Commercial in Confidence

Client Report No. 38512

The Chemical Analysis of Pesticides in Stem Injection Trials

Gary Scott and Stefan Gous

THE JOINT FORCES OF CSIRO & SCION

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The Chemical Analysis of Pesticides in Stem Injection Trials

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Date: **July 2005**
Client: **MAF and FBRC**
Contract No:

CSIRO SCION

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EXECUTIVE SUMMARY

Chemical analysis revealed that all selected insecticides could be detected *in vitro*. However, *in sito* detection was not possible with the exception of Methamidophos. This might be due to two factors, either the injected rates were to low and therefore could not be detected, or the insecticides were not successfully translocated into the foliage of the injected trees.

Methamidophos was also the only injected insecticide that proved to be highly successful in controlling herbivorous insects, as revealed in later phases of this project.

OBJECTIVE

Chemical analysis of uptake and translocation efficacy of stem injected insecticides. Candidate insecticides were injected into *Eucalyptus* trees. Foliage was harvested at 7m above ground level and chemically analysed to determine translocation efficacy.

Results will provide a profile of the rate and efficacy of the uptake. These data will indicate the rate of translocation and the time lapsed after injection until the maximum concentration levels are reached in the foliage. Once this maximum concentration has established, the foliage will be used in feeding bioassays to determine insecticide efficacy against *Uraba. lugens*.

KEY RESULTS

Methamidophos was detected within 24 hours after injection into the xylem of *Eucalyptus nitens*. None of the other injected insecticides translocated to detectable levels by chemical analysis, in the foliage of injected trees.

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Information for Ensis abstracting:

Contract number	
Client Report No.	3851238512
Products investigated	Methamidophos, Deltamethrin, Imidacloprid, Thiacloprid and Spinosad
Wood species worked on	Eucalyptus nitens
Other materials used	Acetonitrile, de-ionised water, acetophenone, dichloromethane, sodium chloride
Location	Rotorua, Kapenga Tree Farm

INTRODUCTION

Insecticides are agents mainly of chemical origin that control insects (Ware and Whitacre, 2004). The chemical analyses of pesticides in plant matter are routine procedures in industry. Most commonly, the analyses are for spray applications to plant matter, whether it be vegetables, seeds for crop protection or eradication of pests from vegetation.

As introduced insect pests become an increasing problem to vegetation, it poses a substantial risk to trees in both the forest and urban environment. These introduced pests seldomely have any native predators to control their numbers. Therefore pesticides are the only alternative method to control or eradicate them. Although many pesticides are commercially available, an important concern would be that pests could possibly develop resistance to any one particular pesticide. Therefore, investigating a range of pesticides for efficacy carries significant importance. If these insects develop resistance, alternative, often more toxic pesticides are required to control and manage them.

As these insect pests encroach into urban areas, the type of pesticides utilised becomes increasingly important to authorities, as these pesticides could possibly have negative impacts on humans and the natural environment. Therefore normal "blanket" pesticide spray application techniques becomes unfavourable. Due to this factor, *in-vitro* application of pesticide, i.e. no physical or direct contact with the pesticide, becomes extremely favourable.

Very few *in-vitro* processes exist for the determination of pesticides by chemical analysis. *In-vitro* analysis becomes increasingly difficult when one considers the dilution factor involved with in-vitro application into a tree.

MATERIALS AND METHODS

Physical properties of selected pesticides

Commercial name: Tamaron

Active ingredient: Methamidophos
IUPAC name: O,S-dimethyl phosphoramidothioate

The appearance of the pure chemical is a slightly hazy straw coloured liquid, with a characteristic pungent odour (rotten eggs)[1]. Methamidophos (Figure 1) has a boiling point of 188°C, but does not have a UV spectrum (Figure 2). Methamidophos is very soluble in water and dichloromethane (DCM), as well as, polyethyleneglycol (PEG). Organophosphates are esters of phosphorus having varying combinations of oxygen, carbon, sulfur and nitrogen.

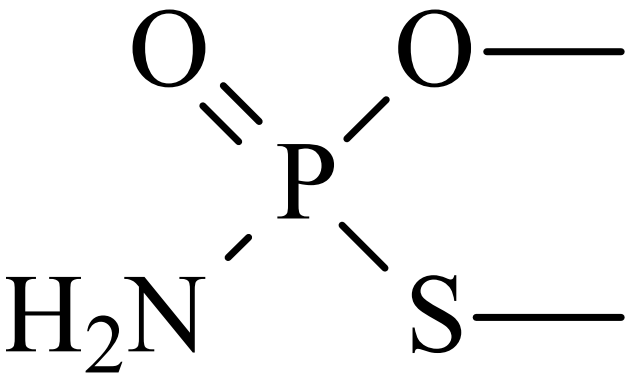

Figure 1: Chemical structure of Methamidophos.

