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Development and validation of a method for the analysis of total amitraz in fruit and honey with quantification by gas chromatography-mass spectrometry

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Abstract

The EU Maximum Residue Limit (MRL) definition for amitraz is ‘the sum of amitraz plus all its metabolites containing the 2,4-aniline moiety, expressed as amitraz’. A rapid and sensitive method has been developed and validated in-house for the determination of total amitraz in pears, strawberries, oranges and honey. Samples were hydrolysed (under acidic followed by alkaline conditions) to convert amitraz to 2,4-dimethylaniline which was then partitioned into 2,2,4-trimethylpentane prior to quantification by GC-MS. The method was validated at 0.02 and 0.1 mg kg⁻¹ amitraz (well below MRL requirements) with a lowest calibrated level (LCL) for 2,4-dimethylaniline of 0.002 mg kg⁻¹ (equivalent to 0.0024 mg kg⁻¹ amitraz). A single partition step yielded recoveries of approximately 60 % (with % CV’s in the range 3.3 – 8.2), which is satisfactory for screening purposes. A second partition increased recoveries by 10 - 20 % making the method suitable for the quantification of residues.

1
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3 **Keywords:** amitraz, 2,4-dimethylaniline, gas chromatography-mass spectrometry
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5 (GC-MS), hydrolysis
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10 **Introduction**

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15 Amitraz is a non-systemic acaricide and insecticide, with contact and respiratory
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17 action. Prior to the partial withdrawal of amitraz-based products, on 12 August 2004
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19 (Annex 1, Council Directive 91/141/EEC), its uses included control of all the growth
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21 stages of mites, insects, flies and aphids in a wide variety of crops including
22
23 pome/citrus/stone/bush/soft fruit, curcubits, capsicums and tomatoes. It was also used
24
25 as an animal ectoparasiticide to control ticks, mites and lice on domestic and farm
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27 animals (The Pesticides Manual, 2003). Following this date the use of amitraz is still
28
29 permitted on pear trees, after harvest, in The Netherlands, UK and Portugal until 30
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31 June 2007. In apiculture amitraz is used to control the honeybee parasite *Varroa*
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33 *destructor* (Korta et al. 2001).
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42 The MRL definition for amitraz is 'the sum of amitraz plus all its metabolites
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44 containing the 2,4 aniline moiety'. In plants amitraz is rapidly degraded to N-(2,4-
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46 dimethylphenyl)-N'-methylformamidine and 2,4-dimethylformanilide (The Pesticides
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48 Manual 2003). Methods that are based solely on the analysis of parent amitraz could
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50 potentially underestimate the concentration of total amitraz in the sample. The
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52 hydrolysis pathway of amitraz (figure 1) (Pierpoint et al. 1997) shows that amitraz is
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54 readily hydrolysed (under acidic conditions) to 2,4-dimethylphenyl formamide, which
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56 can be rapidly hydrolysed to 2,4-dimethylaniline under alkaline conditions. It is
57
58 commonly accepted that 2,4-dimethylaniline is the stable end-point of amitraz
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3 degradation (Taccheo et al. 1988). Methods of analysis should therefore include a
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5 hydrolysis step to meet the MRL definition.
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10 [Insert Figure 1 about here]
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15 Existing methods for the analysis of amitraz are time-consuming and do not always
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17 include the analysis of the metabolites or provide satisfactory confirmation of the
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19 analyte. For example Muñio and Lozano 1993 and Maver and Poklukar 2003 both
20
21 developed methods for the analysis of the parent analyte only in honey using gas
22
23 chromatography-mass spectrometry (GC-MS) detection. Hornish et al. 1984,
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25 Taccheo et al. 1988 and Jiménez et al. 2002 all developed methods involving steam
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27 distillation and/or hydrolysis with a derivitisation prior to detection using an electron-
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29 capture detector (ECD).
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36 The aim of this work was to develop a rapid method which would allow the
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38 quantification of 'total' amitraz and provide confirmation of the identify of 2,4-
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40 dimethylaniline in representative matrices including those for which there is still
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42 permitted use, to ensure compliance with recent legislation.
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49 **Material and methods**

