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## Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

*The Wildlife Incident Investigation Scheme (WIIS) of England and Wales performs pesticide residue analysis in cases of suspected poisoning of wildlife and domestic animals. In this project a wide range of investigations were conducted into new and improved analytical methods for use in the laboratory and the field in support of the WIIS.*

### Objective 1

#### Validation of methods developed as part of the WIIS Investigations

There is an increasing requirement to analyse sample types or determine analytes not previously encountered by the WIIS and for which a need has not been foreseen. In many cases such samples have needed analysis at short notice. Validation of these new methods is important since high profile investigations and/or prosecutions may proceed based on the results. Work to validate several such methods was undertaken for the following applications: mepiquat in kidney, isofenphos in stomach contents, fungicides on treated grain, fluoxastrobin in avian tissues, chlorate in a white powder sample, chlorothalonil in bees, creosote in wood and grass samples and diquat in kidney tissue.

#### Analysis method for phosphine in soil

Suspected incidents involving fumigants such as phosphine to poison animals living in burrows are currently investigated using a field test. Such a procedure is suitable as a 'screening analysis' when the fumigant is still present in the air in high concentrations. However, in some circumstances, incidents may be missed because the gas has dissipated. Experience of analysing foodstuffs has shown that, despite its volatility, traces of phosphine do remain adsorbed to solid material for extended periods. A very sensitive method was successfully developed and validated to detect phosphine in soil, which will improve the capability of the WIIS to investigate such incidents.

#### New analytical methods for pesticides

The WIIS needs to have a range of pesticide analysis methods that is representative of current pesticide use. Where possible, analytes not currently covered by WIIS methods, have been incorporated into existing multiresidue methods, but in some cases completely new procedures were required.

#### Methods for Insecticides

New analysis methods were developed for the following applications: spinosad, spiromesifen and natural pyrethrins in honeybees, indoxacarb in honeybees and vertebrate liver, nicotine (and its main metabolite cotinine) in vertebrate liver, abamectin in honeybees and vertebrate liver.

### *Methods for Fungicides and Herbicides*

A list of herbicides and fungicides to include in the WIIS method development work was previously agreed. An existing method for fungicides was successfully extended to include azaconazole, and new methods were developed for propyzamide and dichlobenil in liver. Difficulties were encountered with dithianon and thiram determination in liver. Both analytes are unstable in the presence of raw liver, and neither could be determined as the intact pesticide. It was possible to detect thiram in liver as its transformation product, carbon disulphide. An existing method for carbon disulphide analysis in vegetable and fruit samples was successfully validated for liver. Unfortunately, a method for detecting the presence of dithianon in WIIS samples proved elusive.

### **Pesticide Metabolites**

WIIS methods have concentrated on the analysis of parent compounds as the best means of identifying the cause of a poisoning incident. However, in some cases, significant degradation of parent compound may have occurred as a result of metabolism or chemical reaction. The WIIS therefore requires methods for the analysis of these compounds to aid forensic interpretation of events, or identify poisoning.

### *Organophosphate metabolites*

Dialkylphosphate (DAP) metabolites of organophosphate pesticides are quite stable and there are reports of their detection in tissues months after death. A new ion chromatography – mass spectroscopy method to determine five DAPs in kidney samples was successfully validated and used to detect residues of mevinphos and diazinon DAPs in wildlife incident tissues. Improvement of the methodology and further work to measure DAP levels in WIIS samples is needed to understand typical DAP patterns in poisoning incidents.

## **Objective 2**

### **Evaluation of Liquid Chromatography – Time-of-Flight Mass Spectrometry (LC-TOF-MS)**

The high mass accuracy, resolution and dynamic range of TOF-MS can provide greater confidence in identification of analytes, even when there is considerable co-elution with matrix compounds. The reduced need to remove co-extractives may allow simpler sample preparation procedures without time-consuming clean-ups. Furthermore, high speed scanning enables acquisition of all masses over a wide mass range for the whole sample chromatogram, allowing retrospective re-analysis of data in the light of new information regarding potential analytes. Searching for transformation products of a suspected pesticide for example. The suitability of LC-TOF-MS for screening WIIS sample extracts was evaluated and sensitivity was found to be sufficient for many pesticides involved in vertebrate incidents, but not for rodenticides. The technique was not sufficiently sensitive to replace LC-MS/MS for screening honeybee samples, where very low detection limits are required for certain compounds. Further work re-analysing WIIS samples from incidents where pesticides have been found by established methods is needed, to assess this technique for identifying related compounds such as transformation products and co-formulants, which could help in interpretation of a poisoning scenario.

### **Accelerated Solvent Extraction**

Accelerated solvent extraction (ASE) is a sample extraction technique which uses pressurised extraction solvent, allowing use of temperatures above its boiling point. It can provide a very exhaustive extraction in a short time, when compared to the similarly exhaustive Soxhlet method. Experiments were undertaken to see if ASE could improve recovery of rodenticides (especially of the indanedione type) from liver. At high temperatures ASE does appear to extract more of these analytes, although this is offset by extraction of far more matrix material. This is likely to raise limits of detection, and require an improved clean-up. The ASE equipment was prone to blockages, and required regular maintenance. It was concluded that the possible benefits of using the ASE are outweighed by its cost and unreliability.

### **Direct Insertion Probe and Direct Exposure Probe for MS analysis of formulations and baits**

The conventional WIIS approach to analysing suspected baits and concentrated pesticide samples is to prepare an extract or solution from the sample and, after dilution, perform liquid or gas chromatography with MS detection. Samples of this type are usually associated with illegal pesticide use and analysis results are often key evidence in prosecutions. Rapid, reliable identification of active ingredients is important. It informs subsequent analyses of related samples and provides evidence for formal investigations. Initial identification of samples could be obtained faster in some cases by using a Direct Insertion Probe (DIP) or Direct Exposure Probe (DEP). With this technique, samples are introduced directly into the MS source then vaporised by rapid heating. The technique does not have the limitations created by the requirements of the gas or liquid chromatographic separation. GC-MS gives good qualitative confirmation in full-scan EI mode but is not amenable to low volatility, polar analytes. LC is the method of choice for polar analytes but LC-MS usually provides only limited mass spectral information and ionisation can be poor for non-polar compounds. Samples that are difficult to analyse by chromatography due to their thermal instability or chemical activity (e.g. acids and bases) can often be analysed directly by DIP or DEP. Both probes were evaluated for analysis of a variety of typical WIIS non-tissue samples, e.g. pesticide formulations such as strychnine and chloralose powders, metaldehyde or methiocarb pellets, rodenticide pellets/treated grain and aldicarb granules. Results showed that these techniques can provide

useful qualitative data for these samples types.

### **Objective 3**

#### **Vibrational spectroscopic methods of analysis – Raman spectroscopy**

WIIS investigations would be aided by rapid tests that provide tentative identification of pesticides in pesticide formulations in storage containers and as applied to “poison baits” or from spillages (e.g. “slug pellets”). Such tests might then require confirmation in the laboratory, but might provide ‘leads’ and expedite legal proceedings. Sampling can be hazardous in such circumstances, so an aid to identifying possible risks is also helpful. Raman spectroscopy is able to identify molecules by measuring wavelength changes in scattered laser-light, at frequencies that interact with the vibrational states of molecules. This method has not been adopted routinely for low-level analytical work because it lacks sufficient sensitivity. However it has potential advantages for analysis in WIIS investigations. Portable Raman devices exist which can be used to perform fast, non-destructive analysis of liquids and solids, without making physical contact with the sample. It is even possible in some circumstances, to make measurements through containers (translucent plastic or glass). This has obvious health and safety benefits. Two commercially available Raman systems were evaluated for their suitability as practical, portable, field-use devices for non-specialist operators to make tentative identifications of pesticide formulations. Results showed that the technology can, for some samples, provide reliable, accurate identification in a few minutes. A sufficiently robust and simple device is currently available, and would be a valuable tool for field use.

### **Objective 4**

#### **Chloralose**

The WIIS method for chloralose is complicated and involves many steps, including derivatisation for GC analysis. An alternative, published method for determining chloralose in liver, with analysis by LC-MS/MS, was evaluated for detection of chloralose in kidney samples. This alternative method is now in regular use by the WIIS.

#### **Development of an improved honeybee pesticides analysis method**

An integrated, targeted, multi-residue procedure for screening honeybees for a wide range of pesticides was developed and validated at a level of 1 ng per bee. This offers improved limits of detection for some analytes, a simplified analytical procedure and more efficient use of small samples. In some cases, small residues found with this screening method will require confirmation using existing methodology.

## **Project Report to Defra**

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8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
- the scientific objectives as set out in the contract;
  - the extent to which the objectives set out in the contract have been met;
  - details of methods used and the results obtained, including statistical analysis (if appropriate);
  - a discussion of the results and their reliability;
  - the main implications of the findings;
  - possible future work; and
  - any action resulting from the research (e.g. IP, Knowledge Transfer).

### **This report covers the following objectives in project PS2539:**

1. Development of methods for pesticides and metabolites not currently covered by WIIS
2. Evaluation of applicability of novel techniques for the WIIS
3. Development of rapid tests for use in the field or laboratory
4. Improvements to existing WIIS analysis methods

## REPORT ON OBJECTIVE 1

### Validation of methods developed by the WIIS as part of Wildlife Incident Investigation Scheme (WIIS) investigations

There is a requirement to devise methods *ad hoc* for WIIS incidents believed to involve unusual chemicals, where no method already exists. These methods require validation since they may be used in high profile investigations and/or prosecutions.

#### **Mepiquat in kidney**

An incident involving suspected mepiquat poisoning of a dog required rapid development of a suitable analytical method. Mepiquat, in common with chlormequat, paraquat and diquat, is a quaternary ammonium compound, and is highly soluble in water. In mammals, it is rapidly excreted via the kidney following exposure, so work concentrated on detecting mepiquat residues in kidney, although some work on stomach contents was also performed as oral ingestion was the most likely route of exposure. A rapid and simple extraction and preparation method for kidney tissue was developed, with determination of mepiquat and chlormequat by liquid chromatography with tandem mass spectrometry (LC-MS/MS). This was validated by fortifying control samples at 1mg/kg and 0.2 mg/kg. Average recoveries in kidney fortified at 1 mg/kg (n=5) were: mepiquat 96% (range 95 - 98%), relative standard deviation (RSD) 1.3%; chlormequat 93% (range 89 - 96%), RSD 2.9%. Average recoveries in kidney fortified at 0.2mg/kg (n=5) were: mepiquat 97% (range 94 - 99%), RSD 2.4%; chlormequat 95% (range 91 - 98%), RSD 2.6%. Recoveries (n=1) for mepiquat in fortified stomach contents were: 99% at 10 mg/kg, 109% at 5 mg/kg, and 97% at 1mg/kg. Limits of detection (LOD) for kidney were estimated to be 0.05 mg/kg for mepiquat chloride and 0.02 mg/kg for chlormequat chloride.

#### **Isofenphos**

Scottish wildlife poisoning incidents reported by Science and Advice for Scottish Agriculture (SASA), reveal the use of carbofuran formulations that contain the organophosphorus (OP) pesticide isofenphos. To ensure that an adequate method is available to the WIIS to detect isofenphos residues, a method currently used to determine OPs was validated for this compound. Stomach contents fortified at 1 mg/kg gave an average recovery (n=5) of 98% (range 93 – 102%), RSD 3.9%.

#### **Fungicides on treated grain**

A method for determining Ergosterol Biosynthesis-Inhibiting (EBI) fungicides on treated grain (including fluquinconazole) using gas-chromatography with mass spectroscopy (GC-MS) was developed and validated at 1 mg/kg. A portion of grain (1 g) was vortex-mixed in 10 ml of methanol (plus one drop of water, which helps to release the more polar residues from the grain matrix) for one minute, followed by ultrasonication for two minutes. An aliquot (2.0 ml) was evaporated to dryness, and re-dissolved in ethyl acetate (1.0 ml) for analysis with matrix-matched standard solutions. For validation, grain was spiked with the analytes in a methanol solution and was left overnight at room temperature for spiking solvent to evaporate fully. It appears that prothioconazole breaks down to prothioconazole-desthio under these conditions. The limit of detection was estimated to be approximately 0.05 mg/kg for each analyte.

**Table 1. Validation results for control sample spiked at 1mg/kg (n=5)**

| Analyte                  | Recovery Range (%) | Average (%)   | RSD (%) |
|--------------------------|--------------------|---------------|---------|
| cyproconazole            | 81 - 86            | 83            | 2.2     |
| difenoconazole           | 78 - 86            | 81            | 4.5     |
| fluquinconazole          | 74 - 79            | 76            | 2.6     |
| flusilazole              | 74 - 86            | 83            | 3.4     |
| imazalil                 | 74 - 90            | 81            | 7.7     |
| myclobutanil             | 80 - 88            | 83            | 3.8     |
| penconazole              | 81 - 88            | 85            | 3.1     |
| prochloraz               | 62 - 77            | 71            | 8.7     |
| propiconazole            | 86 - 95            | 89            | 4.2     |
| prothioconazole          |                    | not detected* |         |
| tebuconazole             | 83 - 89            | 85            | 3       |
| triadimefon              | 82 - 89            | 84            | 3.4     |
| triadimenol              | 87 - 94            | 90            | 3.4     |
| *prothioconazole-desthio | 23 - 26            | 24            | 4.2     |

\*prothioconazole undergoes breakdown to prothioconazole-desthio on grain.