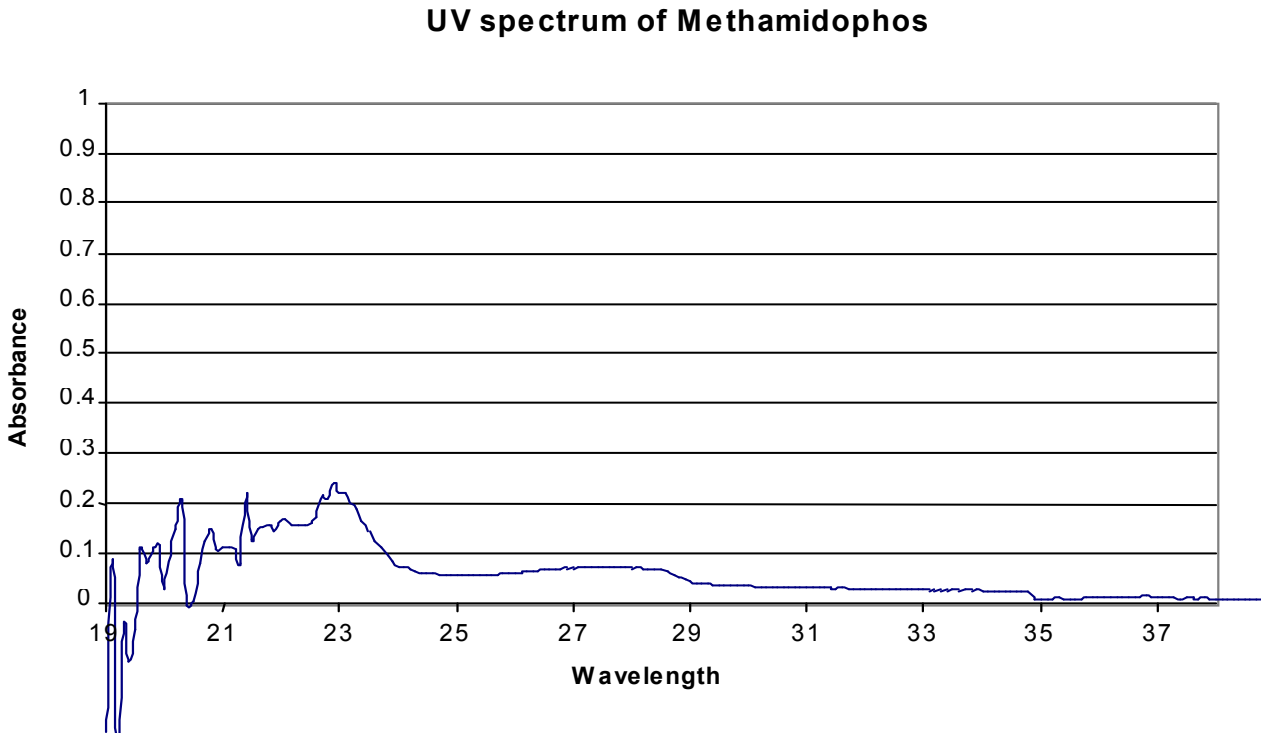

Figure 2. UV: spectrum of Methamidophos.

Mode of action: Organophosphates inhibit enzymes of the nervous system, namely cholinesterase (ChE). The enzyme is phosphorylated when it becomes attached to the phosphorous component of the insecticide, an irreversible bond. This inhibition results in the accumulation of acetylcholine (ACh) at the neuron/neuron and neuron/muscle (neuromuscular) synapses, causing spastic contractions of voluntary muscles and finally paralysis (Ware and Whitacre, 2004).

Commercial name: Decis

Active ingredient: Deltamethrin
IUPAC name: 3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarboxylic acid cyano(3-phenoxyphenyl)methyl ester

The appearance of the pure chemical is a white powder[1]. Deltamethrin (Figure 3) has a melting point 98°C and boiling point of 300°C. In the UV spectrum (Figure 4), two maxima's are observed at 228 and 268 nm. Deltamethrin is photostable, therefore it does not undergo photolysis in sunlight. It has minimal volatility consequently it provides extended residual effectiveness, up to 10 days under optimum conditions.