50 *Chemicals*

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52 Hydrochloric acid (laboratory reagent grade), sodium hydroxide pellets (analytical
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54 reagent grade), sodium chloride (analytical reagent grade) and 2,2,4-trimethylpentane
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56 (isø octane), methanol and acetonitrile (all HPLC grade) were obtained from Fischer
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58 Scientific (Loughborough, UK). A standard of amitraz (96.4 % purity) was obtained
59
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3 from Sigma-Aldrich (Dorset, UK). 2,4-Dimethylaniline (99 % purity) was obtained
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5 from Greyhound Chromatography and Allied Chemicals (Birkenhead, UK).
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10 *Materials*

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12 Representative matrices: strawberries, pears and oranges (labelled as organically
13 produced) were purchased from local retailers and used in both the method
14 development and validation studies. Honey (Yorkshire Blossom) was supplied by the
15 National Bee Unit (Central Science Laboratory, UK).
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24 *Preparation of standard solutions*

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26 A stock solution of 2,4-dimethylaniline was prepared in methanol and stored at -
27 20 °C. Intermediate dilutions were prepared in 2,2,4-trimethylpentane and/or
28 methanol and stored at +4 °C.
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36 A stock solution of amitraz was prepared in acetonitrile – it is unstable and degrades
37 rapidly in methanol (Pierpoint et al. 1997; and Bernal et al. 1997). The stock standard
38 solution was stored at -20 °C. Intermediate dilutions were prepared in 2,2,4-
39 trimethylpentane and stored at +4 °C.
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50 *Sample Preparation*

51 Samples of pears, oranges and strawberries were frozen and comminuted
52 cryogenically in the presence of dry ice (frozen carbon dioxide); sub-samples were
53 stored at -20 °C until required. The sample of honey was well mixed and stored at
54 room temperature.
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Extraction

Sub-samples (20 g) were weighed into Schott bottles (with a septum to seal) and heated at 80 °C for 1 hour in the presence of 100 ml of 0.5 M hydrochloric acid in a water bath, the contents were swirled intermittently. The extract was cooled using a cooling waterbath (10 °C), and the pH adjusted to 11-12 with 5 M sodium hydroxide using a pH meter. The alkaline extract was heated at 80 °C for 1 hour in a water bath; the contents were again swirled intermittently. The extract was cooled using a cooling waterbath (10 °C) and sodium chloride (50 g) was added. The mixture was shaken vigorously until saturation was achieved to allow optimal partitioning of the 2,4-dimethylaniline into the 2,2,4-trimethylpentane (20 ml, shake for 30 seconds, centrifuge at 2350 g for 5 minutes). The (upper) 2,2,4-trimethylpentane layer was removed and an aliquot retained for analysis by GC-MS. A second volume of 2,2,4-trimethylpentane was added to the hydrolysed extract (20 ml) and vigorously shaken (30 seconds), followed by centrifugation (2350 g for 5 minutes). Again an aliquot of the supernatant was retained for analysis by GC-MS. The supernatants were analysed separately. [Note for screening purposes only one partition is required. To increase the percentage recovery a second partition can be undertaken and the extracts pooled.]

GC-MS analysis

Quantification was performed using an Agilent 6890 gas chromatograph connected to a 5973 Agilent Mass Selective Detector operated in selected ion monitoring mode (SIM). The injection (2 µl) was splitless at 250 °C and the detector temperature was set at 280 °C. A fused silica capillary column; Rtx5-MS (stationary phase 5% diphenyl/95% dimethyl polysiloxane stationary phase, 30 m x 0.25 mm i.d. x 0.25 µm film thickness) with the carrier gas (helium) set at 0.9 ml minute⁻¹ (constant flow) was

