Pesticide Residue Analysis of Fruit and Animal Extracts Using Liquid Chromatography-Orthogonal Acceleration Time-of-Flight Mass Spectrometry (LC-oa-ToFMS)

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Introduction

The UK government is committed to ensuring the safety of food and drink available to the consumer. It therefore carries out a comprehensive surveillance programme to monitor the presence/quantity of pesticide residues in foodstuffs on sale throughout the UK. A series of surveys which cover the main food groups of fruit and vegetables, cereals and cereal products and products of animal and fish origin are carried out annually, with the purpose of monitoring being for the following reasons:

1. to check that no unexpected residues are occurring
2. to check that statutory UK maximum residue levels (MRL's) are not exceeded
3. to carry out enforcement programs
4. to check that human dietary intakes of residues are at acceptable levels

In addition to monitoring the presence of pesticide residues in foodstuffs, the government, via the Wildlife Incident Investigation Schemes (WIIS), also investigates deaths of wildlife, including beneficial insects, companion animals and some livestock, where there is strong evidence to indicate that pesticide poisoning may be involved. Incidents that are known to involve pesticides are usually assigned to one of the four following categories:

1. Approved use
2. Misuse
3. Abuse
4. Unspecified use

For both monitoring schemes, the use of analytical techniques and state of the art instrumentation capable of quantitatively screening/confirming ultra-low levels of residues in a variety of matrices is required. Traditionally, GC-MS analysis, using a combination of ionisation and scan methods, has been used since many of the pesticides sought were amenable to GC-MS. However, compounds that are involatile, thermally labile and/or polar, present the residue chemist with a difficult problem particularly at the low ppb reporting levels demanded by the associated regulatory authorities.

Pre and post harvest treatment of fruit and vegetables can involve the application of a virtual pesticide 'cocktail', which will often contain substances of different chemical functionality and physical properties. This can affect the complexity of the analysis, potentially requiring a multi-chromatographic strategy and lengthy sample preparation procedures. However, methodologies that minimise sample preparation, reduce analysis times and provide increased specificity/selectivity in a variety of matrices are preferred. In many cases, the development and use of novel multi-residue methods has significantly increased.

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With the advent of atmospheric pressure ionisation (API) sources coupled with continual improvement in instrumentation design, the residue chemist has been able to apply liquid chromatography-mass spectrometry (LC-MS) techniques for pesticide residue screening. Such technology combines the advantages of both LC and MS e.g. the simultaneous separation and detection of various classes of compounds that are not readily amenable to GC-MS without arduous derivatisation. The versatility and robustness of such ion sources also means that it is also possible to minimise sample preparation and reduce analysis times by employing the multi-residue screening of crude extracts i.e no sample clean-up - just simple filtration followed by direct analysis. Multi-residue screening methods have been developed using tandem quadrupole mass spectrometry (LC-MS/MS) and lower analyte detection limits have been achieved due to the enhanced sensitivity and selectivity afforded by tandem MS. In one example, the quantitative multi-residue analysis of pesticide residues in crude extracts from turnips has been demonstrated[1]. The corresponding target suite of pesticides involved would have normally required a multi-method approach and extensive sample clean-up procedures.

LC-MS is now a widely accepted technique for many analytical applications and, in the case of pesticide residue screening, has been shown to be highly beneficial. As well as being able to use quadrupole technology for LC-MS instrumentation, over the past few years, orthogonal acceleration Time-of-flight (oa-TOF) mass spectrometry has been shown to be extremely powerful for many different analytical applications. The benefits of such technology for LC-MS experiments include:

- Elevated mass spectral resolution (>5000 FWHM)
  - providing a high degree of selectivity (removing matrix related interferences at same nominal mass using exact mass chromatograms)
- Exact mass measurement (< 5ppm RMS)
  - providing a high degree of specificity for confirmation
  - allowing determination of elemental compositions of unknowns
- Efficient instrument duty cycle
  - non-scanning instrument providing high sensitivity with full spectral data
- Fast acquisition mass analyser
  - generating high quality data for narrow LC peaks

As such benefits are available with oa-TOF instrumentation, in this application note we describe a collaborative study that was carried out to investigate the use of LC-oa-TOFMS for pesticide residue screening assays. The study was split into 2 parts:

1. Identification and quantification of 17 target pesticide residues present in crude extracts from organic grapes that had been fortified at 3 reporting levels (0.1, 0.05 & 0.02mg/kg)
2. Identification and confirmation of an unknown poison in red kite and buzzard-liver samples.

