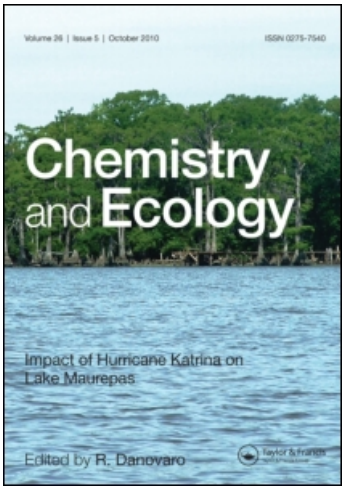

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

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To cite this Article Allchin, C. R. and Law, R. J.(1988) 'The Analysis of Tecnazene (2,3,5,6-Tetrachloronitro-benzene) and its Metabolites in Water and Fish', Chemistry and Ecology, 3: 3, 255 — 264
To link to this Article: DOI: 10.1080/02757548808070845
URL: http://dx.doi.org/10.1080/02757548808070845

Chemistry and Ecology, 1988, Vol. 3, pp. 255–264
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Printed in the United Kingdom

The Analysis of Tecnazene (2,3,5,6-Tetrachloronitrobenzene) and its Metabolites in Water and Fish

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(Received January 28, 1988)

A method for the analysis of Tecnazene (2,3,5,6-tetrachloronitrobenzene) and its major metabolites (2,3,5,6-tetrachloroaniline and 2,3,5,6-tetrachlorothioanisole) is presented. This rapid analytical technique was developed to measure these compounds in samples of water and fish produced during the course of toxicological investigations. Water samples were extracted into hexane and analysed by high resolution capillary gas chromatography using flame ionisation detection (HRGC-FID). Fish samples were extracted into hexane, cleaned up on a short alumina micro-column and also analysed by HRGC-FID.

KEY WORDS: Metabolites; Hexane; flame ionisation.

INTRODUCTION

Tecnazene (2,3,5,6-tetrachloronitrobenzene; 2356-TCNB) is widely used in its formulated form as a fungicide and a sprout inhibitor on stored potatoes. Recent changes in marketing practices have meant that increasing numbers of treated potatoes are being removed from store and washed prior to distribution to retailers. Effluent from potato washing plants may, therefore, contain residual levels of 2356-TCNB, together with its metabolites or breakdown products. These may be discharged to water-courses via surface drains and thus result in elevated environmental concentrations in water and fish.

An assessment of the possible hazards of this practice revealed that little information on the toxicity of Tecnazene to freshwater fish was available. Accordingly, a thorough investigation of its toxicological properties has been carried out at this laboratory. The results of this study are being reported elsewhere (Whale *et al.*, in prep.).

To support this study, a rapid analytical method was required to enable: a) the concentration of Tecnazene in the test tanks to be established so that a concentration profile for the duration of the test could be constructed and thus allow a more accurate estimation of the toxicity, based on the true concentration rather than on a nominal value; and b) the measurement of residue levels in fish to be determined during experiments on the uptake and depuration of Tecnazene by fish from the water column. Like the method for water, a primary requirement of the methodology for the fish samples was speed of analysis, since large numbers of samples can be generated by even a short-term toxicity test and this can easily overload the available analytical resources.

The method preferred by the World Health Organisation for the determination of Tecnazene residues is by gas chromatography with electron capture detection (ECD); a limit of detection of $0.01\,mg\,kg^{-1}$ with a recovery of more than 90% is cited (WHO, 1984). The alternative method presented here has similar limits of detection and recovery rates but uses flame ionisation detection which, although less specific than ECD, has the advantage of being generally more robust and is adequately sensitive for ecotoxicological work. Recent investigations into levels of Tecnazene in environmental samples have shown that flame ionisation detection is suitable for this purpose. The method also makes use of modern high-resolution capillary columns and advanced data handling procedures, to increase sample throughput and to increase the integrity of sample data.

