectra recorded in the NIST Mass Spectral Library:

Pico a 32.779 min: T239 09 B.D



Hit 1 : Chlorpyrifos
C9H11Cl3NO3PS; MF: 833; RMF: 859; CAS: 2921-88-2; Lib: mainlib; ID: 51834.



Custody of samples received:

Samples will be kept at -20 °C for three months after issuing this report, after which time they will be destroyed unless we are informed of any interest in keeping them further during this time period.

Signed.:
Engineering graduate.

Approved by: Rafael Mateo
Head of the Toxicology laboratory

In, at (day) (month) 20..



ANNEX III: FINAL REPORT BY EXPERT APPRAISER

(Attached hereto is an example of a report by the expert appraiser)

DEFINITIVE REPORT PERTAINING TO THE FORENSIC EXAMINATION OF TWO GOSHAWKS (*Accipiter gentilis*) AND SUSPECTED POISONED BAIT WITH REFERENCE NUMBER N___ / ___

Sample data

Carcass A

Species: Goshawk (*Accipiter gentilis*)

Sex: Female

Age: Adult

Seal number and colour: 005013 Green

Carcass B

Species: Goshawk (*Accipiter gentilis*)

Sex: Male

Age: Juvenile (2 years)

Seal number and colour: 005014 Green

Sample A: Bone remains, with sparse muscle tissue attached thereto, of an incomplete bird carcass

Seal number and colour: 005015 Green

Origin: Place name _____, municipal district of _____,

Province of _____

Sender: SEPRONA, ENVIRONMENT OFFICERS

Date of reception: _____

Analysis results

Carcasses A and B

External examination

The specimens sent for analysis are two carcasses of two Goshawks (*Accipiter gentilis*) (carcasses A and B) (Images 1, 2, 3 and 4). One of them is an adult female weighing 1064 grams and the other is a male juvenile weighing 655 grams. Both show an optimum state of nutrition and musculature for the species. The carcasses show a moderate degree of decomposition (2-5 days old, approximately). Palpation showed no signs of contents in the crop.



In the case of carcass A, the plumage and other integuments are in a good state. Carcass B shows broken seventh and tenth primaries in the right wing, third and fourth secondaries on the left wing and two central tail feathers.



Image 1. Ventral view of carcass A



Image 2. Dorsal view of carcass A



Image 3. Dorsal view of carcass B



Image 4. Ventral view of carcass B

Neither carcass has any observable injuries or fractures that might suggest an impact.

Radiological study

An X-ray was taken of the two goshawk carcasses. The X-ray of goshawk carcass B showed three projectiles, one in the lower righthand part of the body, another in the left and another in the left wing (Image 5).

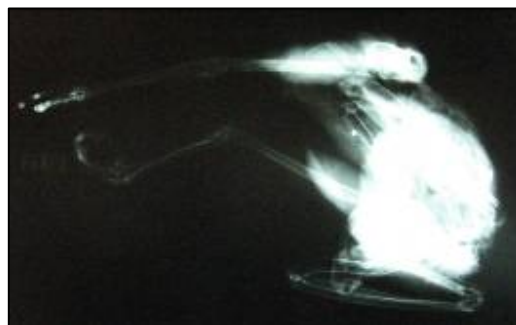


Image 5. X-ray of carcass B



Internal examination

The internal examination of the birds showed that the body fat and subcutaneous fat is normal for the species.

In both cases there is a notable liver and kidney congestion. The liver also seems to be swollen and spongy (Images 10 and 11). The upper digestive tract of both birds shows content inside. The content of carcass A's upper digestive tract weighs 18 grams while carcass B's weighs 2.5 g.

In carcass A the contents of the cranial chamber (proventriculus) and caudal chamber (ventriculus) is soft pink tissue compatible with muscle tissue, with bone fragments and small white feathers. The ventriculus contents of carcass A include the lower righthand end of a bird, apparently a dove/pigeon (Images 6, 7, 8 and 9).

Carcass B's upper digestive tract contains about 1 ml of a light brown liquid.