Using suitable examples in each case, the benefits of using oa-TOF for pesticide residue analysis will be highlighted: - these include the advantages of exact mass measurement, high instrument sensitivity for low level analyte detection and the ability to perform quantitative assays.

Experimental Methods

Fortified organic grape samples (10g sample weight) were extracted using ethyl acetate. A solvent exchange to methanol was performed and the crude extracts obtained (0.2g extract/mL) were simply filtered using an Acrodisc 45µM PTFE syringe filter. The two separate bird of prey liver samples were solvent extracted using methanol to produce crude extracts. No sample clean-up was employed. Both the grape and bird of prey liver crude extracts were then subjected to exact mass LC-MS analysis using the following conditions:

LC Conditions

HPLC :	Waters 2790
Column :	Waters Symmetry C18, 2.1x100mm
Flow rate:	0.3mL/min
Solvent A :	10mM ammonium acetate
Solvent B :	Methanol
Gradient :	0min=95%A 2min=75%A 8min=2%A 12min=2%A 13min=95%A and re-equilibrate
Injection Vol :	10µL

MS Conditions

MS :	Micromass LCT with dual electrospray ion source[2,3] (see Figure 1 and 2)
Ion mode :	ESI+ve (for grape extracts) ESI-ve (for bird of prey extracts)
Capillary :	3000V (ESI+ve) 2500V (ESI-ve)
Cone :	20V
Source temp :	120°C
Desolv. Temp :	350°C
Acquisition mode:	centroid data

Exact Mass Measurement Conditions

Lock Mass : Sulfadimethoxine ($C_{12}H_{14}N_4O_4S$) infused at 10µL/min

Acquisition time : 1 second

Inter-spray time : switching time every 10 seconds

Dual Electrospray Design and Operation

Figure 1 shows the schematic diagram of the Micromass LCT. To facilitate exact mass measurements of LC peaks on oa-TOF, only a single reference 'lock mass' is required. The use a dual electrospray ion source (Figure 2) overcomes practical problems associated with the traditional method of obtaining exact mass measurement of chromatographic peaks i.e infusion of the lock mass compound into the eluant stream post-column. Such problems encountered included variation of reference signal with LC gradients, mass interferences with analytes having the same nominal mass and suppression of the reference compound with high concentrations of analyte response. The use of a dual electrospray ion source for exact mass measurement of LC peaks therefore simplifies the practical difficulty associated with exact mass measurement.

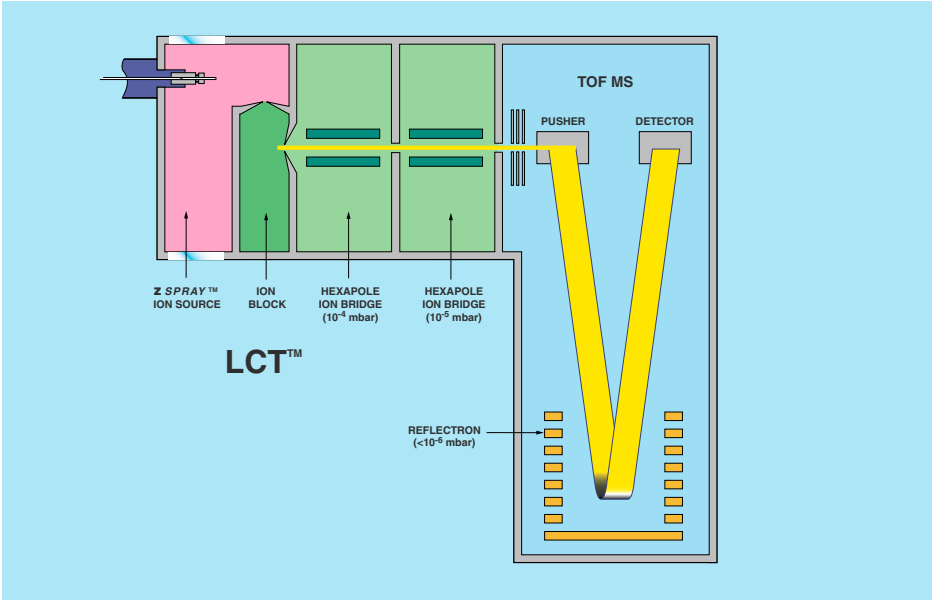

Figure 1. Schematic Diagram of Micromass LCT.