ANALYTICAL METHODOLOGY

Water analysis

Samples of water (500 ml) were taken at mid-depth from the experimental tanks of the continuous flow toxicity testing apparatus

using a siphon tube. The use of a siphon tube has the advantage of reducing to a minimum any disturbance to the fish under test and also allows the sample to be taken from the same place in the tank each time. The water samples were then transferred to a 1l separating funnel and, after the addition of 25 ml of hexane (glass distilled grade, Rathburn Chemicals, Walkerburn, Scotland), were extracted for 15 min using a laboratory shaker. After allowing the two layers to separate, the hexane layer was removed and a second extraction using a further 25 ml of hexane was performed. The combined extracts were dried over sodium sulphate (Analar grade, BDH Ltd, Poole, Dorset) and reduced to approximately 5 ml using a rotary evaporator. This was quantitatively transferred to a 10 ml volumetric flask or stoppered graduated measuring cylinder and n-docosane (Aldrich Chemicals, Gillingham, Dorset) added as an internal standard. The volume of the final extracts was then adjusted to 1, 5 or 10 ml depending on the final concentration expected. If the extract needed to be concentrated after the first evaporation stage, this was performed using a stream of dry air or oxygen-free nitrogen in a Tecne "dri-block" sample concentrator (Radleys, Sawbridgeworth, Herts). One millilitre of the extract was then transferred to an autosampler vial and analysed by HRGC-FID.

Fish analysis

Samples of fish (weight approximately 20 g) were homogenised using an Ultra Turrex homogeniser and mixed with anhydrous sodium sulphate. When dry, the samples were transferred to a glass extraction thimble and extracted with 100 ml of hexane in a Soxhlet apparatus for 2 h. The extracts were allowed to cool and then quantitatively transferred to 100 ml volumetric flasks. Hexane extractable lipids were determined by evaporating a 50 ml portion of the sample to dryness over a steam bath in a fume cupboard, and then further drying in an oven at 105°C for 1 h, expressing the resultant residue as a percentage of the original sample. A suitable aliquot of the hexane extract (between 1 and 10 ml) was then taken and reduced to 1 ml under a stream of nitrogen. The precise volume of sample extract taken was determined by the lipid content; the sample size during the course of this work ranged from 10–30 g.

The samples were then cleaned up on a 3 g column of 5% deactivated alumina using hexane as the eluting solvent (Allchin and Portmann, in press). The first 5 ml from this column contained 2356-TCNB, 2,3,5,6-tetrachloroaniline (2356-TCA) and 2,3,5,6-tetrachlorothioanisole (2356-TCTA). The volume of the eluant was then reduced to <1 ml, docosane added as the internal standard, and the sample transferred to an autosampler vial ready for analysis by HRGC-FID.

GC apparatus

The gas chromatograph used was a Hewlett Packard model HP5730A equipped with a 18740B capillary control module. The column was 25 m × 0.2 mm ID fused silica coated with a 0.3 μm film of 5% phenyl methyl silicone (crosslinked). High-purity hydrogen was used as a carrier gas at an inlet pressure of 7 psi. The injection was in the splitless mode using an HP7671 autosampler and the volume of injection was 2 μl, the split valve being opened after 1 min. The injection port was held at 250°C. The oven temperature at injection was 100°C. This was held for 1 min and then programmed to rise at the rate of 5°C min^{-1} to 300°C. The flame ionisation detector was held at 300°C and high-purity hydrogen (at 30 ml min^{-1}) and air (at 250 ml min^{-1}) were used as fuel gases. High-purity helium (at 50 ml min^{-1}) was used as a make-up gas for the detector. The autosampler control and data acquisition and reduction was via an HP3357 laboratory automation system. Examples of chromatograms of standards and fish tissue extracts are given in Figures 1 and 2.