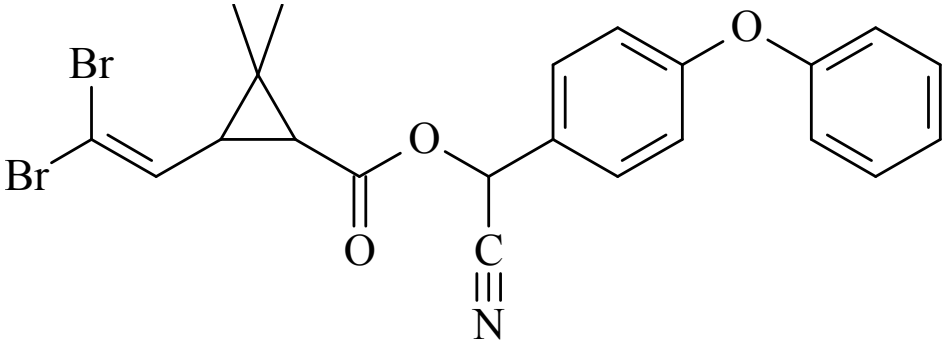

Figure 3: Chemical structure of Deltamethrin.

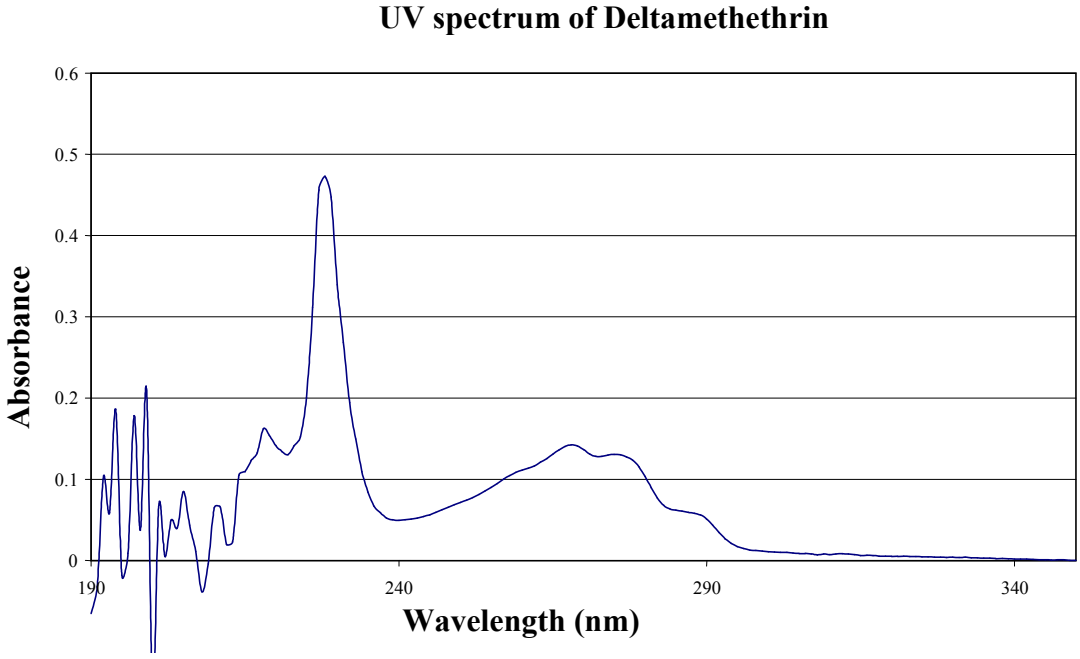

Figure 4: UV Spectrum of Deltamethrin.

Mode of action: The pyrethroids share similar modes of action, resembling that of DDT, they are considered axonic poisons. It works by keeping open the sodium channels in neuronal membranes. There are two types of pyrethroids. Type I, among other physiological responses, have a negative temperature coefficient, resembling that of DDT. Type II, in contrast have a positive temperature coefficient, showing increased kill with increase in ambient temperature. Pyrethroids affect both the peripheral and central nervous system of the insect. They stimulate nerve cells to produce repetitive discharges and eventually cause paralysis. Such effects are caused by their action on the sodium channel, a tiny hole through which sodium ions are permitted to enter the axon to cause excitation. The stimulating effect of pyrethroids is much more pronounced than that of DDT (Ware and Whitacre, 2004).

Commercial name: Confidor:

Active ingredient: Imidacloprid

In pure form, imidacloprid (Figure 5) is a colourless crystalline compound with a melting point of 144°C and an undetermined boiling point[1]. The fact the compound does not have a boiling point is a good indication that it is not volatile and therefore would degrade under high temperature well before boiling point is achieved. An important characteristic of Imidacloprid is the fact that it is not particularly soluble (0.6g/litre) in water at 20°C. A comparable solubility is that 67g of imidacloprid is soluble in a liter of dichloromethane.