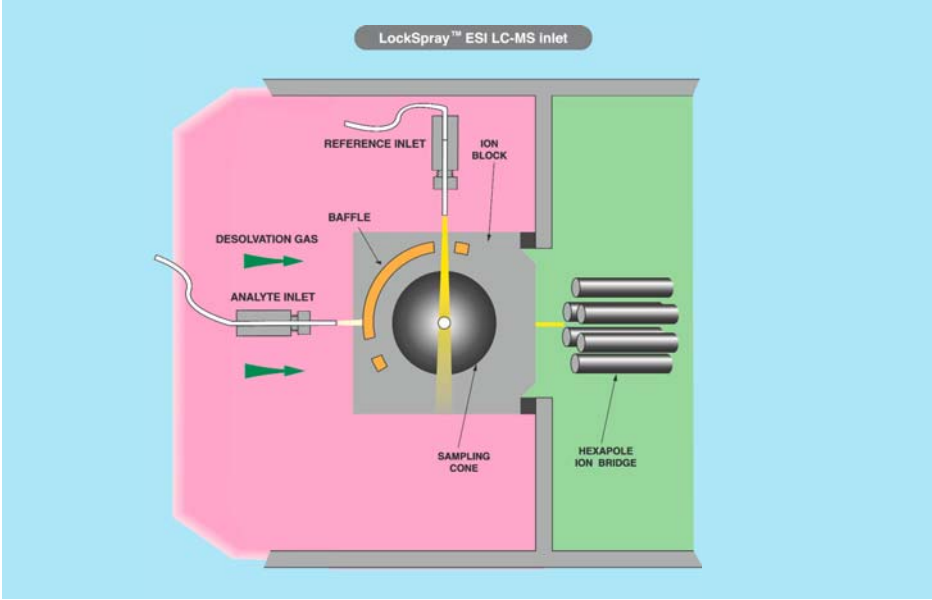

Figure 2. Dual Electrospray Schematic.

Results and Discussion

1. Organic Grape Extracts

Using the chromatographic conditions described, all 17 target pesticides in the crude extracts were separated within the 16 minute run time. Figure 3 shows a typical BPI chromatogram obtained from standard compounds dissolved in mobile phase solvent. The grape samples were crude extracts subjected to no sample clean up (ethyl acetate extraction only), and some signal suppression was observed probably due to the presence of co-extractants in the matrix, but all 17 pesticides were detected in the lowest level matrix matched standard and in the sample extracts.

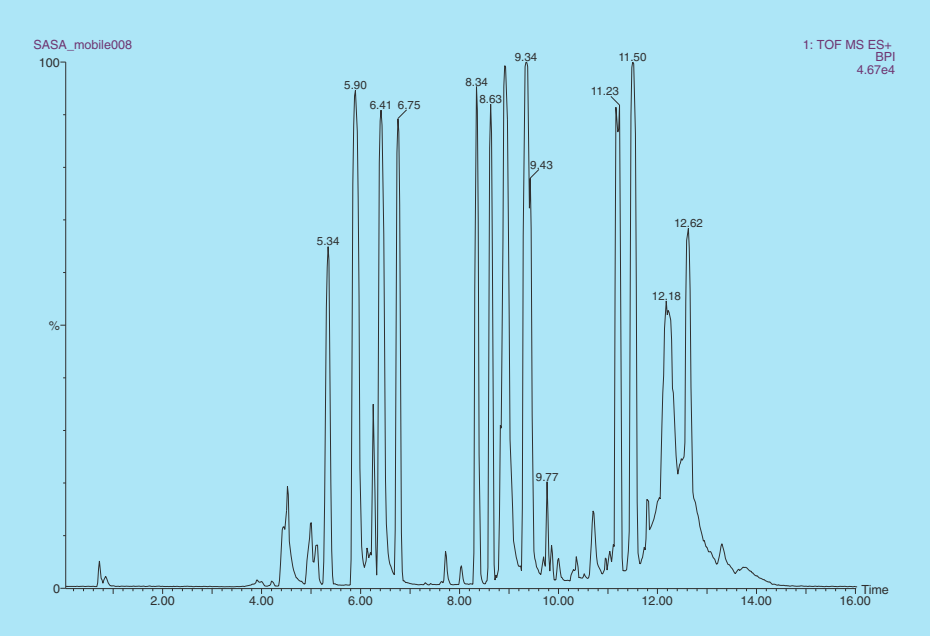

Figure 3. Typical Chromatographic Separation.