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3 used for analysis. The typical oven programme was as follows: initial temperature 50
4 °C, held for 1.5 minutes, programmed to 120 °C at 20 °C minute⁻¹, then programmed
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6 °C, held for 1.5 minutes, programmed to 120 °C at 20 °C minute⁻¹, then programmed
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8 to 200 °C at 15 °C minute⁻¹, then finally programmed to 280 °C at 40 °C minute⁻¹
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10 (held for 5 minutes). The total run time was 17.33 minutes. The ions monitored for
11
12 2,4-dimethylaniline were m/z 106, 120, 121 (figure 2) and for amitraz m/z 121, 132,
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14 162, 293. Quantification was undertaken using m/z 106. The identity of 2,4-
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16 dimethylaniline was confirmed using the criteria specified in the Document N°
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18 SANCO/10232/2006 document 'Quality Control Procedures for Pesticide Residues
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20 Analysis': confirmation of identity for the principal ions are within 70 – 130 % of
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22 those obtained for the standard and data is available for three ions with m/z >100.
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29 [Insert Figure 2 about here]
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34 *Calibration Curves*

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36 The calibration curves obtained for 2,4-dimethylaniline, using matrix-matched
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38 standards and non-weighted linear regression, were linear across the range 0.002 –
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40 0.5 µg ml⁻¹ (figure 3) with correlation coefficients >0.990 for all commodities (table
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42 1), with a signal to noise ratio at 0.002 µg/ml⁻¹ (equivalent to 0.002 mg kg⁻¹ 2,4-
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44 dimethylaniline with a final volume of 20 ml) of >10:1.
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51 [insert Figure 3 about here]
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55 *Calculations (Taccheo et al. 1988)*

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57 To express the results as amitraz a conversion factor must be applied; the conversion
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59 factor between amitraz (molecular weight 293 Da) and 2,4-dimethylaniline (molecular
60

weight 121 Da) must be taken into account. Since one mole of amitraz produces two moles of 2,4-dimethylaniline, then:

$$1 \text{ mg kg}^{-1} \text{ amitraz} = 0.826 \text{ mg kg}^{-1} \text{ 2,4-dimethylaniline.}$$

Results and Discussion

Jiménez et al 2002 recommended an extraction pH of 11 for the analysis of total amitraz in honey, to ensure complete extraction of 2,4-dimethylaniline. Initial experiments conducted in 0.5 M NaOH (aq) showed the complete conversion of amitraz to the 2,4-dimethylaniline under alkaline conditions. However in the presence of matrix it was necessary to also include an acidic hydrolysis stage to ensure complete conversion occurred. During validation, the absence of amitraz parent in the final extract was used as an indication that complete conversion to 2,4-dimethylaniline had occurred during the two-stage hydrolysis. An internal standard was not included in the extraction as this would give no indication to the degree of conversion achieved. 4-chloro-2-methylaniline would be a suitable internal standard to correct for volumetric injection errors.

Initial experiments conducted in 0.5 M NaOH (aq) showed that 2,4-dimethylaniline in hydrolysed alkaline extract could be successfully retained on a solid phase extraction (SPE) (Chromabond HR-P cartridges (3 ml, 200 mg, MN New Application Database), and then eluted with acetonitrile/methanol, 1/1, v/v prior to quantification by HPLC-MS. However due to the high viscosity of the acid-hydrolysed sample extracts the use of SPE cartridges was not practicable even with pre-filtration of the hydrolysate