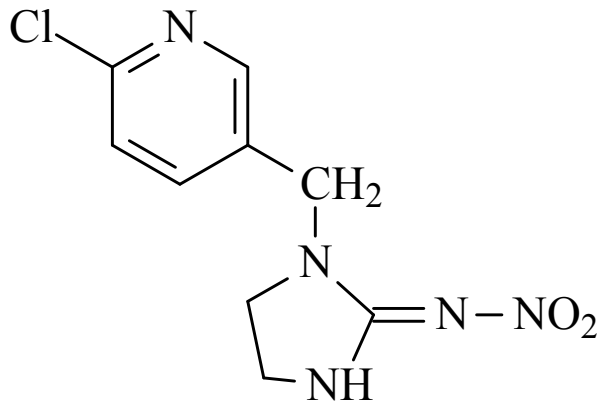

Figure 5: Chemical structure of imidacloprid.

In the UV spectrum of imidacloprid (Figure 6), a maxima's are observed at 269 and 212 nm.

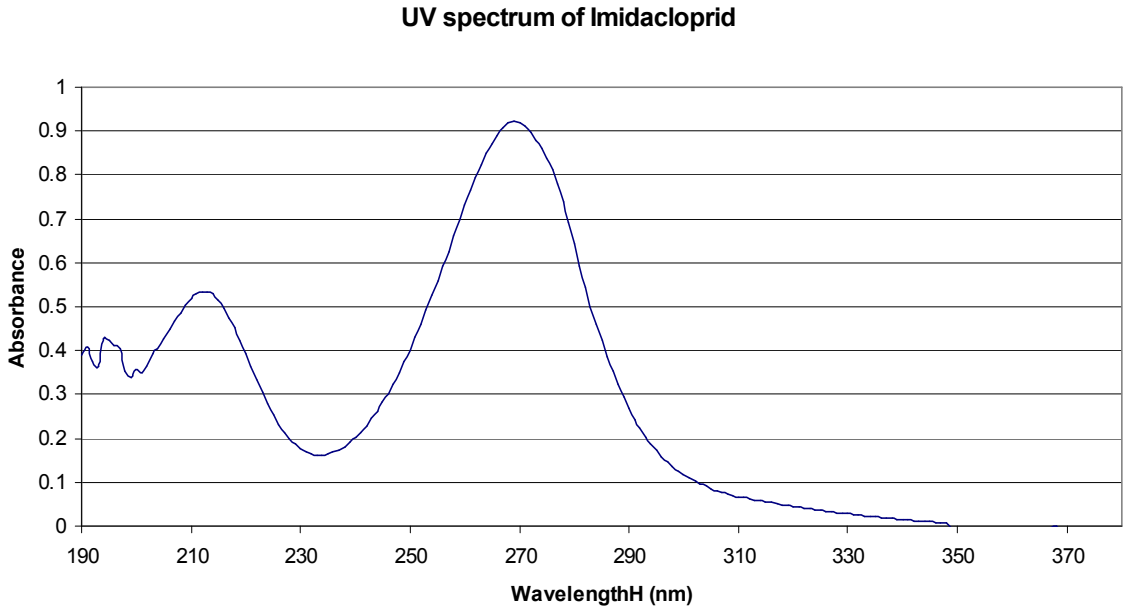

Figure 6: UV Spectrum of Imidacloprid.

Mode of action: The nicotinoids act on the central nervous system of insects, causing irreversible blockage of postsynaptic nicotinergic acetylcholine receptors, resulting in twitching, convulsions, and death, all in rapid order (Ware and Whitacre, 2004).

Commercial name: Calypso:

Active ingredient: Thiacloprid
IUPAC name: N-[3-(6-Chloro-pyridin-3-ylmethyl)-thiazolidin-2-ylidene)-cyanamide

The appearance of the pure chemical is a yellowish powder, with no apparent odour. Thiacloprid (Figure 7) has a melting point of 144°C and an undetermined boiling point and as a result suffers the same fate of imidacloprid at high temperatures. In the UV spectrum of imidacloprid (Figure 8), maxima's are observed at 242 and 200 nm. The solubility of thiacloprid in water is slight higher than that of imidacloprid, reported to be 0.186g per liter of water and a 160g/L in DCM. Despite its slightly higher solubility in water, it is still not very high with respect to solubility in organic solvents.

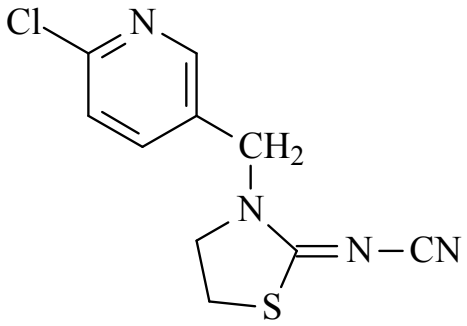

Figure 7: Chemical structure of thiacloprid.