This new method was used successfully to detect the presence of fungicides residues in three grain samples from two incidents. Prothioconazole-desthio (implying the initial presence of prothioconazole) and fluquinconazole were identified in one grain sample, and fluquinconazole and prochloraz were identified in the others.

### **Fluoxastrobin in greenfinch tissues**

A method was developed using liquid chromatography with tandem mass spectroscopy (LC-MS/MS) for the determination of fluoxastrobin in liver and gizzard (including contents). The method was suitable for the very small sample sizes available. Liver and gizzard with contents were ground with anhydrous sodium sulphate and extracted by shaking in dichloromethane (DCM). Aliquots of extracts (25 ml) were evaporated to dryness and re-dissolved in methanol (1.0 ml), filtered through a 0.2 µm PTFE syringe filter, and analysed by LC-MS/MS. Two liver samples spiked at 10 µg/kg gave recoveries of 74 and 82%. The identity of residues was confirmed using ion ratios (transitions 459>427 and 223>126 m/z). The method proved capable of identifying and confirming the presence of fluoxastrobin in the greenfinch at very low levels: 0.56 µg/kg in liver and 2.9 µg/kg in gizzard with contents. Interestingly, the method was also able to detect a residue of 4.3 ng fluoxastrobin on the surface of the finch's beak (in a DCM surface wash). Limits of detection were estimated to be 0.4 µg/kg in liver and 0.2 µg/kg in gizzard with contents. A method was also developed and successfully employed for the determination of fluoxastrobin at higher levels in a grain sample, using the same extraction and sample preparation as described above in 'Fungicides on treated grain', but using LC with UV diode array detection.

### **Identification of chlorate in a white powder sample**

A sample of white powder believed to be a sodium chlorate weed killer formulation, was submitted to the WIIS for identification. Similar samples have occasionally been analysed in the past, but only qualitative, colorimetric chemical tests, prone to interference, were available. A quantitative and highly selective method was developed for the determination of sodium chlorate in this sample. Since chlorate is an anion, application of ion chromatography (IC) with conductivity detection (Dionex 3000) was initially investigated, but the analyte signal to noise was poor. However, using the IC system interfaced with a tandem mass spectrometer (Sciex API2000) provided good sensitivity. Aqueous solutions of the sample were filtered and analysed directly. The ion transition 83>67 m/z was used for calibration, and confirmation was by ion ratio (transitions 83>67 and 83>51). Good linearity of response, and peak symmetry was obtained over the calibration range used. The lowest detectable concentration was estimated to be 5 ng/ml.

### **Chlorothalonil in bees**

A method was developed for the determination of chlorothalonil in bumblebees as part of a WIIS incident. The method employed Soxhlet extraction using diethyl ether, which is routinely used for analysis of bees in the WIIS. A clean-up using silica solid phase extraction (SPE), screening by GC with electron capture detector (ECD), and confirmation by GC-MS was devised, with a view to possibly including chlorothalonil in a multi-residue method for bees that was in development as part of this project. It was found that chlorothalonil elutes from the silica SPE cartridge in the same fraction as the pyrethroid insecticides, and can be simultaneously determined using the GC-ECD screening conditions used for pyrethroids. Alternatively, it could probably be included in the multi-residue screening method described in Objective 4, although the timing of this incident meant that chlorothalonil is not included in the validation data reported in that objective. A procedural recovery of 96% was obtained at the level of 2.5 ng per honeybee (no control bumblebees were available for method development), with estimated limits of detection of about 1 ng per honeybee.

### **Qualitative determination of creosote by GC-MS (Ion Trap) in wood and grass samples**

A method to identify creosote residues on grass and wood samples was developed. Coal tar creosote is a complicated mixture of hydrocarbons (predominantly polyaromatic hydrocarbons). Guillen *et al* [1] identified around 200 separate compounds, including many isomers, in creosote. The relative amounts of these different compounds are variable, making accurate measurement of residues extremely difficult. An old sample of coal tar creosote was obtained to act as a reference material (the product is no longer sold in the UK), and was characterised by GC-MS using an ion trap detector in full-scan mode. DCM was found to be a suitable solvent for the creosote reference material. Extracts of a wood sample and a grass sample were prepared and analysed directly, after suitable dilution.

In order to simplify matters, several representative compounds were determined in the samples, and a semi-quantitative measurement was made, based on those components. The mass spectra of six different compounds were identified in the chromatograms of the reference material and in the two sample extracts, confirming the presence of creosote. Nearly all of these compounds had several structural isomers at similar retention times that produced a characteristic pattern of peaks in the chromatogram. The identified compounds were naphthalene (128 m/z), methyl-naphthalene (142 m/z), dimethyl-naphthalene (156 m/z), trimethyl-naphthalene (170 m/z), phenanthrene or anthracene (178 m/z) and methyl-phenanthrene (192 m/z), and were all present in the description of coal tar pitch distillate by Guillen *et al* (the ions used for semi-quantitative measurement are listed in brackets).

### **Diquat in kidney tissue**

A method used in the WIIS for determination of paraquat was successfully validated for diquat. The method oxidises the quaternary amine analyte, to produce a dipyrindone compound that can be determined by LC with fluorescence detection. Although it was known that the chemistry involved should allow the method to be used

for diquat determination, this had not previously proved possible in practice due to an interfering peak in the chromatograms obtained. The problem peak was eventually traced to one of the reagents, in which the interfering compound was formed over a short period after preparation. To avoid the problem, the reagent (the oxidant) needs to be made freshly before each oxidation is performed. The procedure was subsequently validated for diquat at a level of 0.5 mg/kg in spiked kidney tissue, and gave recoveries in the range 54 to 68% with an average recovery of 64% (n=5), RSD 8.6%. The limit of detection was estimated to be 0.08 mg/kg.

### **Phosphine in soil**

The illegal use of phosphine to poison burrowing animals such as rabbits and badgers is currently investigated using a field test. This field test can detect phosphine gas present in the air inside burrows, or from the remains of decomposing aluminium phosphide tablets. Such an approach is suitable if the incident is investigated within hours of occurring. However, in some circumstances incidents may be missed because the gas has escaped and concentrations in the burrow are too low to detect using the field test. It is known however, that very small amounts of phosphine can be adsorbed onto solid materials during fumigation, which can be detected many days later by gas chromatography after controlled desorption in a sealed container. This approach was investigated for soil samples. Using acid digestion of the soil, and headspace analysis by gas chromatography with a nitrogen/phosphorous detector (GC-NPD), a highly sensitive method for detecting phosphine in soil was developed and validated. Average recovery at 0.005 mg/kg (n=5) was 79% (range 73 – 85%) with an RSD of 5.6%. The limit of detection was estimated to be 0.5 µg/kg.

### **New analytical methods for parent pesticides**

#### **Methods for insecticides in honeybees: spinosad, indoxacarb, spiromesifen, pyrethrins and abamectin**

These insecticides vary from moderately toxic to bees (spiromesifen) to highly toxic (spinosad). All of these compounds have low to very low solubility in water due to their non-polar nature, but are not volatile enough, or are too unstable for GC analysis. As they have similar analytical requirements, it was decided to attempt to develop a multi-residue method with LC-MS/MS as the determination step. Suitable LC-MS/MS methods for abamectin (avermectins B1a and B1b), pyrethrins I and II, spinosad (spinosyn A and D), spiromesifen and indoxacarb were established, monitoring two ion transitions for each analyte.

WIIS honeybee samples are currently extracted using Soxhlet apparatus with diethyl ether. This is an exhaustive extraction technique, giving good recoveries for most analytes, even if only moderately soluble in diethyl ether, but requires long extraction times (several hours) and uses large amounts of water and electricity. As an alternative, shaking the ground and dried sample in a suitable solvent and then filtering, was investigated. Various solvents were tested for their ability to solvate both the analytes of interest and the waxy deposits in the ground honeybee bodies that could contain non-polar pesticide residues. DCM and toluene were found to be the only solvents that adequately dissolved beeswax, and since toluene has a relatively high boiling point and is time-consuming to evaporate, DCM was selected for further work. Diatomaceous earth (Hydromatrix) made a very good drying agent for preparing the sample for extraction as its abrasive properties aided breakdown of the tough honeybee exoskeleton. It was also an effective dispersant, providing a large surface area for contact with solvent during extraction.

Low detection limits were needed for the compounds most toxic to honeybees (spinosad and abamectin) so a clean-up with a concentration step was required. Various solid-phase extraction (SPE) cartridges were evaluated. Normal-phase (alumina, silica and Florisil) clean-up of DCM extract was only partly successful due to inter-analyte variation in retention and overlapping elution of coextractives. Reverse-phase adsorbents were therefore evaluated. DCM is not a suitable solvent in which to apply extracts to reverse-phase adsorbents and it was necessary to dissolve the extract in a more polar solvent. Simply evaporating the DCM aliquot to dryness created a solid waxy deposit that could not be re-dissolved in reverse-phase-suitable solvents (water, acetonitrile or methanol), potentially trapping analytes. Instead an aliquot of DCM extract was co-evaporated with 2ml of acetonitrile until the volume was between 0.5 and 1 ml. Reverse-phase, silica-based C8, C18, C18 (end-capped) and primary-secondary amine, and polymeric divinylbenzene-co-N-vinylpyrrolidone (with and without anion-exchange functionality) were evaluated. The best wax/lipid retention and analyte recovery was achieved using an end-capped C18 cartridge. Simply passing the acetonitrile solution through the cartridge was found to remove between 70 and 90% by weight of the extract residue (for 3 different honeybee samples tested) with high recovery of all the analytes.

The above extraction and clean-up for honeybee samples fortified with these analytes at the equivalent of 5 ng/bee gave the following recoveries (n=3):

|                 |              |                |             |              |             |
|-----------------|--------------|----------------|-------------|--------------|-------------|
| abamectin (B1a) | 94, 81, 84%  | pyrethrin (I)  | 87, 96, 90% | spiromesifen | 85, 94, 99% |
| abamectin (B1b) | 105, 97, 88% | pyrethrin (II) | 84, 94, 86% | indoxacarb   | 89, 90, 88% |

Spinosad was found to be too strongly retained on C18 to be eluted with acetonitrile and could not be determined with this method. However, elution with a further SPE fraction of acetonitrile:methanol (1:1) with 1% triethylamine eluted it completely. Alternatively, spinosad could be detected without the use of a clean-up by simply filtering the acetonitrile-reconstituted extract prepared as described above. Dispensing with the clean-up caused faster contamination of the chromatography column, but this method gave recoveries of 76, 86, 96% for spinosad(A) and 82, 83, 99% for spinosad(D) at 5 ng/bee. The procedure with clean-up gave estimated limits of detection of approximately 0.5 ng/bee for indoxacarb and spiromesifen, 1 ng/bee for pyrethrin(I), pyrethrin(II) and abamectin(B1a), and 5 ng/bee for abamectin(B1b). The filtration only method for spinosad gives estimated limits of detection of about 2 ng/bee for both spinosyn A and D.

The detection limits for abamectin and spinosad might not be low enough to detect all concentrations of interest in poisoned bees. Extoxnet (Oregon State University) quotes an acute contact LD<sub>50</sub> of 2 ng/bee for abamectin, and the Pesticide Manual quotes an acute LD<sub>50</sub> of 2.9 ng/bee for spinosad (e-Pesticide Manual, Version 4.0, British Crop Protection Council). Ideally detection of about one tenth this level would be desirable. Further work is planned to try and improve the detection limits for spinosad and abamectin in honeybee samples. This extraction method and simple wax/lipid removing clean-up step was developed further in Objective 4 of this report, to form a multi-residue screening procedure for honeybees.

### ***Nicotine in vertebrate tissues***

The naturally occurring plant alkaloid nicotine is still used as an insecticide in some commercial horticultural applications and is highly toxic to vertebrates. It is used as either a fumigant (free base form) or as a spray (sulphate salt). It is a rapidly acting poison that is easily absorbed through skin and mucous membranes in its free base form, and more slowly from the stomach where it is present in its ionised form. It is predominantly metabolised by the liver. The nicotine molecule has similar acid-base properties to strychnine, another alkaloid occasionally identified in wildlife poisoning incidents. The strychnine analysis method currently used in WIIS was investigated for its suitability for the determination of nicotine. The method gave poor recoveries and the liquid/liquid partitioning step was often slow, with troublesome emulsions often forming. Consequently an alternative method was developed, with extraction by homogenisation in aqueous 5% trichloroacetic acid followed by cation exchange SPE. Both a LC-MS/MS and a gas chromatography-mass spectrometry (GC-MS) determination for nicotine were devised, but the GC-MS method gave better sensitivity. The GC-MS method was validated for nicotine and its main metabolite cotinine by analysing control samples spiked at 5 mg/kg (nicotine only) and 0.5 mg/kg (nicotine and cotinine). The average nicotine recovery at 5 mg/kg was 96% (range 91 – 99%, n=5) with RSD of 3.3%. Average recoveries at 0.5 mg/kg were: nicotine 82% (range 69 to 98%, n=5), relative standard deviation (RSD) 16.4%; cotinine 84% (range 75 to 94%, n=5), RSD 10.9%. Limits of detection for nicotine and cotinine were estimated to be 0.1 to 0.25 mg/kg. Nicotine response and peak-shape was negatively affected by the slightest deterioration of GC column condition, and matrix contamination from previously injected samples was a problem. Despite this, the method is considered to perform well enough for detection of acute poisoning in vertebrates. The sum of the response for the ions 176, 147 and 98 m/z was used for calculating cotinine recoveries, and the sum of the response for the ions 133 and 161 m/z was used for calculating nicotine recoveries. Summed ion responses gave better data using an ion trap mass spectrometer.