Table 1 lists the 17 pesticides targeted in this experiment along with the exact mass measurements obtained using the dual electrospray ion source. All mass measurements were better than 1.8mDa allowing confirmation of the target compound.

All samples analysed were exact mass measured as the compounds eluted from the column, and exact mass chromatograms were reconstructed using a narrow window (0.05Da) around the mass of interest. Using carbosulfan as an example ($M+H^+$=381.2212), Figure 4 clearly demonstrates the improved selectivity of exact mass chromatograms over nominal mass chromatograms (1Da) by removal of interferences from matrix material at the same nominal mass. This improved selectivity offers lower limits of determination with oa-TOF when compared to low resolution instruments because signal to noise (S:N) is often improved (Figure 5).

Using matrix matched standards to generate calibration curves, the 17 target pesticides listed in Table 1 were quantified in 2 grape samples at the 3 different reporting levels (0.1, 0.05 & 0.02mg/kg depending on the pesticide of interest). Linear calibration curves were applied with no fit weighting and excluding the origin. Using carbosulfan and fenarimol as examples, Figures 6a and 6b show the typical calibration graphs obtained. Good linear curves were obtained with correlation coefficients (r) better than 0.99 achieved over the calibration range.

2. *Bird of Prey Liver Extracts*

The potential of LC-oaTOFMS to determine the identification of unknown analytes was investigated using two avine samples. The livers from a buzzard and a red kite, two protected birds of prey, fortified with an 'unknown' poison were solvent extracted using methanol and then submitted for LC-MS analysis using the conditions described.

For both samples, the chromatogram contained two distinct peaks having retention times of 6.0 and 6.7 minutes. Figure 7 shows an example chromatogram obtained from the buzzard liver sample.

Initial interrogation of the mass spectra for each peak indicated that the compound under investigation in this experiment potentially contained chlorine or bromine elements with a monoisotopic molecular anion at $[M-H]^-$=307. However, as the data acquired was exact mass measured, elemental compositions could be calculated from the mass measurements obtained. Figure 8 shows the exact mass measured spectrum associated with the main chromatographic peak (6.0 minutes) in the buzzard sample along with the generated elemental composition report for the monoisotopic mass.

Pesticide	Formula	Theoretical M+H	Measured M+H	mDa Error
3-hydroxycarbofuran	$C_{12}H_{15}NO_4$	238.1079	238.1095	1.6
Carbendazim	$C_9H_9N_3O_2$	192.0773	192.0758	1.5
Carbofuran	$C_{12}H_{15}NO_3$	222.113	222.1116	1.4
Carbosulfan	$C_{20}H_{32}N_2O_3S$	381.2212	381.2210	0.2
Fenarimol	$C_{17}H_{12}Cl_2N_2O$	331.0405	331.0401	0.4
Fenbutatin-oxide	$C_{60}H_{78}OSn_2$	517.2129	517.2126	0.3
Imazalil	$C_{14}H_{14}Cl_2N2O$	297.0561	297.0566	0.5
Methiocarb	$C_{11}H_{15}NO_2S$	226.0902	226.0910	0.8
Methiocarbsulfone	$C_{11}H_{15}NO_4S$	258.08	258.0810	1.0
Methiocarbsulfoxide	$C_{11}H_{15}NO_3S$	242.0851	242.0849	0.2
Methomyl	$C_5H_{10}N_2O_2S$	163.0541	163.0538	0.3
Myclobutanil	$C_{15}H_{17}ClN_4$	289.122	289.1222	0.2
Penconazole	$C_{13}H_{15}Cl_2N_3$	284.0721	284.0735	1.4
Propiconazole	$C_{15}H_{17}Cl_2N_3O_2$	342.0776	342.0794	1.8
Pyrimethanil	$C_{12}H_{13}N_3$	200.1187	200.1174	1.3
Tebuconazole	$C_{16}H_{22}ClN_3O$	308.1529	308.1541	1.2
Thiabendazole	$C_{10}H_7N_3S$	202.0439	202.0426	1.3

Table 1. Exact Mass Measurement Results.