Percentage recovery

Samples of water used for the toxicity tests were spiked with a range of concentrations of 2356-TCNB in acetone and extracted as previously described. At the 1 $\mu g\,l^{-1}$ level, the recovery was 91% with a relative standard deviation (RSD) of 1.7%, where $n = 9$. The practical limit of detection, using a 500 ml sample and a final hexane volume of 1 ml, was set at 1 $\mu g\,l^{-1}$. However, for environmental samples, increasing the sample volume to 2l and using an ECD and alumina clean-up, can reduce the limit of detection to 0.25 ng l^{-1}. A

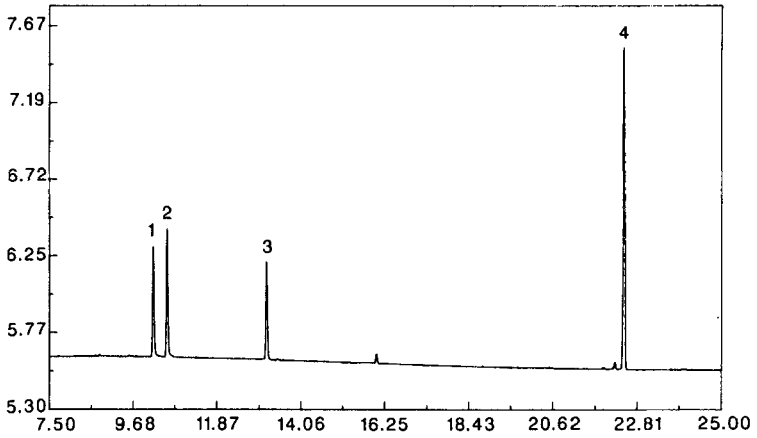

FIGURE 1 Standard solution: 1. 2356-TCNB; 2. 2356-TCA; 3. 2356-TCTA; 4. DOCOSANE.

representative batch of trout from clean water shown to be free of TCNB residues was extracted with hexane spiked with a range of concentrations of 2356-TCNB. The extracts were then passed through the normal clean-up procedure. At the 0.1 mg kg^{-1} level, the recovery was 97% with an RSD of 9.1%, where $n = 3$. The limit of detection for fish depends on the lipid content and weight of

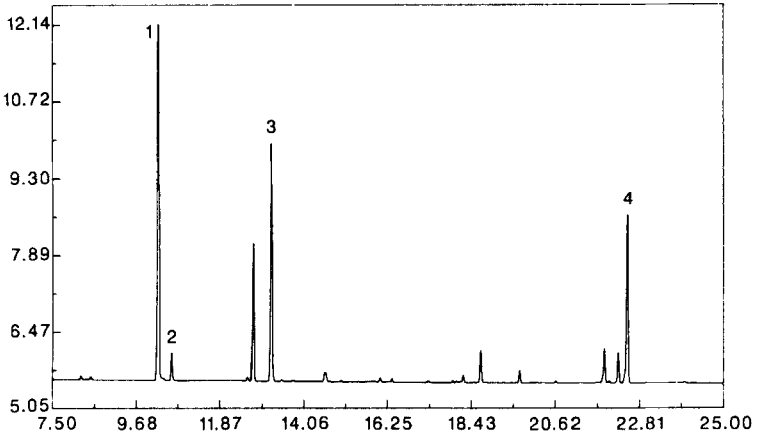

FIGURE 2 Trout (whole) extract (key as Figure 1).

sample, but for routine use a limit of 0.1 mg kg^{-1} was easily achievable, and 0.001 mg kg^{-1} could be obtained by use of an ECD.

Identification by MS

Selected extracts prepared for analysis using HRGC-FID and solutions of authentic standard materials were also analysed by capillary gas chromatography–mass spectrometry on a Finnigan 3200E quadruple mass spectrometer interfaced to and controlled by an Incos 2300 data system. One microlitre aliquots were injected via a cold on-column injector onto a 25 m × 0.3 mm ID fused silica capillary column mounted in a Carlo Erba 4160 gas chromatograph, the column had a crosslinked 1 μm film of 5% phenyl methyl silicone (Hewlett Packard Ltd, Winnersh, Berks) and the end of the capillary column terminated in the ion source of the mass spectrometer. The injections were made at 100°C and this temperature was held for 1 min, then raised at 10°C min^{-1} to 300°C. The mass spectrometer was operated in the electron impact mode at 70 eV, and was scanned from 35 to 300 Daltons with a cycle time of 1 s. The identity of the unknown compounds in fish samples was established first by use of the NBS spectral library resident on the Incos data system, followed by comparison of the spectra and retention times of authentic materials (Aldrich Chemicals).