As a by-product of the work done during development of an LC separation for nicotine using ultraviolet-diode-array detection, an improved chromatographic method for strychnine was established. This raised the possibility of a multi-residue method that would prove suitable for both nicotine and strychnine determination in liver. Initial experiments indicated that the extraction and clean-up procedures developed for nicotine are also suitable for strychnine. However it did not prove possible to develop a simultaneous LC determination step with optimised sensitivity for both analytes, due to their very different reverse-phase retention.

### ***Indoxacarb in vertebrates***

A method for indoxacarb was developed in which liver was extracted by grinding with sodium sulphate drying agent and shaking in DCM. Portions of extract equivalent to 0.5 g of tissue were evaporated onto 100 mg of loose primary-secondary amine solid-phase extraction (SPE) adsorbent, then transferred with acetonitrile rinses to perform a reverse-phase clean-up by passing through a C18 (end capped) SPE cartridge. The eluate was evaporated to dryness under a stream of nitrogen, redissolved in 1 ml of ethyl acetate and analysed by GC-MS using an ion-trap detector with matrix-matched calibration solutions. Reconstructed ion chromatograms for ions 264, 218 and 499 m/z were used to measure recoveries. To validate the method chicken liver was spiked at 0.5 mg/kg. This is well below the acute oral LD<sub>50</sub> values quoted in the e-Pesticide Manual (14<sup>th</sup> Edition) ver. 4.0, (e.g. bobwhite quail 98 mg/kg body weight, female rats 268 mg/kg). Average recovery was 91% (range 77 to 100%, n=5), RSD 9.6%. Limit of detection was estimated to be 0.05 mg/kg.

### ***Abamectin in vertebrate tissues***

Abamectin is of fairly high toxicity to vertebrates, the acute oral LD<sub>50</sub> for rats is 10 mg/kg (e-Pesticide Manual, Version 4.0, British Crop Protection Council). A review of abamectin analysis methods [2] indicated that the most suitable tissue for analysis would be liver. The low volatility of abamectin dictates a LC determination stage. In order to obtain the necessary sensitivity without the complexity of the derivatisation and fluorescence detection



methods described in the literature, an LC-MS/MS approach was investigated. The advantages of unequivocal identification and simplicity of sample preparation outweighed the possible issues of matrix effect. Abamectin consists of  $\geq 80\%$  (by weight) avermectin B1a, with the balance consisting mostly of avermectin B1b. A simple method consisting of extraction of liver samples with acetonitrile, dilution, and direct analysis without clean-up was validated at levels of 0.1 and 1 mg/kg for both avermectin B1a and B1b in liver. Matrix effects were not significant at the extract concentrations used, so solvent-only calibration solutions could be used. Average recoveries at 0.1 mg/kg were: B1a 102% (n=2), RSD of 6.1%; B1b 106% (n=2), RSD of 4.4%. Average recoveries at 1 mg/kg were: B1a 82% (n=2), RSD of 9.5%; B1b 128% (n=2), RSD of 6.1%. The limits of detection for the method were estimated to be about 0.02 mg/kg (B1a) and 0.007 mg/kg (B1b).

## Methods for fungicides and herbicides

### **Azaconazole**

Azaconazole is a fungicide with relatively high toxicity to mammals (acute oral LD<sub>50</sub> dogs 114 – 136 mg/kg, e-Pesticide Manual, British Crop Protection Council). An existing method (DCM shaking extraction followed by high-performance gel-permeation chromatography, then Florisil SPE and determination by GC-MS) for determination of similar fungicides in liver was validated for this compound. Average recovery at 0.5 mg/kg (n=5) was 84% (range 83 – 85%) RSD=1.1%. The limit of detection was estimated to be approximately 0.05 mg/kg.

### **Dichlobenil**

A GC-Electron Capture Detector (ECD) method and confirmatory GC-MS method were developed and validated for determining the herbicide dichlobenil in liver. Samples were extracted with diethyl ether using Soxhlet apparatus and extracts were cleaned up using neutral-alumina SPE cartridges. Average recovery at 5 mg/kg (n=5) measured by GC-ECD was 95% (range 90 – 102%) RSD 4.8%. The LOD was estimated to be approximately 0.5 mg/kg.

### **Propyzamide**

A method was developed in which liver was extracted and cleaned-up in the same manner as described above in the indoxacarb method. Determination was by GC-MS using an ion-trap detector with matrix-matched calibration solutions. Chicken liver was spiked at 5 mg/kg, (cf. the acute oral LD<sub>50</sub> for Japanese quail of 8770 mg/kg body weight and for female rats 5620 mg/kg, e-Pesticide Manual, 14<sup>th</sup> Edition, ver. 4.0). Average recovery (n=5) was 84 % (range 79 to 88%), RSD 4.4 %. Limit of detection was estimated to be 0.5 mg/kg.

### **Epoxiconazole (and other EBI fungicides) in honeybees by LC-MS/MS**

See objective 4 - Development and validation of a rapid extraction and integrated clean-up scheme for honeybee screening.

### **Diathianon**

Dithianon is a fungicide used as a spray on fruit. It is relatively toxic, with Oral Acute oral LD<sub>50</sub> for rats 300, guinea pigs 115 mg/kg, male quail 430, female quail 290 mg/kg (e-Pesticide Manual, ver. 4.0). Published literature indicated that the best tissue for analysis was liver. Two extraction procedures were evaluated. The DCM shaking extraction regularly used in the WIIS, and a QuEChERS (“Quick, Easy, Cheap, Effective, Rugged, Safe”) [3] extraction procedure often used to determine pesticides, including, dithianon in fruit and vegetables. LC-MS/MS conditions for determination of dithianon were developed, monitoring ion transitions 296>264, 296>238 and 296>164 m/z., which gave excellent sensitivity and linearity over a wide range of concentrations. Despite various attempts at extracting and determining dithianon in spiked tissues, a reliable method proved elusive. Recoveries were typically less than 1% at a spiking level of 1 mg/kg using either extraction technique. The LC-MS/MS method devised can be used to measure dithianon in formulation samples and poison baits, but it seems the analyte is too rapidly degraded to be detected in spiked tissues.

### **Analysis of WIIS vertebrate samples for thiram residues**

While the fungicide thiram is of relatively low toxicity to vertebrates, it is widely used in seed treatments and ground sprays, on a diverse range of crops. A need was identified for a method to detect thiram in wildlife tissues or suspected baits and formulations. The e-Pesticide Manual (ver. 4.0) reports an acute oral LD<sub>50</sub> of 2600 mg/kg for rats, 1500-2000 mg/kg for mice, and 210 mg/kg for rabbits; and 673 mg/kg for male ring-necked pheasants, >2800 mg/kg for mallard ducks, and >100 mg/kg for starlings and redwing blackbirds. Assuming that these data are broadly representative for other animals and birds, residues from thiram poisoning incidents might be expected to hundreds to thousands of mg per kg body weight. To allow for possible residue losses after exposure, method development was aimed at detecting levels of at least 10 to 20 mg/kg of tissue or sample. Initial investigations concentrated on the possibility of detecting thiram in stomach contents but experiments indicated that thiram spiked at 20 mg/kg was decomposed before it could be detected. Radio-labelled thiram studies described in an International Programme on Chemical Safety (IPCS) pesticide monologue (No. 853; <http://www.inchem.org/>) indicated that the largest residues might occur in liver tissue. Also, an EC paper on thiram (Health & Consumer Protection Directorate-General, Thiram, 6507/VI/99-Final, 27 June 2003,

<http://ec.europa.eu/>) suggests that the livers of dogs exposed to thiram can contain small amounts of the intact analyte. Consequently, it was decided to develop a method to identify thiram in liver, rather than stomach or gizzard contents.

Conditions for detection of thiram, using LC-UV-DAD were established. Excellent chromatography was achieved using a simple methanol/water gradient. This method could be used to identify thiram in suspected baits or formulations. Solubility of thiram in DCM, and octanol/water partition coefficients indicated that the standard WIIS shaking extraction for liver should be suitable. However, when chicken or pig liver samples were spiked at 20 mg/kg, no thiram was detected in the extracts. Further investigation showed that thiram was decomposed by contact with liver tissue. This instability in liver made it rather unlikely that thiram could be found as an intact residue under the typical conditions of a wildlife incident, where there are often several days between poisoning and reception of tissue samples by the WIIS.

The problem of dithiocarbamate instability is also an issue for the routine pesticide screening of fruit and vegetables. The pragmatic approach adopted for this, is to completely decompose residues of parent fungicides and their intermediate transformation products, to produce carbon disulphide. This is then measured by GC-MS. It was decided to evaluate this method for liver tissue samples. Use of this method by the WIIS would not give information about the particular dithiocarbamate fungicide(s) originally present, but in combination with other circumstantial evidence, such as LC-UV-DAD analysis of associated bait or formulation samples, it could be useful, and it might provide some explanation of cause of death.

#### ***Carbon disulphide determination in liver samples***

A well established analytical method for detecting the dithiocarbamate fungicide breakdown product, carbon disulphide (CS<sub>2</sub>), was evaluated for liver samples. Spiked liver samples are extracted with iso-octane in the presence of 1.5% (w/v) tin (II) chloride in 5 molar hydrochloric acid solution at 80 °C. All dithiocarbamate fungicide residues are decomposed to CS<sub>2</sub> under these conditions. Analysis was by GC-MS (Ion Trap) operated in full-scan mode from 40 to 80 amu. The ion used for quantitation was 76 m/z. Spiking tissue with thiram at 11 mg/kg (n=4) the average recovery of CS<sub>2</sub> was 85% (range 81 - 91%), RSD 5.2%.

### **Pesticide Metabolites**

#### **Organophosphate metabolites - dialkylphosphates**

##### ***Introduction***

Although largely replaced by newer, safer classes of pesticide, organophosphates (OPs) are still found as residues in animals, birds and poison baits submitted to the WIIS. Since OPs are fast acting poisons, the sample of choice is stomach or gizzard contents, where residues are normally highest. However, total amounts found in these samples are highly variable and are sometimes less than would be expected from extrapolation of acute oral LD50 data. OP residues also decrease as a result of sample decomposition prior to analysis. Data reported in Project PR1150 (WIIS method development 2000 to 2003) show that the level of detectable OP residues on animal tissue remains stable until decomposition starts. Then levels decrease rapidly, presumably as they are metabolised by bacteria into other compounds. Dialkylphosphates (DAPs) are transformation products of OPs. They are produced by metabolism, and by hydrolytic chemical reaction. DAPs are acidic, polar, water-soluble compounds, and are often monitored in the urine of workers occupationally exposed to OP. There is also some evidence in the literature that DAPs can be detected in the body tissues of poisoning victims, long after the parent OP has become undetectable [4]. Clearly it would be advantageous for the WIIS to have a method for detecting DAPs in the tissues of OP-poisoned animals and birds. Existing DAP analysis methods involve either liquid chromatography or derivatisation prior to gas chromatography. The derivatisation methods used tend to be complicated, and the LC methods involve ion-pairing reagents which also create compatibility issues for other analyses on the same equipment. Since DAPs are acids and are ionic at neutral pH, a method based on ion chromatography (IC) was investigated.

##### ***Experimental***

It was possible to find commercial sources for reference materials of five of the six potential DAPs formed by common OPs. The compounds obtained cover most of the potential transformation products from OPs routinely found in WIIS samples. If the sixth compound becomes available, it should be possible to add it to the method reported here. Due to the polar nature of DAPs, they are primarily excreted via the kidney. For this reason, and because it generally contains less fat than liver, kidney was chosen for analysis. Pig kidney was spiked at 0.5 mg/kg with O,O-dimethylphosphate (DMP), O,O-diethylphosphate (DET), O,O-dimethylthiophosphate (DMTP), O,O-diethylthiophosphate (DETP) and O,O-diethyldithiophosphate (DEDTP) to estimate extraction efficiency. As well as analysing spiked kidney samples, five kidney samples from animals and birds where OP residues had been detected were analysed. Samples were homogenised in acetonitrile, then centrifuged. Portions of extract were evaporated to dryness and the residue re-suspended in water, filtered, and analysed by ion chromatography – tandem mass spectrometry (IC-MS/MS) with electrospray ion source in negative ion mode. The IC was

performed on a Dionex AS24 high capacity anion exchange column in isocratic mode. A Metrohm chemical suppression system was used. Injection volume was 1 ml.

**Table 2. Dialkylphosphate ion transitions**

| DAP           | DMP     | DEP     | DMTP    | DETP    | DEDTP   |
|---------------|---------|---------|---------|---------|---------|
| Trans 1 (m/z) | 125>63  | 153>79  | 141>126 | 169>95  | 185>79  |
| Trans 2 (m/z) | 125>79* | 153>124 | 141>95* | 169>141 | 185>111 |

\*interference from ethyl analogue

### Results

Average recoveries from spiked pig kidney were in the range 42 to 76%. Stability of instrument response was acceptable, and sensitivity was good. Limits of detection were estimated to be 0.02 mg/kg in kidney, without correction for recovery.