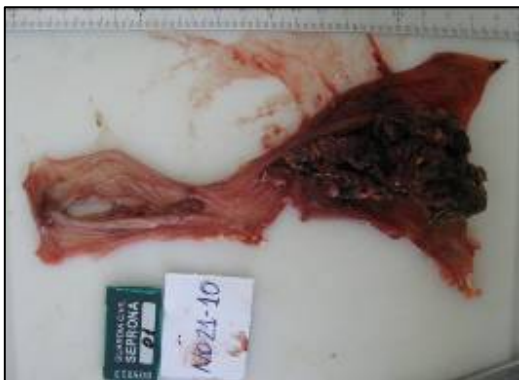


Image 6. Contents of the upper digestive tract of carcass A



Image 7. Suspect granulation of the upper digestive tract of carcass A



Image 8. Contents of the upper digestive tract of carcass B



Image 9. Detail of the contents of the upper digestive content of carcass B



Image 10. Congestive liver of carcass A



Image 11. Congestive liver of carcass B

The contents of both birds showed greyish irregular granules about 1 mm in diameter.

During the internal examination of goshawk carcass B, two lead projectiles about 2 mm in diameter (of the three observed in the radiological study) were removed. These were located in the caudal part of the proximal epiphysis of the right tibiotarsus and the caudal face of the left femur, among the musculature of the region. Both projectiles were surrounded by fibrous and scar tissue, showing no signs of haemorrhage or acute inflammation around them.

Sample A

Examination of the sample

The sent sample consists of the incomplete carcass of a bird, apparently a dove/pigeon. The carcass comprises some bones, sparse dehydrated muscle tissue sticking to the bones, the heart and the remains of soft tissue of the coelom.

The carcass conserves only the last three cervical vertebrae, both humeri, both coracoids, the thoracic vertebrae, part of the synsacrum, the right femur, six incomplete ribs of the lefthand side and the cranial half of the carina and sternum. Visible inside the incomplete coelom is the heart and soft dark red tissue of friable consistency compatible with part of the liver. Adhering to the surface of the tissues and dehydrated musculature of the visceral surface, of the coelom, is a suspicious greyish-blue irregular granulation about 1 mm in diameter.



Image 12. Sample A



Image 13. Suspicious granulation of sample A

Chemical-toxicological analysis

The following samples were sent for toxicological analysis:

N021/10 A – Suspicious granulation taken from the contents of the higher digestive track of goshawk carcass B.

N021/10 B – Liquid taken from the upper digestive tract of goshawk carcass B.

N021/10 C – Contents of the upper digestive tract (2.5 g) of goshawk carcass B.

N021/10 D – Liver of goshawk carcass B.

N021/10 E – Suspicious granulation taken from the contents of the upper digestive tract of goshawk carcass A.

N021/10 F – Contents of the upper digestive tract (18 g) of goshawk carcass A.

N021/10 G – Liver of goshawk carcass A.

N021/10 H – Suspicious granulation taken from sample A.

N021/10 I – Heart, liver-like soft tissue, bone and dehydrated muscle tissue of sample A.

Toxin detected: Carbofuran, terbufos and fenamiphos.

Concentration:

a) Sample A (N021/10 A): 37.60 µg/g of carbofuran and 136.5 µg/g of terbufos.

b) Sample C (N021/10 C): 20.74 µg/g of carbofuran. 61.56 µg/g of terbufos and 9.98 µg/g of fenamiphos

c) Sample E (N021/10 E): 4.15 mg/g of carbofuran. 379.16 µg/g of terbufos and 21.26 µg/g of fenamiphos.



- d) Sample F (N021/10 F): 18.83 µg/g of carbofuran. 23.13 µg/g of terbufos and 14.73 µg/g of fenamiphos
- e) Sample H (N021/10 H): 4.48 mg/g of carbofuran. 750.52 µg/g of terbufos and 58.35 µg/g of fenamiphos.
- f) Sample I (N021/10 I): 1.37 mg/g of carbofuran. 802.13 µg/g of terbufos and 529.49 µg/g of fenamiphos.