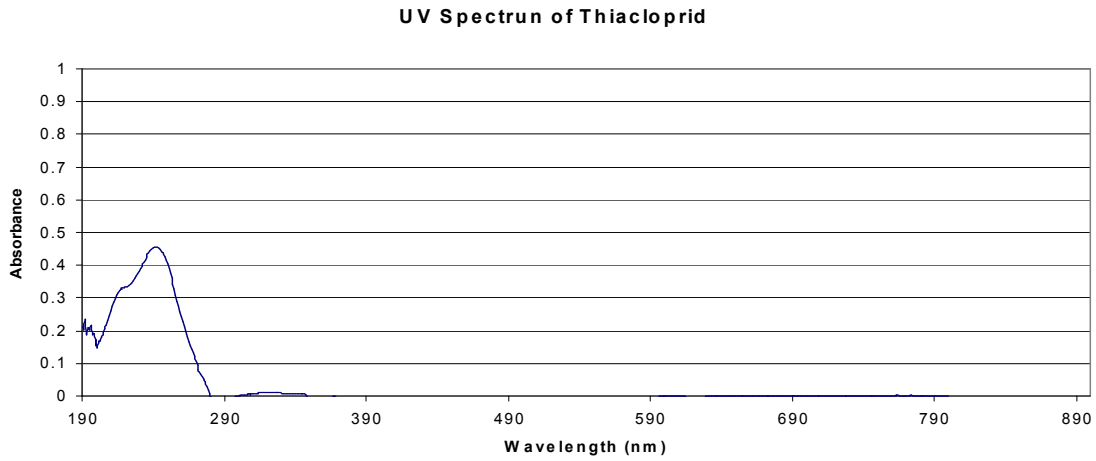

Figure 8: UV spectrum of Thiacloprid.

Mode of action: The nicotinoids act on the central nervous system of insects, causing irreversible blockage of postsynaptic nicotinergic acetylcholine receptors, resulting in twitching, convulsions, and death, all in rapid order (Ware and Whitacre, 2004).

Commercial name: Success' Naturalyte'

Active ingredient: Spinosad

One isomer of spinosad (Figure 9) is a liquid and the other is a crystal and the boiling point of this compound is undetermined[2]. In the UV spectrum of Spinosad (Figure 10), maxima's are observed at 242 and 200 nm. Spinosad is a fermentation metabolite of the actinomycete *Saccharopolyspora spinosa*, a soil-inhabiting microorganism. Spinosad is a mixture of spinosyns A and D (thus its name, spinosAD).

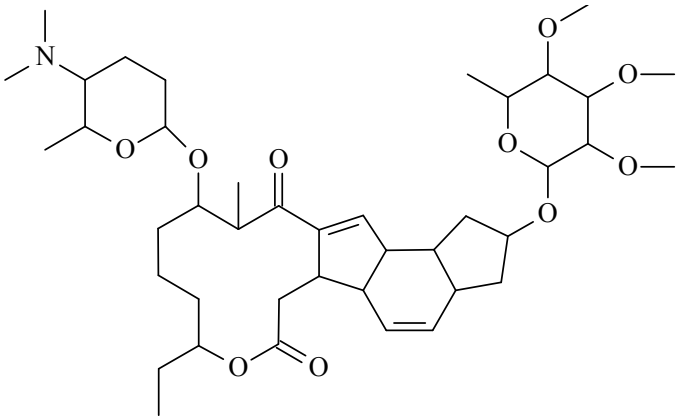

Figure 9: Chemical structure of Spinosad.

Mode of action: Spinosad acts by disrupting binding of acetylcholine in nicotinic acetylcholine receptors at the postsynaptic cell. Very similar in action to Confidor and Calypso.

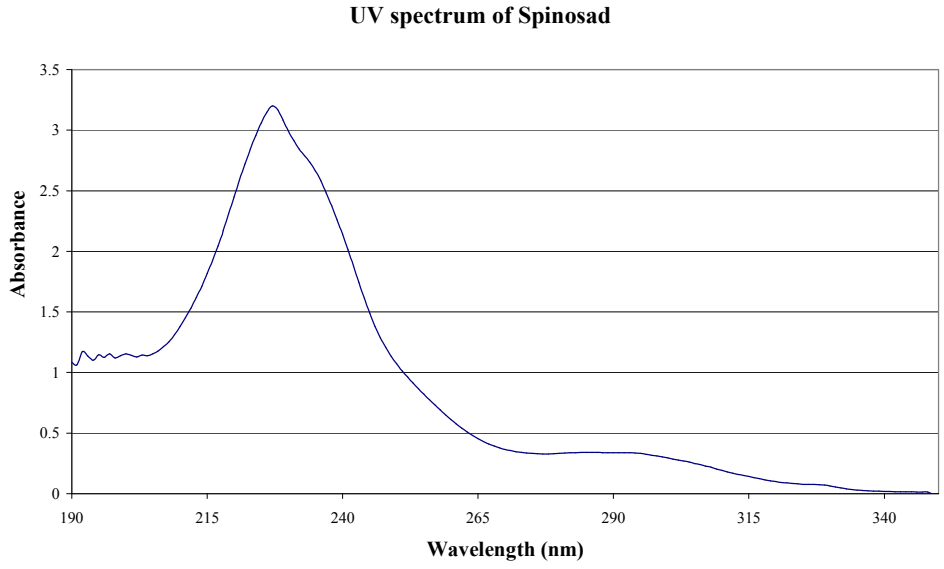

Figure 10: UV spectrum of Spinosad.