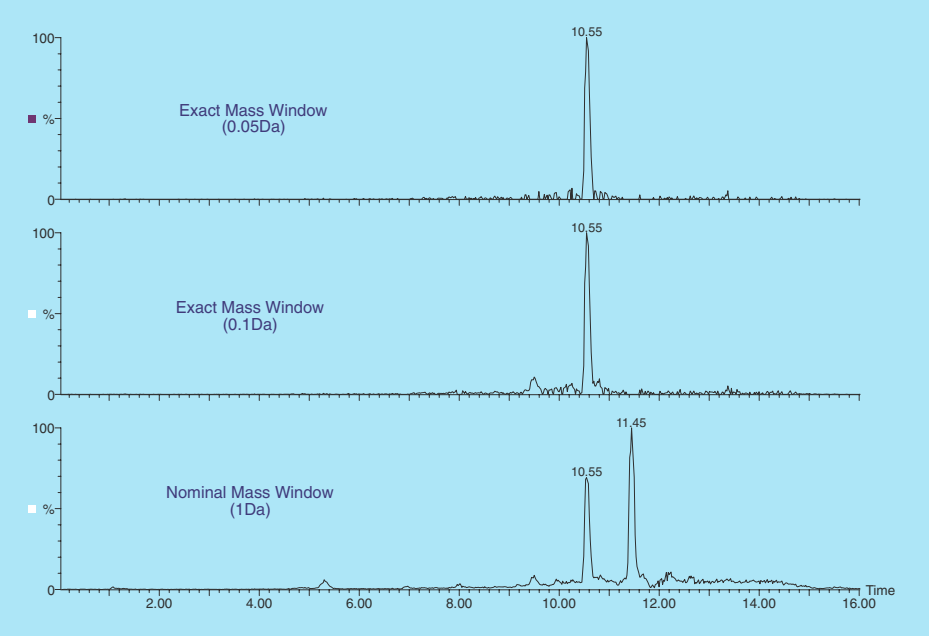

Figure 4. Exact Mass vs Nominal Mass Chromatograms for Carbosulfan.

From the exact mass measurement and elemental composition results, the unknown pesticide was correctly identified as chloralose ($C_8H_{11}Cl_3O_6$, $[M-H]^-$=306.9543 Da). Figure 9 shows the two isomeric structures of α and β-chloralose which correspond to the two chromatographic peaks eluting at 6.0 and 6.7 minutes, respectively.

The monoisotopic exact mass measurements obtained for both avine samples were:

Buzzard sample = 306.9555 (1.2mDa, 3.9ppm error)
Red Kite sample = 306.9546 (0.3mDa, 1ppm error)

Chloralose is used as a rodenticide against mice, for indoor use only. It does, however, have a more sinister and deliberate use as a poison to illegally kill birds of prey and other animals. Traditional methods of chloralose analysis involved lengthy sample preparation and clean-up procedures followed by derivatisation before determination using gas chromatography with electron capture detection (GC-ECD). Instead, by using LC-MS, a simple extraction procedure alone was required before analysis, thus minimising the overall analytical methodology. Furthermore, the use of oa-TOFMS in this study shows that exact mass measurement can provide powerful information when identifying and confirming unknown analytes by generating candidate elemental compositions.

As a further example of exact mass measurement from this study, complementary confirmatory evidence was provided by taking into account the ^{35}Cl and ^{37}Cl isotopes. From the exact mass measured spectrum shown in Figure 8, the possible elemental compositions were calculated using the relative contribution of the ^{35}Cl and ^{37}Cl isotopes. Table 2 shows the exact mass measurement data and candidate empirical formula for the buzzard liver extract.