Acute oral median lethal dose: 5-13 mg/kg in rats and 0,48-0.51 mg/kg in ducks for carbofuran, 1.6 mg/kg in rats and 15 mg/kg in quail for terbufos and 8 mg/kg in rats and 1.68 mg/kg in ducks for fenamiphos (Toxnet).

Formulations:

- a) Carbofuran: Not currently marketed in Spain as a biocide or phytosanitary product but was marketed up to 2007 (LCEur\2007\1034) as a ground-application insecticide concentrate in microgranules with active principle concentration of 5% and as concentrated suspension with concentration of 20%.
- b) Terbufos: Not currently marketed in Spain as a biocide or phytosanitary product but was marketed as a ground-application insecticide in microgranules with active principle concentration of between 2 and 5%.
- c) Fenamiphos: Marketed in Spain as ground-application insecticide in the form of microcapsules and emulsifiable concentrate with active principle concentration of 24% and 40% respectively.

Action Mechanism: Carbofuran is a carbamate pesticide, and terbufos and fenamiphos are organophosphate compounds. Both families of insecticides are low-persistence cholinesterase inhibiting insecticides. Cholinesterases are enzymes involved in the correct transmission of nerve impulses and their inhibition upsets the nervous system, causing death by respiratory arrest. This action mechanism makes these compounds very quick-acting neurotoxins capable of producing death after exposure of a few minutes. Organophosphate compounds usually cause death after exposures of 10 to 30 minutes with doses higher than LD50 and between 30 minutes and six hours at lower doses, although this may be prolonged up to 12 or even 24 hours for some latent cholinesterase inhibitors; death by exposure to carbamates usually occurs after exposure of 5-30 min. (Hill, 1995). Exposure to organophosphates in birds and mammals produces survival-threatening muscle paralysis, reducing their capacity of moving about depending on the doses and toxin they have been exposed to (Hill, 2003).

Interpretation: On the basis of the high toxicity of the compounds and the detected concentrations in the stomach contents, we conclude that the goshawks were intoxicated and the presence of bait with the same compounds suggests the poisoning was deliberate.

Definitive Conclusions

Both goshawk carcasses, in optimum bodily condition with sound plumage and normal body fat deposits, and the presence of food in the upper digestive tract, suggest that the death of the



animals was acute after ingesting some food, since any chronic process would have produced a telltale loss of bodily condition.

In goshawk carcass A the absence of projectiles, of fractures and other internal or external lesions rules out shooting or impact as direct cause of death.

In goshawk carcass B the presence of projectiles in the X-ray shows that the bird had been shot at. But the internal examination of this carcass showed that these projectiles are surrounded by healthy scar tissue, proving that the lesions were old and the shooting was not the cause of death.

The generalised congestive condition of some of the internal organs of both carcasses is fairly nonspecific but is nonetheless compatible with such processes as intoxication with anticoagulant substances (cumarin derivatives) or high doses of acetylcholinesterase inhibitors (carbamates and organophosphate compounds).

The necropsy findings, recorded in the preliminary report, together with the results of the toxicological analysis and the information culled by judicial agents show that the goshawks' death was produced by acute intoxication with **carbofuran, terbufos and fenamiphos**.

The presence of a large amount of granules in sample A (incomplete bird carcass), indicates the intentionality of introducing these particles into the carcass. The results of the toxicological analyses of said death A (N021/10 H and N021/10 I), confirm that particles introduced into the carcass contain **carbofuran, terbufos and fenamiphos**, which are the same compounds as those found in the contents of the upper digestive tract of both goshawk carcasses.

The conclusion that can be drawn from all the above is that the cause of death was primary intoxication as a direct consequence of consuming poisoned bait with the purpose of illegal non-selective predator control.

Place _____, on _____ (day) _____ (month) _____ (year)
Signed. Graduate.

Member of the professional association of veterinarians (*Colegio Oficial de Veterinarios*) of _____ with membership number _____.