Chemical analysis:

Sample preparation

Mature foliage samples were randomly cut from the branches of injected trees at approximately 7m above ground level. Samples were placed in plastic bags, labled and kept under refrigeration at 4 degrees Celsius until processed for the bio-assays. Where possible, only mature foliage was used in all bio-assays.

The base and tip of the leaf was cut off and 5g of material cut and mixed into a 250ml round-bottomed flask.
The solution utilised for extraction and the solvent for the standards was a mixture of acetonitrile and de-ionised water in a 4:1 ratio. A 100 ppm solution of acetophenone (10ml) was added to the 5g of leaf material in the 250ml round-bottomed flask. A further 40ml of extraction solution was added to make up a final volume of 50ml.

This mixture was then refluxed for 2 hours at 80^0C. Once the mixture cooled to room temperature, the extract (dark green solution) was decanted into a separation funnel. The extract was washed with an aliquot (15ml) of saturated sodium chloride solution. The mixture was then gently shaken for 2 minutes and an aliquot of acetonitrile (10ml) was added and the gentle shaking repeat.

The organic layer was collected in a 100ml round-bottomed flask. The extraction was repeated twice and the organic layers combined.
Anhydrous sodium sulphate was added to the round-bottomed flask and shaken well. This was allowed to stand until sodium sulphate settled to the bottom of the flask, and then the solution was filtered and concentrated to less than 4ml. This solution was then accurately reconstituted to 4ml, and transferred to a 4ml autosampler vial.

Samples analysed for methamidophos extracted in dichloromethane because they were directly injected into the GC.

Instrumentation:

High Performance Liquid Chromatography (HPLC)

HPLC was performed with a system which composed of a Water's 600 pump, a Water 717 autosampler, Hewlett Packard 2487 UV detector with dual channel capability and Turbochrom (Perkin Elmer) networked software as a system controller and data processor.

Analytical conditions were as follows:
Alltech Apollo C18 column (250mmx4.6mm i.d, 5µm; Alltech NZ) and Phenomonex C18 guard column was utilised at a flow rate of 0.8ml/min. The mobile phase started with a 50% acetonitrile gradient / 50% deionised water followed by 20 min linear gradient to 80% acetonitrile / 20% deionised water and the final composition was maintained for 10 min.

The standards were authenticated by LC-MS based on molecular mass of the respective pesticides.

Gas Chromatography – Flame Ionised Detector (GC-FID)

GC was performed on an Agilent (6890N) gas chromatograph with an integrated autosampler and Chem Station® software (Agilent Technologies). The gas chromatograph was equipped with a split-splitless injector and a capillary column coated with a 0.25 µm layer of HP5-A phase (Agilent). All analyses were done with helium as the carrier gas, at linear velocity of 38 cm per second at a column temperature of 60°C. The injector temperature was 250°C and samples were injected in the splitless mode. Samples (2 µL) were analysed using a temperature program of 5°C per minute from 60 (hold for 1 min) to final temperature of 250°C. The detector temperature was set at 250°C.

Methamidophos standard was authenticated by GC-MS (Figure 11).