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3 using glass wool, celite or depth filters and the use of positive pressure the sample
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5 extracts could not be loaded onto the column within a reasonable time.
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10 A liquid/liquid partition of the 2,4-dimethylaniline into 2,2,4-trimethylpentane from
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12 the aqueous phase (saturated with sodium chloride) negated the necessity for the SPE
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14 clean-up. No concentration of the extract was required prior to quantification by GC-
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16 MS.
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21 To comply with EU guidelines (SANCO/10232/2006), the validation of amitraz in
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23 each matrix incorporated the analysis of five replicate recoveries at two spiking levels
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25 (the reporting limit (RL) and five times the RL), and included duplicate analysis of
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27 blank material. The EU MRL's are variable ($0.05 - 0.5 \text{ mg kg}^{-1}$) depending on the
28
29 commodity. In-house validation experiments were undertaken at 0.02 mg kg^{-1}
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31 (equivalent to $0.0165 \text{ mg kg}^{-1}$ 2,4-dimethylaniline) and 0.1 mg kg^{-1} amitraz
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33 (equivalent to $0.0826 \text{ mg kg}^{-1}$ 2,4-dimethylaniline) for each commodity (pears,
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35 oranges, strawberries and honey). A summary of the validation results are shown in
36
37 Table 1. Mean recoveries obtained using a single partition were in the range 55 – 73
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39 % with % CV's in the range 4.4 – 10.9. When both partitions were taken into account
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41 the mean recoveries were in the range 66 – 96 % with % CV's in the range 3.3 – 9.4.