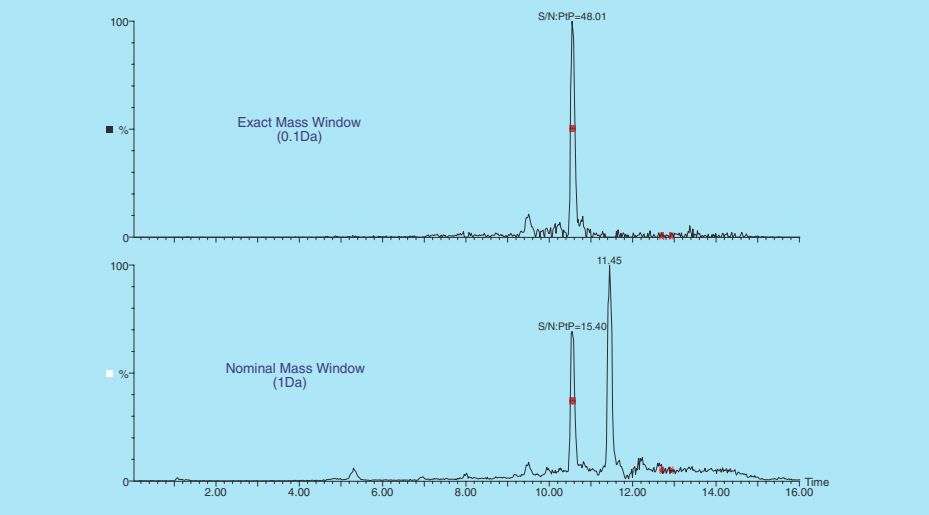

Figure 5. Signal to Noise Comparison of Exact and Nominal Mass Chromatograms.

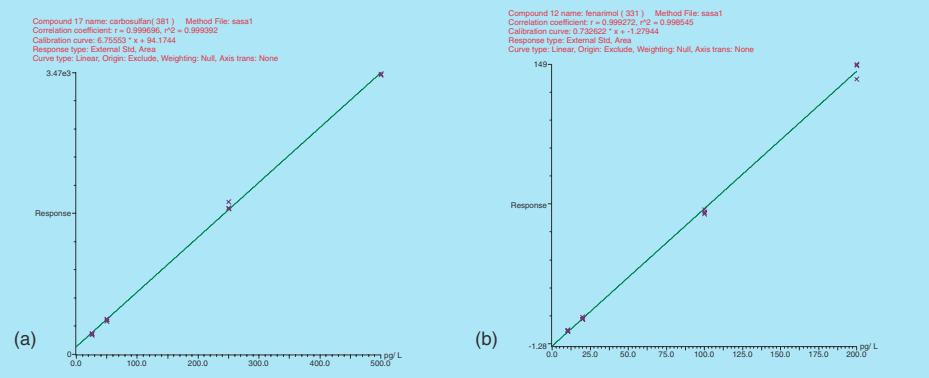

Figure 6a and 6b. Quantitation Calibration Graphs for Carbosulfan and Fenarimol.

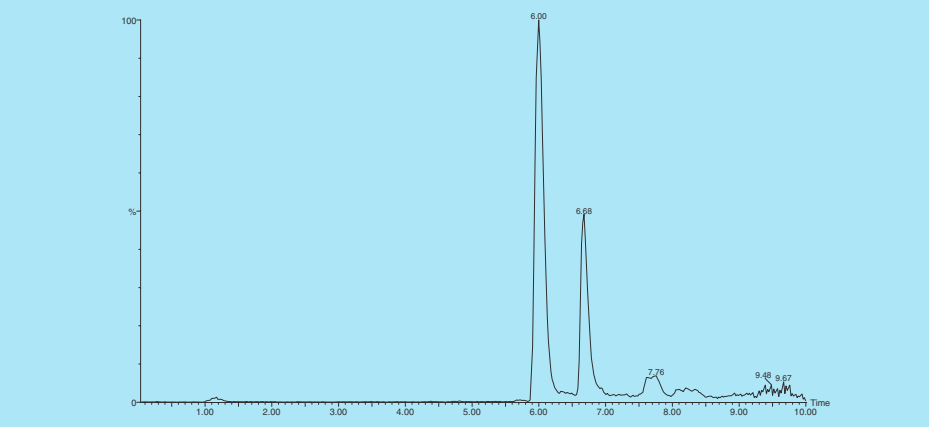

Figure 7. Chromatogram Obtained for Buzzard Sample.

Conclusions

The applicability of LC-oaTOFMS for multi-residue pesticide analysis in fruit and animal extracts has been demonstrated. In part 1 of the study, 17 target pesticides were identified and quantified in crude extracts from fortified organic grape samples. Samples were submitted for LC-MS analysis without any clean-up which significantly reduced the complexity of the analytical methodology. A single generic multi-residue LC-MS method was applied to all the analytes under investigation.

Using 'real-time' exact mass measurements, all 17 pesticides were successfully identified and confirmed within the complex matrix. Using suitable examples, data produced highlighted the benefit of being able to reconstruct exact mass chromatograms (0.05Da) in comparison to nominal mass chromatograms (1Da). The enhanced specificity allowed matrix related interferences to be removed which minimised the potential of any erroneous result, e.g. false positives. The use of oa-TOF with its inherent full spectral MS sensitivity simultaneously allowed all 17 pesticides to be detected at the ppb level. Using matrix matched standards, all 17 target pesticides were also successfully quantified at 3 reporting levels.