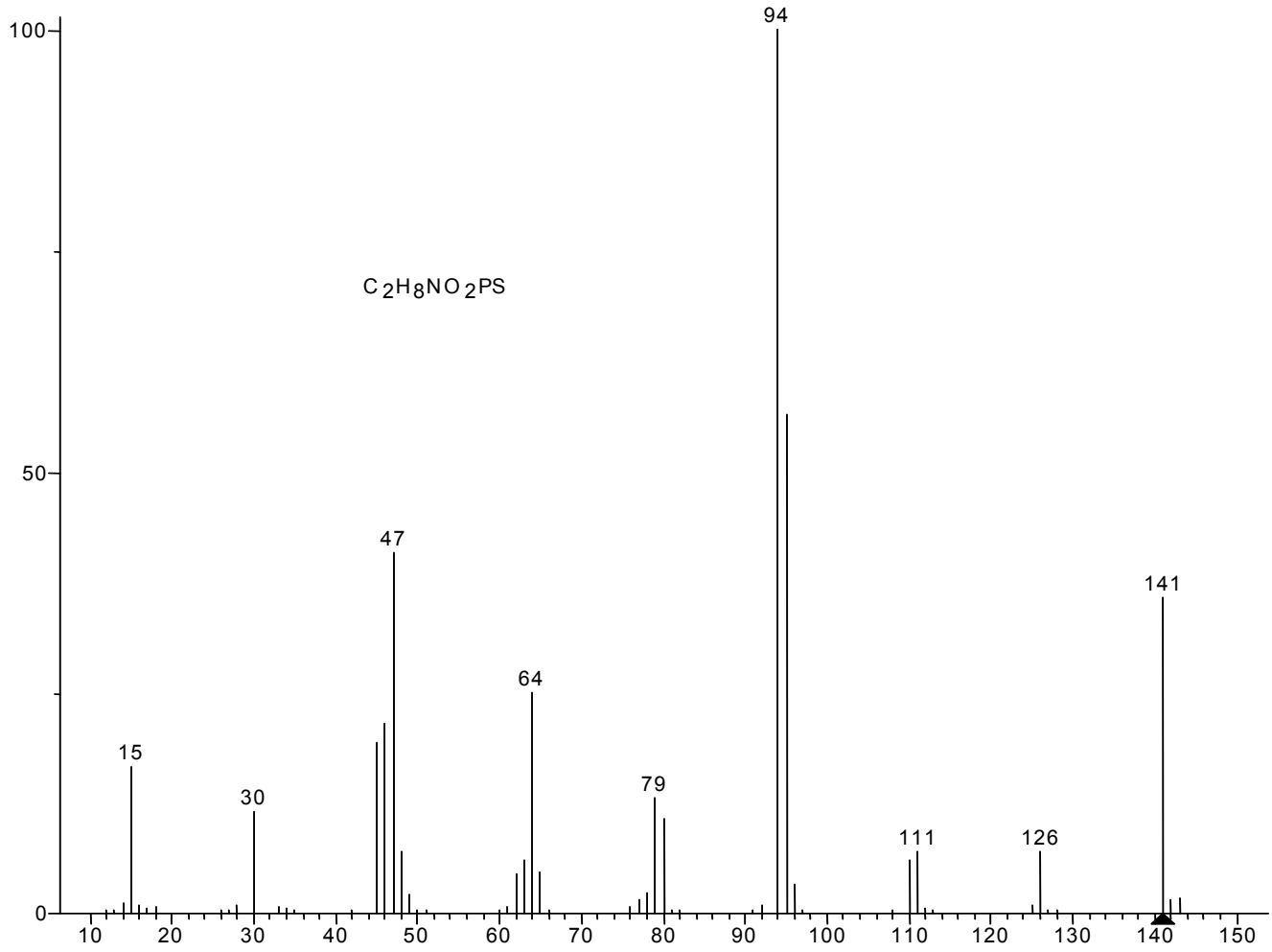

Figure 11: Electron impact (EI) mass spectrum of methamidophos.

Ultra Violet- Visible (UV-Vis) Spectroscopy

UV-Vis spectroscopy was performed on a Varian Cary UV-Vis Spectrophotometer 300 Bio with Cary WinUV software. All pesticides were analysed utilising double beam acquisition, at approximately 1 ppm, but concentrations were adjusted until absorbencies of the pesticides were below an absorbance of one. Imidacloprid, Thiacloprid and spinosad were dissolved in acetonitrile, with acetonitrile as the reference blank. Deltamethrin and methamidophos were dissolved in dichloromethane, with a dichloromethane blank.

RESULTS AND DISCUSSION

Confidor

Imidacloprid was detected in the working standards prepared for analyses of plant material. When an analysis of plant material was done the pesticide was not detected. The absence of imidacloprid from plant extracts was confirmed by LC-MS (selective mass detection).

Calypso

Thiacloprid was detected in the working standards prepared for analyses of plant material. When an analysis of plant material was done the pesticide was not detected. The absence of thiacloprid from plant extracts was confirmed by LC-MS (selective mass detection).

Success' Naturalyte'

Spinosad was detected in the working standards prepared for analyses of plant material. When an analysis of plant material was done the pesticide was not detected. The absence of spinosad from plant extracts was confirmed by LC-MS (selective mass detection).

Decis

Initial trials were conducted with Decis (deltamethrin) by HPLC-UV, the pesticide was injected into multiple trees at varying concentrations and sampled at different time intervals to determine the rate of uptake by the tree from the first detection to the time the respective pesticide could longer be detected.

Decis was not detected at the lower concentrations, but only detected at the highest concentration (Table 1).

Table 1: Detection of Decis.