RESULTS AND DISCUSSION

During the course of this work, it became apparent that as well as identifying Tecnazene the method was isolating additional compounds from the fish samples which appeared to be related to the parent compound. Analysis by gas chromatography–mass spectrometry identified these compounds as 2,3,5,6-tetrachloroaniline (2356-TCA) and 2,3,5,6-tetrachlorothioanisole (2356-TCTA). The mass spectra of 2356-TCNB, 2356-TCA and 2356-TCTA are shown in Figure 3. 2356-TCA has previously been reported (Heikes, 1980) during studies on the residues of 2356-TCNB in potatoes and 2356-TCTA has been reported (Cairns *et al.*, 1987) as being a precursor to the corresponding sulfoxides and sulfones found in parsnips grown in soil treated with pentachloronitrobenzene.

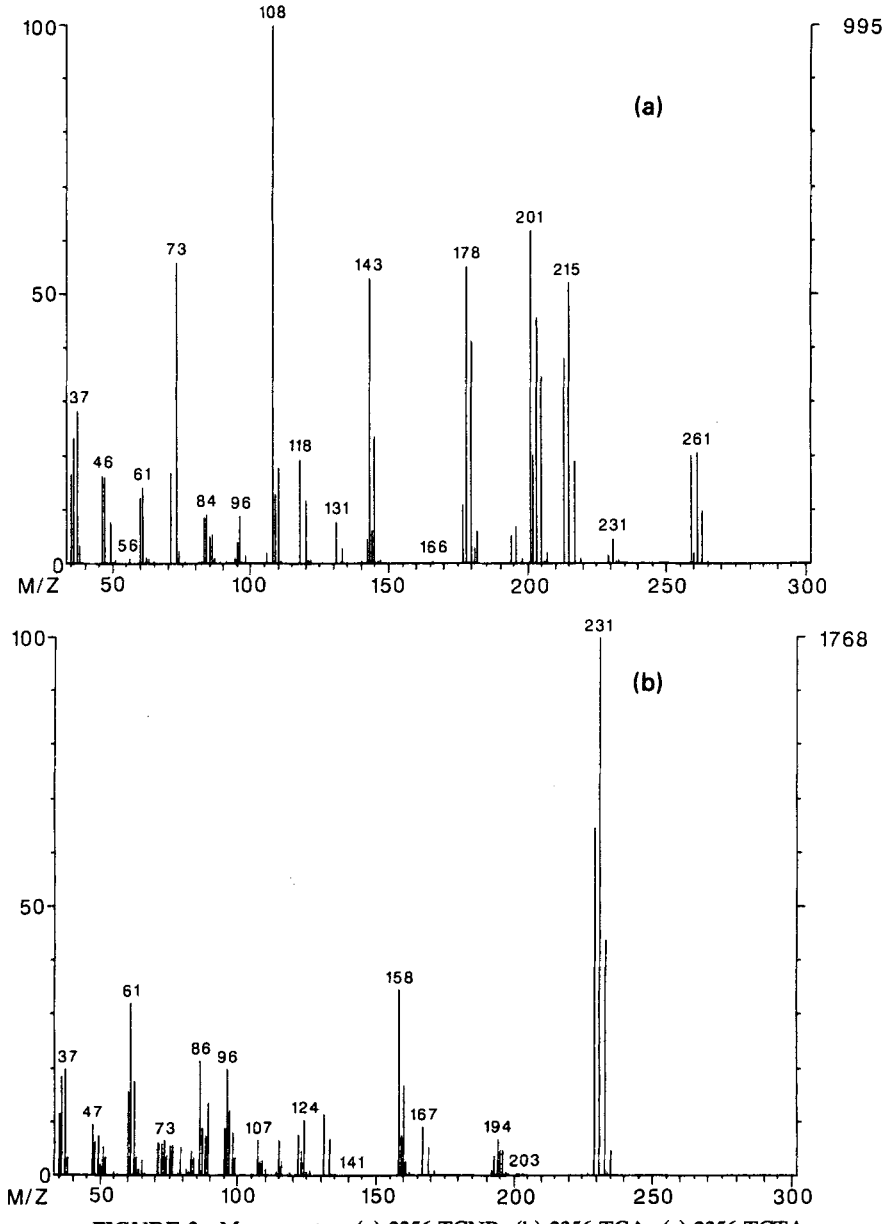

FIGURE 3 Mass spectra: (a) 2356-TCNB; (b) 2356-TCA; (c) 2356-TCTA.

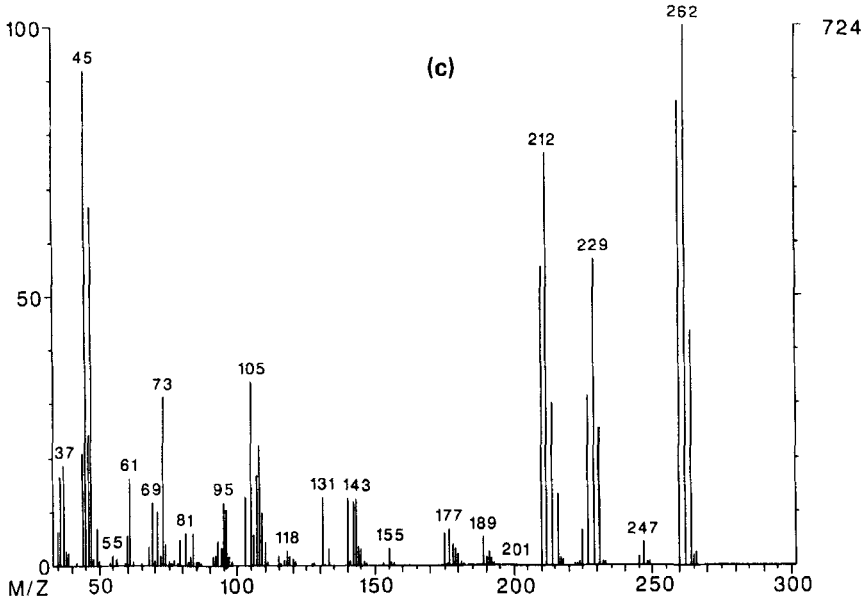

FIGURE 3 (*Continued*)