In part 2 of the study, exact mass measurement data from two avine liver samples allowed an unknown poison to be correctly identified as chloralose, a rodenticide often used illegally to kill birds of prey.

Complementary evidence was also provided from the exact mass measurement of the chlorine isotopic ions and the molecular anion isotope pattern in the full scan mass spectrum.

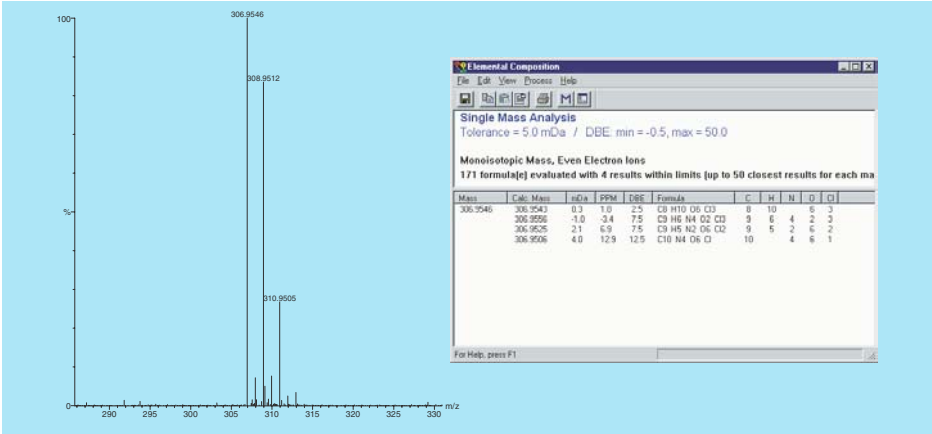

Figure 8. Exact Mass Spectrum of Unknown Pesticide (Buzzard Sample).

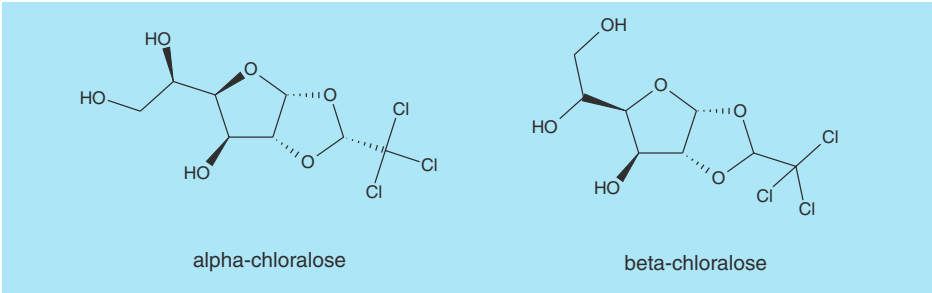

Figure 9. Two Isometric Structure of Chloralose.

Measured Mass	% Relative Abundance	Theoretical Mass	Error (mDa)	Error (ppm)	Formula
306.9546	100	306.9543	0.3	1.0	$C_8H_{10}{}^{35}Cl_3O_6$
		306.9535	1.1	3.6	$C_9H_{10}{}^{35}Cl^{37}Cl_2O_5$
308.9512	83.5	308.9513	-0.1	-0.5	$C_8H_{10}{}^{35}Cl_2{}^{37}ClO_6$
		308.9505	0.7	2.2	$C_9H_{10}{}^{37}Cl_3O_5$
310.9505	26.8	310.9492	1.3	4.1	$C_7H_{10}{}^{35}Cl_3O_7$
		310.9484	2.1	6.8	$C_8H_{10}{}^{35}Cl^{37}Cl_2O_6$
		310.9539	-3.4	-11.0	$C_{10}H_7{}^{35}Cl^{37}ClO_7$

Note - correct assignment

Table 2. Exact Mass Measurements for Isotope Peaks of Chloralose.

Collaborative studies will continue to further investigate the use of oa-TOF for the analysis of environmental samples and include the analysis of a variety of fruit and vegetable extracts, the use of different HPLC separations including fast chromatography and the analysis of 'real' biological extracts containing incurred pesticide residues.

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