Vol. Injected per tree (ml)	Detection after 5hrs	Detection after 10hrs	Detection after 20hrs	Detection after 40hrs
5	no	no	no	no
10	no	no	no	no
20	no	no	no	no
40	no	no	yes	yes

After the first trial, the next phase was to quantify the levels of the respective pesticides and to determine the duration required for translocation of the pesticide to the leaves. Therefore, the experiment was repeated.

Decis was not detected in any of these samples at any of the levels injected in this trial.

It was considered to increase the injection volumes of Decis because the dilution factor, from the point of injection in the stem to the translocation into the leaves, was so great that levels in the leaves were below the detection levels of the HPLC. Based on this reasoning, a trial was commenced for increasing the volume of Decis injected into the tree (between 40 ml and 60 ml per tree). Decis was not detected under these condition (by HPLC-UV) and therefore these samples were analysed by LC-MS as a separate study to confirm it's absence.

LC-MS offers better selectivity, therefore greater sensitivity for a compound in a complex matrix (such as a leave) because it utilises a detector, which is mass selective. This mechanism thus allows one to select the formula mass of deltamethrin and as a result "filter" the signal. Unfortunately LC-MS is not as good for quantitation as HPLC-UV, therefore was never considered for being a fundamental analytical technique for determination of pesticides in leaves, but served to confirm and unequivocally identify the presence of the pesticide.

Decis was not observed by LC-MS.

Tamaron

Tamaron (methamidophos) was analysed by GC-FID because a UV spectrum was not observed. The pesticide was injected into multiple trees at varying concentrations and sampled at different time intervals to determine the rate of uptake by the tree from the first detection to the time the respective pesticides could longer be detected.

Tamaron was detected in all samples at these respective concentrations and at the respective intervals.

After the first trial, the next phase was to quantify the levels of the tamaron and to determine the duration required for translocation of the pesticides to the leaves. Therefore, the experiment was repeated.

It was determined that tamaron took approximately 24 hours to translocate to the leaves of the respective trees (Figure12). Thereafter, it took approximately 72 hours before accurate chemical detection was not possible (tamaron concentration was below instrumentation or methods detection limit).

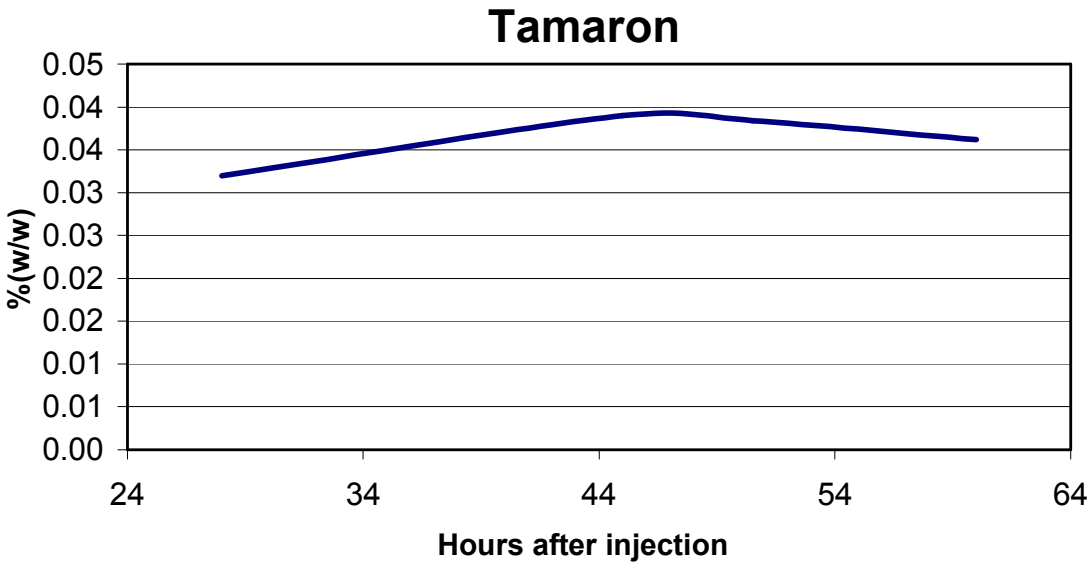

Figure 12: Initial 64 hours of translocation rate.

RECOMMENDATIONS AND CONCLUSIONS

Chemical analysis revealed that *in sito* detection of the injected insecticides were not always possible. Therefore it is suggested that the chemical properties of potential future injected pesticides need to be thoroughly reviewed before utilised.

It is strongly recommended that more work should be done on water soluble insecticides with the emphasis on organophosphates. This group of pesticides appears to have a significant advantage when used in direct injection applications, over most other compounds. This is largely due to it's translocation capability in the plant vascular system and high toxicity to most known herbivorous insects.

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