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43 Indications were that a third partition would not significantly improve the recovery of
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45 the analyte obtained.
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55 [insert Table 1 about here]
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3 Mean recoveries of 55 - 73 %, obtained using a single partition, are considered
4 sufficient to permit cost effective screening of samples. The second partition provides
5 an additional recovery of approximately 10 – 20 %. Although in this case these data
6 were measured at/or around the limit of detection and may have some error associated
7 with it, during actual analysis this problem would not exist, as the two supernatants
8 would be combined. Combination of the two supernatants would give a final volume
9 of 40 ml and accordingly the limit of detection would be decreased two-fold. As the
10 sensitivity of the method is excellent this would have no consequence on the detection
11 of the lowest calibrated level used which was $0.002 \mu\text{g ml}^{-1}$, which gives a signal to
12 noise ratio > 10:1. No interferences were observed during the analysis of blank
13 sample material (figure 2).
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32 Current analysis methods, involving steam distillation and/or derivitization with
33 heptafluorobutyric acid to enable detection by GC-ECD, are time-consuming. The
34 hydrolysis method developed and validated here is much simpler than existing
35 methods and is applicable to different matrices, as demonstrated. It is possible for an
36 analyst to analyse 30 samples per day using the rapid screening method and 20
37 samples per day if a double liquid/liquid partition is used for confirmatory
38 quantitative analysis.
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53 Affairs) for funding and M. Hetmanski for helpful discussions.
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38 and the withdrawal of authorisations for plant protection products containing this
39 active substance (notified under document number C(2004) 332)
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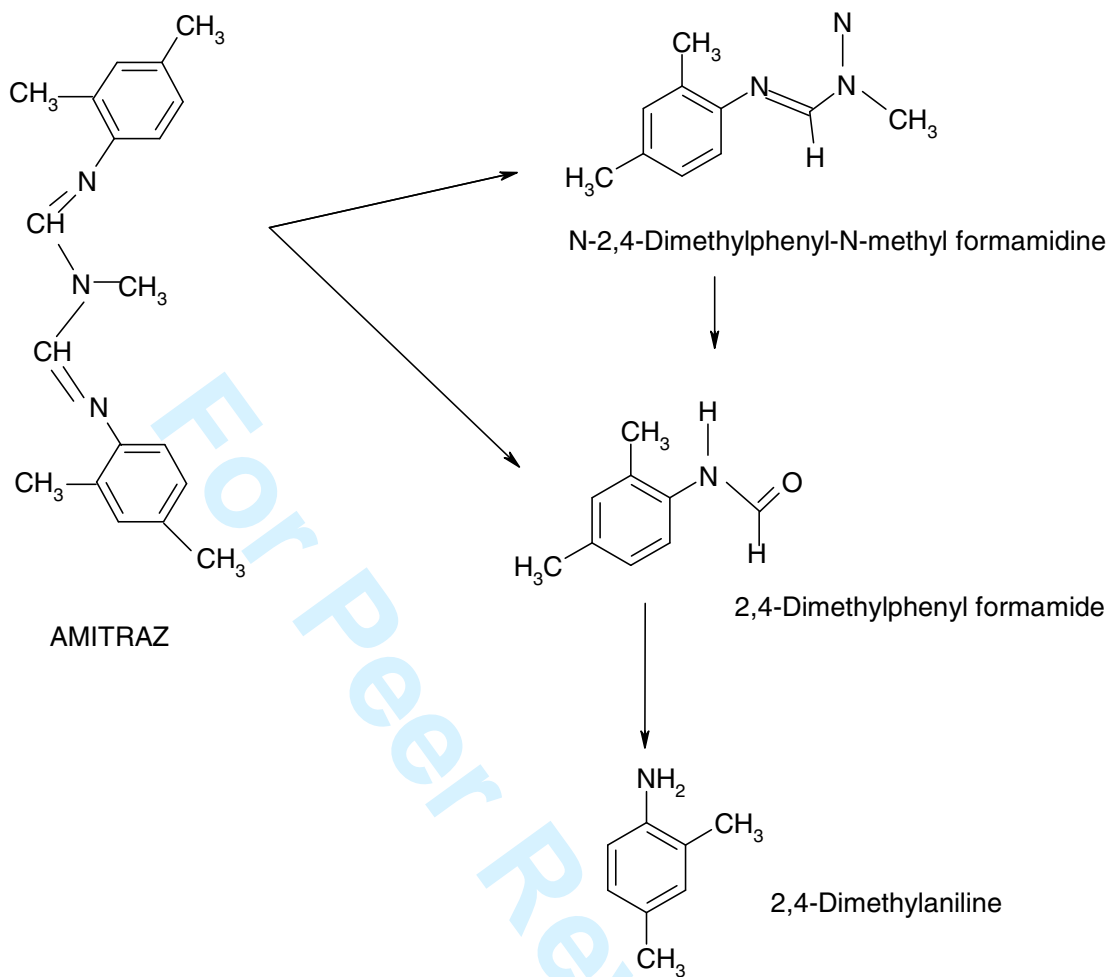
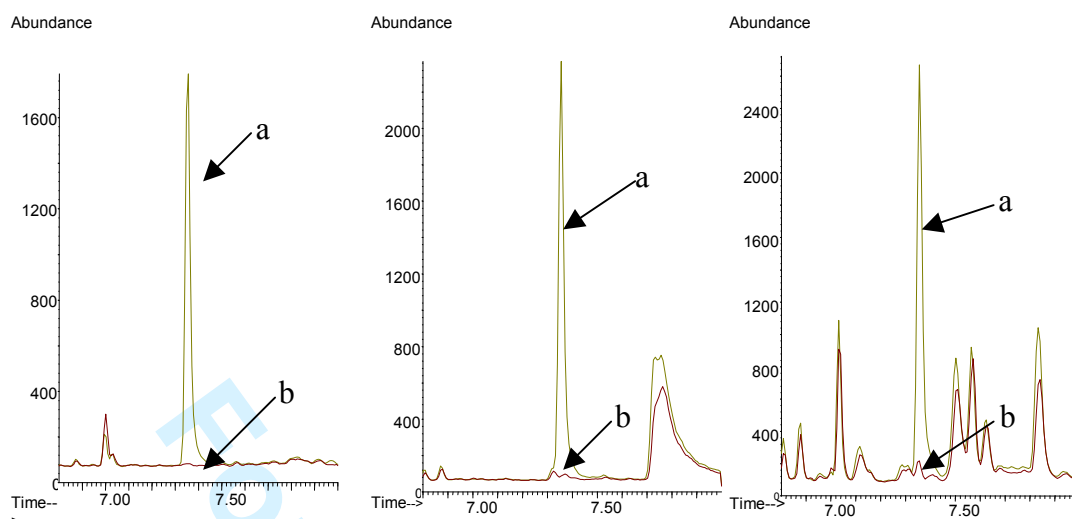