**Table 3. Recoveries spiked at 0.5 mg/kg**

| DAP   | Ion transition | R <sup>2</sup> | Recovery  |         |         |
|-------|----------------|----------------|-----------|---------|---------|
|       |                |                | Range (%) | Ave (%) | RSD (%) |
| DMP   | 125>63         | 0.9702         | 33 - 53   | 42      | 17      |
| DEP   | 153>79         | 0.9110         | 61 - 90   | 76      | 17      |
| DMTP  | 141>126        | 0.9337         | 60 - 71   | 68      | 6.7     |
| DETP  | 169>95         | 0.8453         | 55 - 69   | 63      | 8.7     |
| DEDTP | 185>111        | 0.7979         | 22 - 60   | 47      | 31      |

**Table 4. Analysis of WIIS samples known to have been exposed to OPs**

| Species      | Parent residue |        |       | Metabolite (mg/kg kidney) |          |          |
|--------------|----------------|--------|-------|---------------------------|----------|----------|
|              | Compound       | Tissue | mg/kg | DMP                       | DETP     | DEP      |
| crow (c)     | mevinphos      | GC     | 56    | 0.31 (b)                  | <0.02    | <0.02    |
| dog (c)      | mevinphos      | SC     | 390   | 0.15 (a)                  | <0.02    | 0.11 (b) |
| red kite (d) | mevinphos      | GC     | 3     | 3.89 (a)                  | <0.02    | <0.02    |
|              | diazinon       | GC     | 0.11  | <0.02                     | <0.02    | <0.02    |
| raven (d)    | diazinon       | GC     | 10    | <0.02                     | 0.04 (a) | 0.33 (b) |
| red kite (d) | diazinon       | GC     | 47    | <0.02                     | 4.92 (a) | 1.00 (a) |

(a) confirmed by ion ratio, (b) not confirmed by ion ratio, (c) 2008 sample, (d) 2009 sample

### Discussion

The method presented is very simple and provides recoveries that are comparable with those from much more complicated methods in the literature. Co-elution of DMP and DEP and DMTP and DETP means that care must be taken in interpreting data. Fragmentation of the ethyl compounds in the source give rise to an ion of the same mass as the ion used in one of the transitions for the co-eluting methyl compounds. This produces the same daughter ion (see Table 2; 79 m/z, DMP and 95 m/z, DMTP). It may be possible to improve the chromatography by using a gradient elution, making interpretation easier.

There is very little data for DAP residues in kidney in the scientific literature, but one source [5] reports finding DEP at 0.05 to 0.29 ppm (mg/kg) in kidneys and livers of pigeons exposed to parathion (0.6 mg/kg) and diazinon (2 or 3 mg/kg). Acutely poisoned animals and birds are often exposed to levels much higher than this, judging by residues found in stomachs and gizzards, so the limit of detection for this method should be adequate for most WIIS samples. Analysis of WIIS samples from known OP incidents show that this method can detect real, incurred residues, even after two years of storage. In all cases, the identities of the confirmed residues agree with the theoretical DAPs produced from the known OP involved.

### Conclusion

These initial results are very encouraging, especially finding OP metabolites in the kidneys of poisoned animals and birds, which shows that the pesticides found in their stomachs and gizzards were certainly absorbed. Further work to establish typical DAP levels from OP poisoning and improve the methodology should be undertaken.

## REPORT ON OBJECTIVE 2

### Time Of Flight Mass Spectroscopy (TOF-MS)

The latest bench-top TOF-MS systems with mass accuracy of up to two parts per million (ppm), three to four orders of magnitude of dynamic range and high resolving power and scan rates, can potentially offer several advantages over widely used quadrupole or ion-trap MS technology. Higher mass accuracy, resolution and dynamic range can provide greater confidence in identification of analytes, making identification possible even when there is considerable co-elution with matrix compounds. The reduced need to remove co-extractives may allow the use of simpler sample preparation procedures, perhaps without time-consuming clean-ups. In the case of LC or GC hyphenated TOF, fast scanning allows acquisition of a wide mass range (e.g. 50 to 1000 amu) over the entire chromatographic separation, without loss of sensitivity. By comparison, quadrupole and tandem-quadrupole instruments are only sensitive enough for residue analysis when operated in a highly targeted way, acquiring only data for pre-selected analytes of interest. The TOF-MS enables acquisition of a 'complete' data set, allowing un-targeted screening and retrospective re-analysis of the data if required. This could offer an advantage for WIIS samples, where the pesticides responsible for poisoning incidents are unknown, or when compounds other than the suspected target compounds may be present.

It was proposed that an LC-TOF-MS system be assessed as a means of screening vertebrate and honeybee sample extracts for a range of pesticides, including neonicotinoid insecticides, OP insecticides, carbamate insecticides and anticoagulant rodenticides.

#### **Experimental**

Crude extracts of honeybees and liver samples were spiked with pesticides at various concentrations that were representative of the levels required to be detected by WIIS screening methods (limits of detection). Some extracts that had undergone a clean-up were also spiked at the same levels for comparison. Only cleaned-up honeybee extracts could be re-dissolved in solvent suitable for LC, so only results for these are reported.

A gradient LC programme using 10 mM ammonium acetate (solvent A) and methanol (solvent B) at 0.2 ml/min was used on an Agilent 6224 TOF Mass Spectrometer with dual electrospray ionisation (ESI) ion source was used in positive and negative modes. A mass range of 50 to 1000 amu was acquired at one scan per second in both positive and negative ion modes.

#### **Results**

Optimum ionisation modes and identity of the ions formed for each compound were established first, using high concentration single component standard solutions. The majority of analytes currently included in WIIS screening standard solutions gave best results in positive ion mode. The exceptions to this rule were the anticoagulant rodenticides and alpha-chloralose, which gave best response in negative ion mode. Some of the analytes studied produced adducts in the source, depending on the chemistry of the mobile phases used (see ion species, (Table 5).

The measured mass for an analyte increasingly deviated from the theoretical mass as signal to noise decreased. A narrow and relatively selective mass window for signal extraction could produce an extracted ion chromatogram (EIC) with good signal to noise at one calibration level, but at the next lower level the analyte might be undetectable unless a wider mass window is used. This results in less selective data extraction, and includes more signal from coextractives. In practice, some compromise has to be struck between selectivity of mass window and limit of detection. A mass accuracy window of 20 ppm was used for the results reported below (Tables 5 and 6).

#### **Discussion**

Limits of detection found for vertebrate livers without clean-up were generally adequate for screening WIIS samples for organophosphates, carbamates, and neonicotinoids, but not quite sufficient for the more demanding rodenticides, even with the currently used clean-up. LODs were not found to be low enough for screening honeybee samples. Abamectin, chloralose, fipronil, natural pyrethrins, spinosad, indoxacarb and triazole fungicides also were detectable in vertebrate liver solutions at suitable levels for screening. The matrix removed by clean-up seemed not to make a substantial difference to detection limits. A number of pyrethroid insecticides were investigated but only tetramethrin gave acceptable results in liver matrix. Metaldehyde and phorate were not detected in any matrix solutions at the levels analysed. Despite the mass accuracy of up to 2 ppm, measured masses often deviate from the theoretical at low levels, and for a many analytes, the optimised ES source conditions produce very little fragmentation. This means that a

**Table 5. Detection limits for a range of analytes in honeybee and liver matrix**

| Analyte              | MW (Da)  | Ion m/z  | Ion species          | Lowest detected level in matrix    |                      |                                |                                 |
|----------------------|----------|----------|----------------------|------------------------------------|----------------------|--------------------------------|---------------------------------|
|                      |          |          |                      | Honeybee ng/bee (c/u) <sup>a</sup> | Pig L mg/kg (no c/u) | Pig L mg/kg (c/u) <sup>a</sup> | Lamb L mg/kg (c/u) <sup>b</sup> |
| acetamiprid          | 222.0672 | 223.0745 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| aldicarb             | 190.0776 | 213.0668 | [M+Na] <sup>+</sup>  | >5*                                | 0.1                  | 0.1                            | 0.2                             |
| aldicarb sulfone     | 222.0674 | 223.0747 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| aldicarb sulfoxide   | 206.0725 | 207.0798 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.1                             |
| avermectin B1a       | 872.4922 | 895.4814 | [M+Na] <sup>+</sup>  | 1                                  | 0.2                  | 0.1                            | 0.2                             |
| avermectin B1b       | 858.4766 | 881.4658 | [M+Na] <sup>+</sup>  | >5*                                | 2                    | 2                              | 2                               |
| azaconazole          | 299.0228 | 300.0301 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| azinthos-methyl      | 317.0058 | 318.0131 | [M+H] <sup>+</sup>   | 5                                  | 0.2                  | 0.1                            | 0.1                             |
| bendiocarb           | 223.0845 | 224.0918 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.1                             |
| benfuracarb          | 410.1875 | 411.1948 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| carbaryl             | 201.0790 | 202.0863 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.1                             |
| carbofuran           | 221.1052 | 222.1125 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| carbosulfan          | 380.2134 | 381.2207 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| chloralose           | 307.9621 | 306.9548 | [M-H] <sup>-</sup>   | 5                                  | 1                    | 1                              | 1                               |
| chlorfenvinfos       | 357.9695 | 358.9768 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| chlorpyrifos         | 348.9263 | 349.9336 | [M+H] <sup>+</sup>   | 5                                  | 0.5                  | 0.1                            | 2                               |
| clothianidin         | 249.0087 | 250.0160 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| cyproconazole (pk 1) | 291.1138 | 292.1211 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.2                             |
| cyproconazole (pk 2) | 291.1138 | 292.1211 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.2                             |
| diazinon             | 304.1011 | 305.1084 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| dichlorvos           | 219.9459 | 220.9532 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 1                               |
| difenoconazole       | 405.0725 | 406.0798 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| dimethoate           | 228.9996 | 230.0069 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| epoxiconazole        | 329.0731 | 330.0804 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| fenitrothion         | 277.0174 | 278.0247 | [M+H] <sup>+</sup>   | 5                                  | 0.5                  | 0.5                            | 1                               |
| fenthion             | 278.0200 | 279.0273 | [M+H] <sup>+</sup>   | 5                                  | 0.2                  | 0.1                            | 0.1                             |
| fipronil             | 435.9387 | 453.9720 | [M+NH4] <sup>+</sup> | >5*                                | 0.5                  | 0.2                            | 1                               |
| fipronil sulfone     | 451.9336 | 469.9669 | [M+NH4] <sup>+</sup> | 5                                  | 0.2                  | 0.1                            | 0.2                             |
| flusilazole          | 315.1003 | 316.1076 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| fonofos              | 246.0302 | 247.0375 | [M+H] <sup>+</sup>   | 5                                  | 0.2                  | 0.1                            | 1                               |
| fosthiazate          | 283.0466 | 284.0539 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| furathiocarb         | 382.1562 | 383.1635 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 1                               |
| heptenophos          | 250.0162 | 251.0235 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| imazalil             | 296.0483 | 297.0556 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| imidacloprid         | 255.0523 | 256.0596 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.1                             |
| imidacloprid -5OH    | 271.0472 | 294.0364 | [M+Na] <sup>+</sup>  | 1                                  | 0.2                  | 0.1                            | 0.1                             |
| imidacloprid olefin  | 253.0367 | 254.0440 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.2                             |
| indoxacarb           | 527.0707 | 528.0780 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.1                             |
| malathion            | 330.0361 | 331.0434 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| metaldehyde          | 176.1049 | 194.1381 | [M+NH4] <sup>+</sup> | >5*                                | >1*                  | >1*                            | >1*                             |
| methiocarb           | 225.0824 | 226.0897 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| methomyl             | 162.0463 | 163.0536 | [M+H] <sup>+</sup>   | >5*                                | 0.2                  | 1                              | 1                               |
| methomyl             | 162.0463 | 347.0819 | [2M+H] <sup>+</sup>  | >5*                                | 0.2                  | 1                              | 1                               |
| mevinphos            | 224.0450 | 225.0523 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.1                             |
| myclobutanil         | 288.1142 | 289.1215 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 1                               |
| oxamyl               | 219.0678 | 242.0574 | [M+Na] <sup>+</sup>  | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| penconazole          | 283.0643 | 284.0716 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.1                             |
| phorate              | 260.0128 | 261.0201 | [M+H] <sup>+</sup>   | >5*                                | >1*                  | >1*                            | >1*                             |
| phosalone            | 366.9869 | 367.9942 | [M+H] <sup>+</sup>   | 1                                  | 0.1                  | 0.1                            | 0.2                             |
| pirimicarb           | 238.1430 | 239.1503 | [M+H] <sup>+</sup>   | 5                                  | 0.1                  | 0.1                            | 0.1                             |

|                   |          |          |                                   |     |     |     |     |
|-------------------|----------|----------|-----------------------------------|-----|-----|-----|-----|
| pirimiphos methyl | 305.0963 | 306.1036 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| prochloraz        | 375.0308 | 376.0381 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| propiconazole     | 341.0698 | 342.0771 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| propoxur          | 209.1052 | 210.1125 | [M+H] <sup>+</sup>                | 5   | 0.1 | 0.1 | 0.2 |
| pyrazophos        | 373.0861 | 374.0934 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| pyrethrin I       | 328.2038 | 329.2111 | [M+H] <sup>+</sup>                | 1   | 1   | 1   | 1   |
| pyrethrin II      | 372.1937 | 373.2010 | [M+H] <sup>+</sup>                | >5* | 1   | 1   | 2   |
| spinosyn A        | 731.4608 | 732.4681 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| spinosyn D        | 745.4765 | 746.4838 | [M+H] <sup>+</sup>                | 5   | 0.1 | 1   | 1   |
| spiromesifen      | 370.2144 | 388.2477 | [M+NH <sub>4</sub> ] <sup>+</sup> | >5* | 0.5 | 0.1 | 1   |
| tebuconazole      | 307.1451 | 308.1524 | [M+H] <sup>+</sup>                | 5   | 0.1 | 0.1 | 0.1 |
| tetramethrin      | 331.1784 | 332.1857 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| thiacloprid       | 252.0236 | 253.0309 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| thiamethoxam      | 291.0193 | 292.0266 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| thiodicarb        | 354.0490 | 355.0563 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |
| triadimefon       | 293.0931 | 294.1004 | [M+H] <sup>+</sup>                | 5   | 0.1 | 0.1 | 0.1 |
| triadimenol       | 295.1088 | 296.1161 | [M+H] <sup>+</sup>                | 5   | 0.2 | 0.1 | 0.2 |
| triazophos        | 313.0650 | 314.0723 | [M+H] <sup>+</sup>                | 1   | 0.1 | 0.1 | 0.1 |

\*>5 ng/bee or >1 mg/kg indicates that the analyte was not detected at investigated levels in matrix

Clean-up: a = general reverse-phase C18(EC), PSA, as described elsewhere in this report, b = QuEChERS extraction [3], without PSA dispersive clean-up.

**Table 6. Detection limits for rodenticides in liver extracts**

| Analyte         | MW (Da) | Ion m/z  | Ion species        | Lowest detected level in matrix |  |
|-----------------|---------|----------|--------------------|---------------------------------|--|
|                 |         |          |                    | Badger liver (mg/kg) (no c/u)   | Badger liver (mg/kg) (n-Al c/u) <sup>a</sup> |
| brodifacoum     | 522.083 | 521.0758 | [M-H] <sup>-</sup> | 0.01                            | 0.01   |
| bromadiolone    | 526.078 | 525.0707 | [M-H] <sup>-</sup> | 0.01                            | 0.01   |
| chlorophacinone | 374.071 | 373.0637 | [M-H] <sup>-</sup> | 0.01                            | 0.005  |
| coumatetralyl   | 292.11  | 291.1026 | [M-H] <sup>-</sup> | 0.001                           | 0.001  |
| difenacoum      | 444.173 | 443.1652 | [M-H] <sup>-</sup> | 0.025                           | 0.025  |
| diphacinone     | 340.11  | 339.1026 | [M-H] <sup>-</sup> | 0.025                           | 0.005  |
| flocoumafen     | 542.171 | 541.1632 | [M-H] <sup>-</sup> | 0.002                           | 0.002  |
| warfarin        | 308.105 | 307.0976 | [M-H] <sup>-</sup> | 0.002                           | 0.002  |

<sup>a</sup> neutral alumina SPE clean-up (current WIIS method)

further qualitative confirmation analysis using a different analytical technique would usually be required for WIIS samples, where findings are frequently used as evidence in prosecution.

LC-TOF uses a different approach for method development to the more widely used LC-tandem quadrupole methodology, where optimisation of the analysis depends on identifying parent and daughter ions (ion transitions) for analytes in advance of data acquisition. Since the TOF instrument acquires data for *all* of the ions within its mass range, theoretically it is only necessary to set up a suitable LC method - the optimisation of the MS method is essentially data processing, post acquisition. In practice, data needed to be acquired in both positive and negative ion modes, which meant analysing all solutions in duplicate. Setting up an optimised multi-residue method to extract the correct data from these files is a time-consuming, iterative process. Where possible, the best approach is to standardise the acquisition method, so that to a large extent, the data processing methods can also be standardised. Much time and effort is required to set up the optimum analysis conditions for a large number of analytes. Furthermore, any changes in equipment may require this effort to be repeated.

TOF spectrometry produces very large data sets. In practice, a huge proportion of the data acquired is redundant, and it can be quite challenging to extract all of the pertinent data, and then process it into a summarised form that can be reported. Powerful computers are required to manipulate the large files involved, and data storage can be a problem when a run of 40 injections can easily create data files with a combined size of over 20 Gigabytes.

The ability to retrospectively search acquired data for analytes not originally targeted in screening is an attractive feature of TOF-MS. To do this for 'unknowns' (e.g. pesticide metabolites or transformation products) the empirical formula of a putative analyte needs to be established, and ideally both positive and negative ionisation data should be available to search. The masses of possible positive and negative ions (including adducts) have to be

calculated and extracted ion chromatograms (EICs), with varying mass windows, examined until a likely chromatographic peak is found. This assumes that the sample chosen could potentially contain the analyte (e.g. correct tissue to find a particular metabolite), that the extraction method that was used could potentially extract this analyte, and that the LC method that was used was appropriate. In the case of metabolites, where reference materials are not available, confirming the identity of a suspected residue is difficult. Usually there are many different theoretical empirical formulas that fit the data, despite the high mass accuracy of TOF-MS. For some analytes, confidence in identification could possibly be improved by altering ionisation conditions in the source to try and produce and identify several fragments at the same retention time, but this requires re-analysing the sample. The presence of identified residues would provide qualitative information, but in the cases where nothing was found, it would be difficult or impossible to draw any conclusions due to the uncertainties of an un-validated method. The process would be simpler if reference materials were available, which could subsequently be analysed under the same conditions as the samples initially were. If that were the case, the extraction method used to generate the historical data could also be validated for the new analytes.

### **Conclusion**

LC-TOF-MS would be sensitive enough to screening WIIS vertebrate samples for most routine analytes, but not all. The sensitivity is not sufficient to detect rodenticides in liver, or determine neonicotinoids in honeybees. Furthermore, for sufficient confidence in residue identification, a further confirmatory analysis would usually be required. However, the technique could be useful for building a collection of data from WIIS samples that could be retrospectively searched to identify patterns of suspected pesticide transformation product residues, or other active or non-active ingredients from pesticide formulations, which may provide useful forensic information. A suitable extraction procedure that can extract polar metabolites and transformation products, as well as non-polar analytes would be needed. Analysis of samples from known WIIS poisoning incidents might then give some indication of how often metabolites, transformation products or diagnostically useful pesticide co-formulants are likely to occur.

### **Accelerated Solvent Extraction of rodenticides from liver samples**

It has been the experience of the WIIS and other researchers [6] that some liver samples give very variable and low recoveries of some rodenticides when using the traditional DCM/acetone extraction. This is particularly the case for the indandione rodenticides diphacinone and chlorphacinone. A Dionex ASE200 accelerated solvent extraction (ASE) system was evaluated for its ability to improve recovery of 'bound' rodenticide residues from liver tissue. ASE uses pressurised extraction solvent, allowing use of temperatures above its boiling point. This can provide a very exhaustive extraction in a short time. Less solvent is needed than in the currently used 'shaking' extraction, which has environmental benefits.

Ideally, liver samples containing incurred residues would be used to evaluate the extraction efficiency of the ASE. Unfortunately, tissues available from previous WIIS incidents were of insufficient size for the required replicate analyses and control samples. Artificially spiking tissues that are available in sufficient quantity (e.g. chicken liver) also presents problems. It is possible that the solvent used to apply the analytes to the sample could affect the way they are adsorbed, making it easier or harder to extract them. A procedure was developed that involved solvent-less or 'dry' spiking of tissue. Glassware was spiked with the solution of rodenticides and the solvent evaporated, before adding the chopped liver sample. The sample was then stirred over the dry residue to adsorb it. In initial experiments, analysis of residues on the glassware after spiking showed that the transfer of analyte to the liver was acceptably quantitative (Table 7). So that the exact amount of rodenticides adsorbed during 'dry' spiking was known for the ASE experiments, the glassware used was analysed each time. An adjusted spiking level could then be calculated, and used to ascertain recoveries by ASE and the current WIIS 'shaking' extraction method.

A variety of extraction parameters can be adjusted on the Dionex ASE system, but those that affect extraction efficiency most, in order of importance, are solvent composition, temperature, time and to a much lesser extent, pressure. The extraction conditions were optimised to reduce the number of experimental variables as much as possible. For final experiments, only temperature was varied. To make easier comparison with the WIIS 'shaking' extraction, the same solvent system, acetone:DCM (30:70) was used. Hydromatrix (Varian) diatomaceous earth was used as a drying agent. In order to remove the uncertainty introduced by clean-ups, a high spiking level (1 mg/kg) was chosen so that simple solvent-swap and dilution would allow detection by LC-MS/MS. An improved LC separation was developed so that the coumarins and indandiones could be analysed in one analytical procedure.

It was found that considerably more fat was extracted by the ASE at all temperatures, than is seen with the current 'shaking' method, the amount increasing with rising temperature. This was seen as immiscible fat floating on the surface of the extract solutions. It is unknown whether the current clean-up would be sufficient to cope with these 'dirtier' extracts without a loss of sensitivity. Some of the recoveries measured are high and it is uncertain to what degree the extra co-extracted material in the 100 °C ASE extracts might have affected the

response of the mass spectrometer – despite matrix-matched standard solutions being used.

**Table 7. Efficiency of transfer of analytes from pestle & mortars**

| Analyte         | 'Dry' spiking: transfer from pestle & mortar (%), n=6 |         |         |
|-----------------|---|---------|---------|
|                 | Range (%)   | Ave (%) | RSD (%) |
| brodifacoum     | 82 - 92   | 89      | 4.3     |
| bromadiolone    | 93 - 98   | 97      | 2.0     |
| chlorophacinone | 91 - 98   | 96      | 2.8     |
| coumatetralyl   | 98 - 99   | 99      | 0.4     |
| difenacoum      | 87 - 95   | 92      | 3.7     |
| difethialone    | 78 - 92   | 88      | 6.2     |
| diphacinone     | 93 - 98   | 96      | 1.4     |
| flocoumafen     | 84 - 97   | 92      | 5.5     |
| warfarin        | 99 - 100  | 99      | 0.7     |

Recoveries are quite variable using either technique (Table 8). Recoveries using ASE do appear to be higher than those obtained using shaking extraction. Unfortunately, the complexity of the ASE meant that it was less reliable than the simple shaking method. Blockages were common and replacement parts are expensive. The possibility of failed extractions using ASE is a particular problem for the WIIS, as a failed extraction would not always be repeatable due to the small sample sizes often received.

**Table 8. Summary of recoveries adjusted for spike transfer**

| Analyte         | Recoveries, adjusted for spike transfer |         |         |            |         |         |                    |         |         |
|-----------------|---|---------|---------|------------|---------|---------|--------------------|---------|---------|
|                 | ASE 60 °C                               |         |         | ASE 100 °C |         |         | Shaking extraction |         |         |
|                 | Range (%)                               | Ave (%) | RSD (%) | Range (%)  | Ave (%) | RSD (%) | Range (%)          | Ave (%) | RSD (%) |
| brodifacoum     | 89 - 96                                 | 92      | 3.9     | 98 - 120   | 112     | 11      | 40 - 93            | 58      | 37      |
| bromadiolone    | 62 - 82                                 | 74      | 14      | 73 - 88    | 81      | 9       | 46 - 84            | 57      | 28      |
| chlorophacinone | 45 - 57                                 | 52      | 12      | 72 - 122   | 91      | 30      | 47 - 60            | 55      | 8.9     |
| coumatetralyl   | 88 - 119                                | 100     | 16      | 76 - 92    | 85      | 10      | 87 - 96            | 92      | 4.2     |
| difenacoum      | 78 - 90                                 | 85      | 7.6     | 91 - 104   | 99      | 7       | 49 - 83            | 66      | 19      |
| difethialone    | 83 - 87                                 | 84      | 2.2     | 95 - 119   | 109     | 11      | 62 - 83            | 75      | 12      |
| diphacinone     | 59 - 79                                 | 69      | 14      | 77 - 98    | 89      | 12      | 49 - 69            | 61      | 12      |
| flocoumafen     | 66 - 81                                 | 72      | 11      | 92 - 129   | 107     | 18      | 39 - 95            | 59      | 38      |
| warfarin        | 89 - 101                                | 95      | 6.6     | 81 - 93    | 90      | 9       | 71 - 89            | 79      | 8.8     |

### Conclusion

The higher temperature ASE does appear to extract more of the difficult analytes diphacinone and chlorophacinone, although this is offset by the production of extracts with far more co-extracted material. This is likely to raise limits of detection without development of an improved clean-up. It was concluded that the possible benefits of using the ASE are outweighed by its cost and unreliability.

### Direct Exposure and Direct Insertion Probes for mass spectrometric determination of WIIS samples

#### Introduction

These techniques allow introduction of sample material directly into the source of a mass spectrometer without the usual chromatographic separation step. This enables the analysis of compounds which would not normally be amenable to the chromatographic separation. Use of these techniques should allow acquisition of full-scan Electron-Impact (EI) spectra from analytes which are too non-volatile/thermally unstable for gas chromatography. It was also anticipated that for some applications, a qualitative result could be obtained much faster using these techniques than by using LC or GC-MS.