Figure 1. Hydrolysis pathway of amitraz

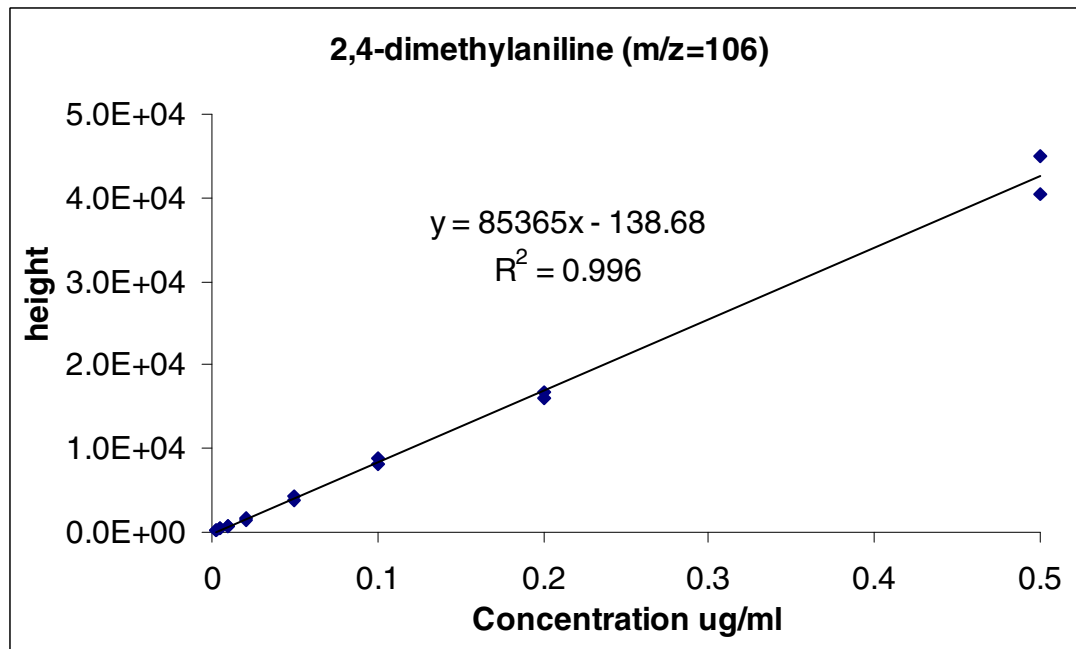


i) ion m/z 106

ii) ion m/z 121

iii) ion m/z 120

Figure 2. Quantitation and confirmation ion chromatograms for 2,4-dimethylaniline for a) a standard at $0.02 \mu\text{g ml}^{-1}$ in strawberry matrix and b) blank strawberry matrix, retention time 7.36 minutes



25 Figure 3. Matrix-matched calibration curve for 2, 4-dimethylaniline in the range
26 $0.002 - 0.5 \mu\text{g ml}^{-1}$ in strawberry matrix
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Matrix type	Correlation coefficient	spiking level mg kg ⁻¹	Mean (%)/ % CV		
			partition 1	partition 2	total
pear	0.991	0.02	61 (8.2)	10 (22.4)	71 (6.1)
		0.1	61 (6.2)	15 (11.8)	77 (7.4)
strawberry	0.996	0.02	61 (4.4)	9 (14.8)	71 (3.3)
		0.1	64 (5.7)	10 (13.3)	73 (5.2)
orange	0.998	0.02	73 (4.5)	23 (8.4)	96 (4.7)
		0.1	63 (10.9)	13 (8.3)	77 (9.4)
honey	0.997	0.02	60 (5.3)	12 (0.0)	72 (3.7)
		0.1	55 (7.3)	11 (11.1)	66 (7.4)

Table 1. Mean validation results (n=5) for amitraz measured as 2,4-dimethylaniline