A Thermo GCQ GC-MS system which has a vacuum interlock that is compatible with the Thermo Direct Exposure and Direct Insertion Probes (DIP and DEP) was used. Direct Insertion and Direct Exposure probes on loan from Thermo were evaluated with the GCQ Ion Trap mass spectrometer for analysis of pesticide residues in suitable WIIS samples. These techniques are usually applied to qualitative analysis of relatively pure samples, not trace levels of analyte in large amounts of matrix, so experiments were focussed on formulations and suspected poison baits. The two probes work in slightly different ways to volatilise the sample for ionisation. For DIP, a few hundred micrograms of sample are placed in a tiny silica sample tube, which is inserted into the end of a temperature-controlled probe. The probe is inserted into the mass spectrometer through the vacuum interlock which prevents the analyser losing vacuum. The tip of the probe rests against a specially designed ion-volume in



the source (where ionisation of analytes occurs). Once inserted, the probe is heated at a controlled rate (from 10 °C/min to 100 °C/min) up to a maximum temperature of 450 °C. A typical temperature ramp lasts for about 5 minutes. For DEP, one or two microlitres of sample solution in volatile solvent is applied to a small rhenium wire loop at the tip of the probe using a microsyringe, and the solvent allowed to evaporate. The probe is inserted into the mass spectrometer in the same way as the DIP. The rhenium wire is rapidly heated by passing increasing electrical current through it at a controlled rate (typically 20 to 500 mA/s) up to a maximum current of 1000 mA. This heats the wire, vaporising the sample residue. At 1000 mA the filament temperature is approximately 1000 °C. During the heating process with either probe, the mass spectrometer is switched on and data is acquired. Solutions of sample can also be analysed using the DIP, but as is the case with the DEP, the solvent must be evaporated away from the sample before the probe is inserted into high vacuum, or sputtering of sample and loss of vacuum can occur as the solvent abruptly boils.

## Results

A variety of analytes commonly encountered by the WIIS in formulations and baits were investigated using both the DIP and the DEP. The analytes are listed below:

|              |                 |             |            |
|--------------|-----------------|-------------|------------|
| aldicarb     | chloralose      | fenthion    | oxamyl     |
| bendiocarb   | chlorophacinone | flocoumafen | paraquat   |
| brodifacoum  | coumatetralyl   | metaldehyde | strychnine |
| bromadiolone | difenacoum      | methiocarb  | warfarin   |
| carbofuran   | diphacinone     | methomyl    |            |
| carbosulfan  | diquat          | mevinphos   |            |

Typically, 100 to 200 ng of analyte reference material were used in experiments with either probe to establish the following analytical methods: DIP, 60 °C for 20 s, followed by a 100 °C/min ramp to 450 °C and hold for 60 s; and DEP, 0 mA for 30 s, followed by a 100 mA/s ramp to 1000 mA and hold for 60 s.

In all cases, diagnostic full-scan EI mass spectra were acquired, that gave good matches to known spectra from WIIS data or National Institute of Standards and Technology (NIST) libraries. Bromadiolone was found to breakdown to some degree, using either probe. Strychnine only produced a recognisable mass spectrum as the free base, although paraquat and diquat salts gave excellent spectra. Difenacoum produced a particularly abundant ion at 444 m/z, and was chosen for some extra DEP-MS/MS and DIP-MS/MS experiments, in an

**Table 9. Analysis results of real WIIS formulation and bait samples**

| Analyte     | Matrix                                    | Residue found using conventional methods | DEP result  |
|-------------|---|--|---|
| methomyl    | bread – suspected bait                    | 1000 mg/kg                               | DEP-MS, confirmed   |
| strychnine  | apple – suspected bait                    | 140 mg/kg                                | DEP-MS, confirmed   |
| metaldehyde | slug pellets                              | 4.4 % (w/w)                              | DEP-MS, one ion only  |
| carbosulfan | feathers                                  | 1900 µg                                  | DEP-MS, confirmed   |
| aldicarb    | black granules - formulation              | 12% (w/w)                                | DEP-MS, confirmed   |
| chloralose  | white powder - formulation                | 12% (w/w)                                | DEP-MS, confirmed   |
| difenacoum  | grain – suspected rodenticide formulation | 12 mg/kg                                 | DEP-MS, not confirmed<br>DIP-MS not confirmed<br>DIP-MS/MS, confirmed |

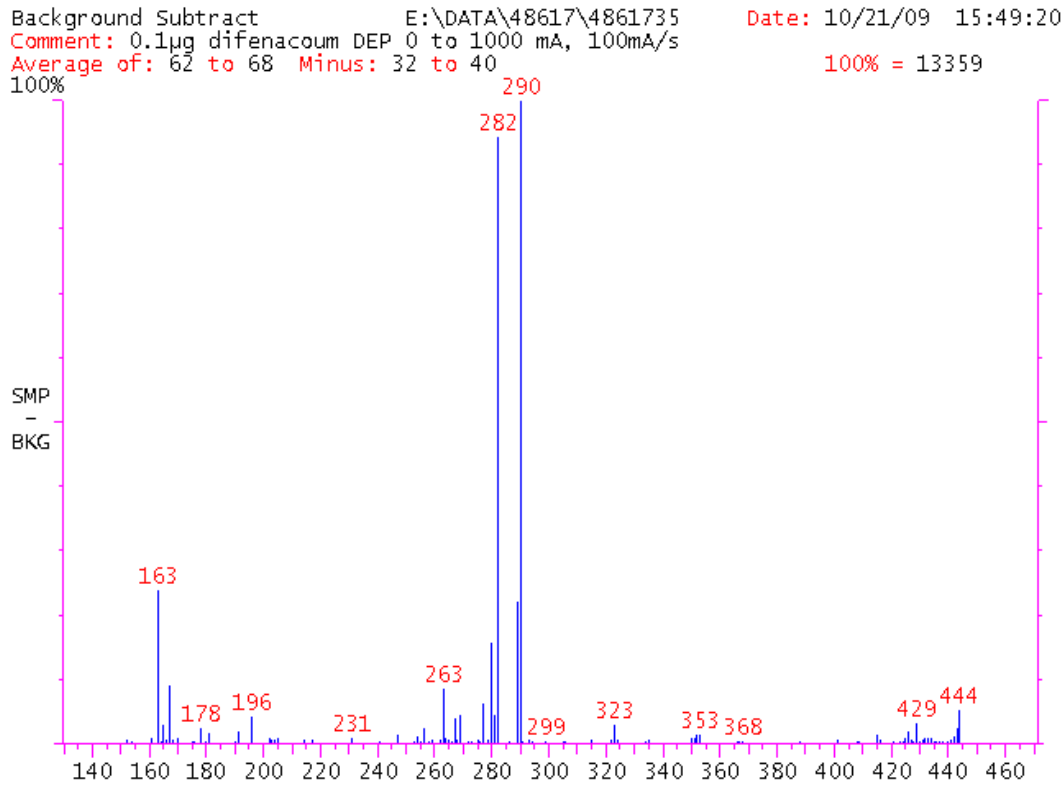
attempt to lower detection limits. A variety of real WIIS sample solutions were analysed by DEP and DIP. These had previously been analysed using conventional WIIS methods. The results of these experiments are summarised above (Table 9).

## Discussion

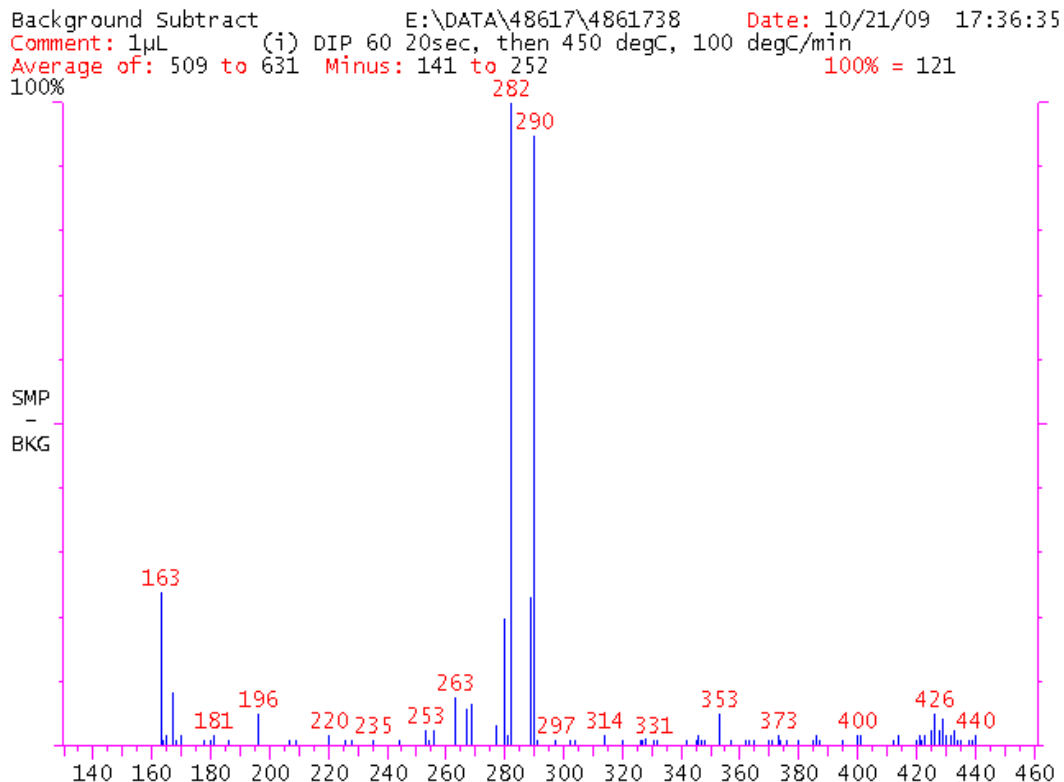
The total analysis time for one DIP experiment from sample loading to analytical result, was found to be about 15 minutes. Whereas more time was needed for sample preparation for DEP (a solution of sample has to be prepared), for a series of experiments, DEP could be faster. This was because the probe could be removed as soon as data acquisition was complete. The DIP probe needed to cool sufficiently to allow withdrawal from the vacuum interlock without melting the seals. In theory the rate of cooling could be accelerated with a coolant gas, but it was not practical to set up the required gas lines for this evaluation. Analysing batches of samples was much faster using DEP than DIP, where ten per hour was possible, depending on the volatility of the sample solution. The speed of heating using the DEP gave much sharper and more obvious 'peaks' but virtually no separation of high and low volatility components. In practice, the faster analysis time and faster heating rate and extended temperature range, as well as the simpler sample loading, made the DEP a more productive technique, and this became the default approach.

Generally, the DIP and DEP data acquired for the WIIS samples gave good matches with those produced by standard reference materials. Difficulties were encountered with metaldehyde, partly due to its high volatility, but

**Fig.1 Difenacoum standard solution, DEP-MS/MS**



**Fig.2 Solution from WIIS sample known to contain difenacoum, DIP-MS/MS**



also due to its having a mass spectrum consisting of very low intensity, low mass ions and one high abundance ion of mass 45 m/z. The 45 m/z ion was detected, but would not be considered enough for confirmation of the presence of metaldehyde. The difenacoum residue results are interesting. The residue was too low for DEP-MS or DIP-MS to detect in the presence of matrix/background. However, DIP-MS/MS was apparently able to confirm the residue (see Figs 1 and 2, above). It was estimated that the DIP-MS/MS detected about 2 ng of difenacoum.

The speed of vacuum recovery after probe insertion required a perfectly functioning vacuum interlock ball valve. Although acceptable for GC-MS, it was found that new seals greatly improved matters. The repeated fluctuation

in vacuum, even with a newly serviced vacuum interlock, caused the diffusion pump oil to contaminate the source to some extent, producing a characteristic ion at 446 m/z, but this did not prevent acquisition of useful data. Loading very small amounts of sample material into the tiny silica tubes for the DIP required considerable manual dexterity. Often, static would cause contamination of the outside of the tube, which then had the potential to contaminate the tip of the probe. Sample loading was much easier using the DEP. The wire loop could only hold a droplet of about two microlitres of solution, but larger volumes could be loaded in microlitre steps allowing the solvent to evaporate in between steps. The rhenium wire loop is detachable, enabling spare loops to be loaded with sample in advance of analysis. It was easy to add too much analyte to either probe when concentrated samples were being analysed. This could saturate the detector, and but was not found to interfere with qualitative analysis, when analyte to matrix ratios were high. For detection of small residues (less than 10% in matrix) this could be a problem. It was found that the source ion volume became contaminated with sample very quickly and required frequent changing. Evidence of this was low-level carry-over between samples. Fortunately ion volume replacement can be performed quickly and easily without venting the analyser, via the vacuum interlock. The slower sample introduction method used by DIP should allow the possibility of some temporal analyte/matrix separation if sufficient differences in volatility exist. This was seen to some extent with volatile analytes, but slow temperature ramps usually resulted in very broad analyte 'peaks' with poor signal to noise. Often the analytes which could take advantage of this effect were sufficiently volatile to begin vaporisation just by being in close proximity to the ion volume (heated to 200 °C) after probe insertion. In these cases, and using either probe, the analyte could easily have disappeared from the probe tip before data acquisition had been started. It was found to be prudent to leave the probe tip a couple of centimetres away from the ion volume until vacuum was steady and acquisition could begin.

### **Conclusion**

The DIP and DEP in conjunction with the GCQ Ion Trap MS can provide a useful, rapid confirmation or screening tool for certain, suitable WIIS samples. Sensitivity in carefully optimised MS/MS mode was even good enough to identify difenacoum in a relatively dilute pesticide formulation of the kind frequently encountered in the WIIS. The full-scan EI data obtained using DIP or DEP for analytes normally determined by LC-MS/MS, can provide valuable added qualitative confirmation.

## **REPORT ON OBJECTIVE 3**

WIIS investigations would be aided by availability of rapid tests that could provide a tentative identification of the presence of pesticides in samples of pesticide formulation, both in storage containers and as applied to 'poison baits' and from spillages (e.g. slug pellets). Such tests would require later confirmation in the laboratory, but might enable a faster response and expedite legal proceedings. In addition sampling can be hazardous in such circumstances, so an aid to identifying possible risks is also helpful.

### **Vibrational spectroscopic methods of analysis: Raman spectroscopy**

#### **Introduction**

Spectroscopic methods using light at frequencies that interact with vibrational states of molecules include infrared, near-infrared and Raman spectroscopy. Raman spectroscopy offers some advantages over the other techniques for types of samples encountered in the WIIS; water does not usually interfere, and Raman can in some cases identify substances directly through clear or translucent containers. It offers the possibility of fast, non-destructive, qualitative analysis of concentrated samples such as formulations and poison baits, with minimal sample contact. A Raman spectrometer measures in-elastically reflected light from the interaction with vibrational states of sample molecules. It consists of a monochromatic light source (usually a laser), which is aimed at the surface of the sample. Scattered light returns via a filter that removes the predominant, elastic Rayleigh radiation. A light-sensitive detector (often a charge-coupled device (CCD), similar to those in digital cameras) then measures the wavelengths and intensity of the reflected light, which is characteristic of the sample. Modern laser diode and semiconductor CCD technology has allowed the miniaturisation of Raman spectrometers to make compact, portable devices without unduly sacrificing performance. Suitable, commercially available devices were evaluated as rapid testing devices for WIIS field investigations.

#### **Results**

Three devices with similar specifications were identified for evaluation: the Inspector Raman (DeltaNu), the First Defender (Ahura Scientific) and the R3000HR (Ocean Optics). Both the R3000HR and Inspector Raman systems consist of a hand-held probe connected to a laptop computer for acquisition control and library searching. The First Defender is a fully self-contained system with an onboard computer. The initial evaluation was that that the R3000HR and Inspector Raman systems would be quite cumbersome to set up in a field situation, requiring booting of computers and loading of software, as well as the maintenance of two sets of batteries (probe and laptop). Claimed battery life for the systems (excluding laptops) was five hours for the Inspector Raman and First Defender, but only two hours for the R3000HR. There was also a risk of damage to the probes and laptops by wet weather or impact during operation in the field. In contrast, the First Defender was waterproof, shockproof,

and physically smaller than the combined units of the other systems. The simplicity of the software user-interface on the First Defender, compared to the laptop-based systems, was also apparent. First Defender was operated using a simple keypad, with menus on a small, colour LCD screen, rather like a mobile telephone menu. Keys are large with a positive action, and easy to use with gloves on. The R3000HR and Inspector Raman systems offered a high degree of control over acquisition parameters, which could be used to optimise scanning and improve data by experiment. The First Defender had fewer customisable parameters, with more automatic optimisation built into the default scanning modes. The Inspector Raman and First Defender were loaned to Fera for more detailed evaluation.

Both instruments had a 'point and shoot' data acquisition mode, where the laser is aimed at a sample a few millimetres away, and a 'vial' mode, where a sub-sample is transferred to a vial and placed in an internal vial holder to be scanned. The Inspector Raman had a manual, variable-focus vial holder, which had to be physically swapped for the 'point and shoot' attachment, whereas the First Defender had an integral vial holder. It quickly became apparent in experiments with either system, that obtaining reliable spectra in 'point and shoot' mode depended on the steadiness and accuracy of the user's aim over the duration of the scan (milliseconds to seconds). There is an optimum focal point that takes repeated practice to find reliably. The Inspector Raman has an optional microscope and clamp attachment (NuScope) that enables accurate positioning of the laser for precise scanning, but use of this facility in the field would not be practical. Evaluation of the Inspector Raman was hampered by software problems, which were eventually traced to corrupt setup files. This problem coupled with the small size of the user-created library (no pesticide library was included with the evaluation instrument) made assessment of library-searching accuracy difficult. The First Defender was supplied with a very large library of spectra (over 7600 entries). Library searching was a simple matter of pressing a button after data acquisition.

**Table 10. Scan results for a variety of samples**

| Sample                                 | Sample correctly identified (yes/no) |                 |                |                 |
|--|--------------------------------------|-----------------|----------------|-----------------|
|  | 'Point and Shoot' mode               |                 | 'Vial' mode    |                 |
|  | First Defender                       | Inspector Raman | First Defender | Inspector Raman |
| chloralose formulation                 | yes                                  | yes             | yes            | yes             |
| paraquat formulation                   | yes                                  | yes             | yes            | yes             |
| glyphosate formulation                 | yes                                  | yes             | yes            | yes             |
| sodium chlorate formulation            | yes                                  | yes             | yes            | yes             |
| ethylene glycol                        | yes                                  | yes             | yes            | yes             |
| chlorpyrifos formulation               | yes                                  | (a)             | yes            | (a)             |
| chlorothalonil formulation             | yes                                  | (a)             | yes            | (a)             |
| trifluralin formulation                | no                                   | (a)             | yes            | (a)             |
| carbofuran reference material          | yes                                  | yes             | yes            | yes             |
| simulated bait – strychnine on chicken | yes                                  | (a)             | n/a            | n/a             |
| simulated bait – paraquat on chicken   | yes                                  | (a)             | n/a            | n/a             |
| WIIS bait – methomyl on bone           | yes                                  | (a)             | n/a            | n/a             |

(a) Software problems prevented the library search working - no assessment made. n/a – 'vial' mode not applicable

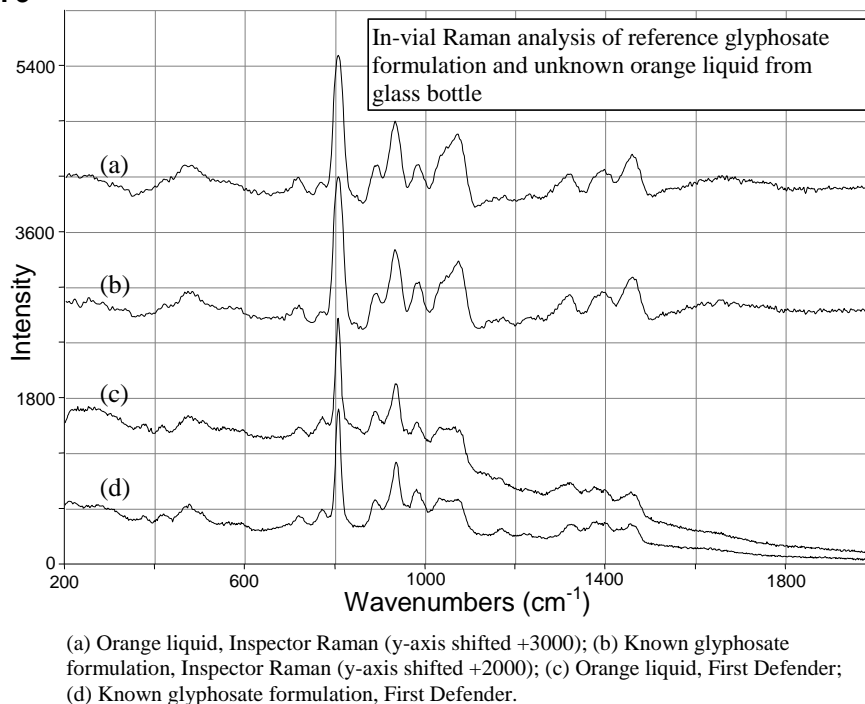
If a match or matches were found, the results are saved with the data, and can be exported as a PDF report. First Defender data files, library files and reports are stored on an internal Compact Flash card, which can be removed and read by a computer. The First Defender and Inspector Raman data files can be exported in 'standard' SPC format and processed using a variety of proprietary spectrum viewing software packages. Calibration of Inspector Raman was performed with an external polystyrene sample using the 'vial' mode, and was simple to do, although varying ambient light conditions would require frequent re-acquisition of 'background' signal, when in 'point and shoot' mode. The First Defender has an automatic internal calibration, which can be verified by scanning a polystyrene sample in 'vial' mode.

The Raman technique did have limitations. Background or analyte fluorescence could interfere with spectrum acquisition, and intensely coloured solid samples could absorb too much laser energy and burn, preventing spectrum acquisition. The 'point and shoot' ability to acquire spectra through sample container walls only worked with the thinnest plastic containers, such as plastic bags. Plain glass can produce fluorescence that swamps the Raman signal, and amber glass, dirty or thick-walled plastic containers absorb too much laser energy. Raman response is compound specific, and some molecules give only weak spectra or none at all. Despite this, in many cases, Raman spectroscopy was able to correctly identify pesticides in a variety of WIIS samples. A summary of the results obtained is shown above (Table 10).

### Example of Raman detector application - orange liquid in bottle

A sample from a previous incident was re-analysed with both Raman detectors (in 'vial' mode). The sample consisted of a bottle of orange liquid, in which a small amount of paraquat (about 0.03% w/v) had been detected by conventional analysis. Raman analysis took only minutes to conduct and indicated that the sample contained a high concentration of glyphosate, but did not detect paraquat (Fig.3). The paraquat residue found previously was only a minor component of a mixture. But since field evidence had suggested the involvement of paraquat in that incident, and associated birds were found to have been poisoned with aldicarb, no further work had been requested on that sample at that time, and the presence of glyphosate was missed. This shows how useful an *additional* Raman scan could be for quickly identifying major components of some samples, in the field or in the laboratory. Obviously the paraquat residue would have been missed using Raman alone, but that is not the type of application the instruments were designed for. The instruments evaluated are primarily intended for qualitative analysis of pure or nearly pure substances, but can in some circumstances detect tens of percent of one substance in the presence of another; aqueous solutions of glyphosate being an example of this.

Fig. 3



### Conclusion

Portable Raman technology would provide a useful, additional tool for field or laboratory WIIS investigations. In practice, it would be expected that a high proportion of scans would return ambiguous or 'no library match' results. But for a significant number of WIIS sample types that are routinely encountered in 'high-priority' incidents - highly concentrated pesticide formulations and poison baits, portable Raman devices can offer rapid identification, and could save valuable time in processing an incident, as well as provide more qualitative, confirmatory evidence. Of the systems evaluated, First Defender would be most suitable for WIIS applications since it is a very rugged system, designed specifically for field use.

### REPORT ON OBJECTIVE 4

Development and validation of a rapid extraction and integrated clean-up scheme for honeybee screening to improve limits of detection and reduce the need for analysis by multiple methods to cover all analytes of interest. This is an extension of the experiments started in Objective 1, 'Methods for insecticides in honeybees: spinosad, indoxacarb, spiromesifen, pyrethrins and abamectin'.

The current procedure for analysing honeybees in WIIS consists of extracting a sample of approximately 40 insects by Soxhlet extraction using diethyl ether as solvent, followed by five separate, pesticide-class-specific clean-ups. Each clean-up is performed on a separate portion of the extract. When submitted samples consist of less than 40 insects, proportionally less sample is available per analysis, which compromises limits of detection. The Soxhlet extraction procedure has high extraction efficiency, but takes in excess of eight hours to perform and uses large amounts of water (for cooling), electricity (for heating) and solvents.

A new extraction procedure which involved grinding the sample with diatomaceous earth drying agent (Hydromatrix) and shaking in DCM for one hour, was validated, along with a new analysis strategy which reduced

the number of clean-up procedures required to cover analytes of interest. After extraction, solvent was evaporated to dryness with loose primary-secondary amine solid-phase extraction (SPE) adsorbent and the residue re-suspended in acetonitrile (ACN). The suspension was then transferred to a C18 SPE cartridge and

**Table 11. Average recoveries at 1 ng/bee**

| Analyte          | Rec. Range (%)          | Ave. Rec. (%) | RSD (%) | Class <sup>(a)</sup> | analysis | Analyte             | Rec. Range (%)      | Ave. Rec. (%) | RSD (%) | Class <sup>(a)</sup> | analysis |
|------------------|-------------------------|---------------|---------|----------------------|----------|---------------------|---------------------|---------------|---------|----------------------|----------|
| acetamiprid      | 90 - 95                 | 93            | 2.1     | 1                    | LC       | fipronil            | 87 - 93             | 91            | 2.6     | 7                    | GC       |
| aldrin           | 65 - 82                 | 73            | 10      | 2                    | GC       | fipronil sulfone    | 86 - 95             | 90            | 3.8     | 7                    | GC       |
| azaconazole      | 79 - 96                 | 90            | 8.8     | 3                    | LC       | flusilazole         | 72 - 82             | 76            | 5.9     | 3                    | LC       |
| azinphos methyl  | 70 - 99                 | 88            | 12      | 4                    | GC       | fluvalinate pk1     | 79 - 100            | 90            | 10      | 6                    | GC       |
| bendiocarb       | 55 - 71                 | 66            | 9.7     | 5                    | LC       | fluvalinate pk2     | 84 - 103            | 91            | 8.9     | 6                    | GC       |
| benfuracarb      | 50 - 97                 | 80            | 20      | 5                    | LC       | fonofos             | 60 - 74             | 67            | 8.3     | 4                    | GC       |
| bifenthin        | 84 - 89                 | 87            | 2.4     | 6                    | GC       | fosthiazate pk1     | 78 - 93             | 85            | 7.2     | 4                    | GC       |
| carbaryl         | 89 - 97                 | 93            | 3.6     | 5                    | LC       | fosthiazate pk2     | 77 - 98             | 86            | 10      | 4                    | GC       |
| carbofuran       | 81 - 95                 | 88            | 6.6     | 5                    | LC       | furathiocarb        | 51 - 76             | 65            | 16      | 5                    | LC       |
| carbosulfan      | 82 - 95                 | 85            | 8.9     | 5                    | LC       | HCH-gamma           | 67 - 77             | 71            | 6.0     | 2                    | GC       |
| chlorfenvinphos  | 83 - 94                 | 87            | 4.6     | 4                    | GC       | heptachlor epoxide  | 68 - 88             | 79            | 9.2     | 2                    | GC       |
| chlorpyrifos     | 83 - 89                 | 85            | 2.6     | 4                    | GC       | heptachlor          | 55 - 72             | 63            | 11      | 2                    | GC       |
| clothianidin     | 89 - 95                 | 91            | 2.5     | 1                    | LC       | heptenophos         | 28 - 70             | 53            | 29      | 4                    | GC       |
| cyfluthrin pk1   | 74 - 98                 | 87            | 14      | 6                    | GC       | imazalil            | <i>not found</i>    |               |         | 3                    | LC       |
| cyfluthrin pk2   | 74 - 87                 | 82            | 6.4     | 6                    | GC       | imidacloprid        | 89 - 99             | 94            | 4.0     | 1                    | LC       |
| cyfluthrin pk3   | 71 - 105                | 83            | 15      | 6                    | GC       | imidacloprid olefin | 31 - 43             | 37            | 13      | 1                    | LC       |
| cyfluthrin pk4   | 62 - 73                 | 72            | 10      | 6                    | GC       | Imidacloprid, 5-OH  | 36 - 45             | 39            | 9.9     | 1                    | LC       |
| cyhalothrin-λ    | 68 - 86                 | 78            | 9.3     | 6                    | GC       | malathion           | 77 - 83             | 80            | 3.9     | 4                    | GC       |
| cypermethrin pk1 | 72 - 97                 | 85            | 13      | 6                    | GC       | methiocarb          | 63 - 89             | 81            | 13      | 5                    | LC       |
| cypermethrin pk2 | 75 - 82                 | 74            | 7.5     | 6                    | GC       | mevinphos           | 45 - 61             | 51            | 13      | 4                    | GC       |
| cypermethrin pk3 | 67 - 89                 | 78            | 11      | 6                    | GC       | myclobutanil        | 82 - 92             | 85            | 5.7     | 3                    | LC       |
| cypermethrin pk4 | 72 - 87                 | 79            | 8.9     | 6                    | GC       | penconazole         | 51 - 62             | 54            | 12      | 3                    | LC       |
| cyproconazole    | 45 - 74                 | 62            | 21      | 3                    | LC       | permethrin pk1      | <i>interference</i> |               |         | 6                    | GC       |
| DDD-pp           | 87 - 93                 | 89            | 2.6     | 2                    | GC       | permethrin pk2      | 81 - 91             | 86            | 4.5     | 6                    | GC       |
| DDE-pp           | 82 - 85                 | 84            | 1.6     | 2                    | GC       | phorate             | 40 - 63             | 52            | 18      | 4                    | GC       |
| DDT-pp           | 78 - 102                | 93            | 11      | 2                    | GC       | phosalone           | 87 - 94             | 91            | 3.1     | 4                    | GC       |
| deltamethrin     | 72 - 93                 | 84            | 12      | 6                    | GC       | pirimicarb          | 66 - 88             | 77            | 11      | 5                    | LC       |
| diazinon         | 70 - 81                 | 75            | 5.7     | 4                    | GC       | pirimiphos-methyl   | 83 - 86             | 84            | 1.2     | 4                    | GC       |
| dichlorvos       | 0, 0, 0, 8 and 2.5% (b) |               |         | 4                    | GC       | prochloraz          | 36 - 45             | 42            | 9.2     | 3                    | LC       |
| dieldrin         | 72 - 106                | 88            | 14      | 2                    | GC       | propiconazole       | 56 - 81             | 71            | 16      | 3                    | LC       |
| difenoconazole   | 59 - 65                 | 62            | 3.9     | 3                    | LC       | propoxur            | 74 - 83             | 79            | 4       | 5                    | LC       |
| dimethoate       | 82 - 98                 | 87            | 7.6     | 4                    | GC       | pyrazophos          | 88 - 98             | 92            | 4.6     | 4                    | GC       |
| endrin           | 80 - 90                 | 84            | 5.0     | 2                    | GC       | tebuconazole        | 65 - 79             | 73            | 8       | 3                    | LC       |
| epoxiconazole    | 62 - 90                 | 75            | 15      | 3                    | LC       | tefluthrin          | 67 - 79             | 74            | 6.2     | 6                    | GC       |
| fenitrothion     | 82 - 90                 | 86            | 3.8     | 4                    | GC       | tetramethrin        | 86 - 102            | 94            | 7.0     | 6                    | GC       |
| fenpropathrin    | 81 - 96                 | 88            | 7.4     | 6                    | GC       | thiacloprid         | 92 - 101            | 95            | 3.9     | 1                    | LC       |
| fenthion         | 83 - 86                 | 84            | 1.8     | 4                    | GC       | thiamethoxam        | 80 - 94             | 87            | 6.2     | 1                    | LC       |
| fenvalerate pk1  | 69 - 94                 | 83            | 13      | 6                    | GC       | triadimefon         | 79 - 97             | 89            | 9.1     | 3                    | LC       |
| fenvalerate pk2  | 118 - 150               | 130           | 11      | 6                    | GC       | triadimenol         | 70 - 82             | 73            | 8       | 3                    | LC       |

<sup>(a)</sup> Pesticide Class (number of recovery replicates in parentheses): 1 = neonicotinoid (n=5), 2 = OC (n=5), 3 = EBI fungicide (n=4), 4 = OP (n=5), 5 = carbamate, 6 = pyrethroid (n=5), 7 = phenylpyrazole (n=5)

<sup>(b)</sup> dichlorvos lost during spiking and sample preparation steps

eluted with further ACN to remove wax and other highly non-polar material. In the early stages of this project, a normal-phase, silica SPE clean-up was developed that provided separate fractions suitable for determination of pyrethroids, fipronil (and breakdown products) and neonicotinoid insecticides. A separate clean-up was used for the determination of organochlorine insecticides. GC with various compound-specific detectors, as well as LC-MS/MS, was used in the determination step. However, the method has been further refined, so that all of the GC analyses are replaced with one GC-MS analysis, which is able to reach at least 1 ng/bee for all analytes without the extra silica clean-up. The extra clean-up step has been abandoned for neonicotinoids, too, with only a slight

deterioration in LC-MS/MS signal to noise for some ion transitions for some compounds. This method has been re-validated at 1 ng/bee for the compounds affected by the changes, and the results, along with the original validation for carbamates and fungicides, are shown below (Table 11). Only five insects are required to achieve detection limits of 1 ng/bee for GC-MS compounds, and a further five insects for LC-MS compounds (for identification of GC-MS and LC-MS compounds, see Table 11). Further insects may be required for confirmation of small residues using existing methods.

So far, the method has only been validated at 1ng/bee (n=5). This level is very close to the limits of detection for many of the analytes, which is why may explain why RSDs are quite high for some. This level was chosen to test the absolute performance of the method, and although a toxicologically relevant level for some of the analytes (e.g. fipronil: acute LD<sub>50</sub> 5 ng/bee and gamma-HCH acute LD<sub>50</sub> 11 ng/bee, e-Pesticide Manual v.4), it is arguably lower than necessary for many of the analytes in a method intended for investigating acute poisoning incidents. The only analytes that were not detected were imazalil and dichlorvos. The loss of dichlorvos, which is highly volatile, probably occurs during solvent evaporation steps. It appears that imazalil is not recovered from the clean-up; the response of matrix-matched standard solutions indicates that the chromatography and detection method are suitably optimised. An interfering peak prevented measurement of the first permethrin isomers peak, but this would not prevent detection of permethrin, since it would be expected that both peaks would be present as a residue.

### **Conclusion**

An alternative, multi-residue screening procedure for pesticides in honeybees is presented. As with all multi-residue approaches, a certain amount of compromise must be accepted – the method is more effective in determining some compounds than others, and will only detect those pesticides which are targeted in the method. With the exception of dichlorvos and imazalil, it should be sufficient to detect poisoning of honeybees by the pesticides listed. It is recommended that this approach is used for the initial pesticide screen of WIIS honeybee incident samples, unless dichlorvos or imazalil, or pesticides not in Table 11 are suspected to be involved. Further work to include chlorothalonil, and the analytes, indoxacarb, spiromesifen, pyrethrins and abamectin could be undertaken, although spinosad would require the approach described in Objective 1.

Another popular extraction and clean-up technique which incorporates a step involving PSA adsorbent in ACN is the “QuEChERS” [3] extraction. Although that method was not originally conceived for waxy samples, and might not be expected to perform well at recovering very non-polar analytes from waxy bee matrix (e.g. pyrethroids), it may be worthwhile evaluating the complete QuEChERS extraction and clean-up procedure as a further simplification of the WIIS honeybee analysis procedure.

### **Chloralose by LC-MS/MS**

A published LC-MS/MS method [7] for determining chloralose in liver was evaluated for kidney samples. The chloralose method currently used in WIIS is time-consuming and involves Soxhlet extraction of kidney samples, followed by size-exclusion chromatography clean-up, derivatisation, then SPE clean-up, before GC-ECD or MS detection. The published method is simpler, with no sample extract clean-up step, relying on matrix-matched calibration solutions to compensate for the sample matrix effects commonly seen in LC-MS/MS. The potential problem with this approach is that WIIS samples are highly variable, and representative control samples suitable for preparing matrix-matched calibration solutions are not always available. Using the same kidney sample for recovery experiments and matrix-matched calibration solutions, the method reliably detected chloralose at 5 mg/kg with an average recovery of 93% (range 87 – 98%, n=5), and RSD of 4.9%. At 1 mg/kg average recovery was 100% (range 91 – 128%, n=5), with an RSD of 16%. LOD was estimated to be about 0.5 mg/kg, which is about the same as the LOD for the current GC method. Matrix effects were examined by comparing chloralose response in nine 0.2 µg/ml matrix-matched calibration solutions (0.05 g/ml matrix; equivalent to 4 mg/kg) made with nine different kidney samples from wildlife incidents. Results obtained suggested that matrix effects did add some uncertainty to measurements, with an RSD of 16% and a maximum difference in response between two kidneys of about 30%. However, accurate measurements of residues from kidneys, which may be partly decomposed and not complete, are less important to the WIIS than reliable, unequivocal identification.

Examination of the last 10 years of WIIS chloralose residue data for kidney samples ('97 to '07) revealed that out of 63 kidney samples from animals poisoned by chloralose, 53 contained levels of 10 mg/kg or higher. Residues of this size would need to be diluted before they could be measured, and this would considerably reduce any matrix effect – if not remove it entirely. Five kidney residues were between 1 and 5 mg/kg and only one was less than 1 mg/kg (0.9 mg/kg). This method is therefore sufficiently sensitive for detection of chloralose at the levels typically detected in poisoned animals. The time taken to prepare samples for analysis is significantly reduced, and there are savings in reagent costs and reductions in energy requirements (electricity and water).

The method has been adopted for screening WIIS samples, using solvent-only calibration solutions.

## Fera/CRD Methods Compendium

The following new methods/validation data have been added to the Methods Compendium:

Abamectin in liver  
Alkylphosphates in kidney by IC-MS/MS  
Azaconazole in liver  
Chloralose in kidney by LC-MS/MS  
Chlorate determination in weed killer formulations by Ion Chromatography-tandem Mass Spectrometry  
Chlorothalonil in honeybees  
Dichlobenil in liver  
Diquat in kidney by oxidation with LC-fluorescence  
Dithiocarbamates in liver by carbon disulphide measurement  
Fluoxastrobin in avian liver tissues  
Fungicides on treated grain  
Indoxacarb in liver  
Isofenphos in stomach contents  
Mepiquat/chlormequat in kidney  
Multi-residue analysis procedure for honeybees  
Nicotine and cotinine in liver  
Phosphine in soil  
Propyzamide in liver  
Spinosad, indoxacarb, spiromesifen, pyrethrins and abamectin in honeybees



## References to published material

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9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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