Although residues of the lower chlorinated nitrobenzenes have been reported in Mississippi river fish (Yurawecz and Puma, 1983) and the identification of twelve chlorinated nitrobenzenes in fish from the River Main (Steinwandter, 1987) has also been reported, as far as the authors are aware, this is the first time that 2356-TCA and 2356-TCTA have been reported in fish tissues exposed to 2356-TCNB. Recently, 2356-TCA and 2356-TCTA have also been found in the final effluent of a sewage treatment works which receives effluent from a potato washing plant (D. Whitmarsh, pers. comm.). It seems, therefore, that residues of Tecnazene and related compounds may be widespread in the aquatic environment. Further studies are underway and will be reported at a later date.

It has recently come to the authors' attention that residues of Tecnazene and its metabolites may have been incorrectly identified in the past as HCH isomers by laboratories analysing environmental samples and using methods which involved little or no sample clean-up, and packed column chromatography, without any further

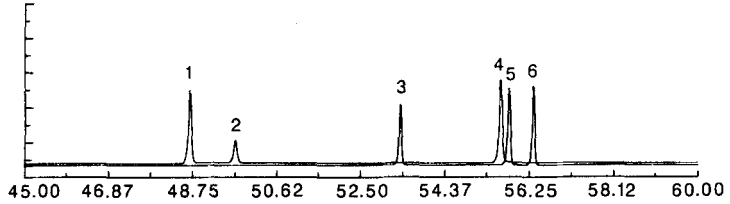

FIGURE 4 Computer reconstructed chromatogram showing in order of elution: 1. 2356-TCNB; 2. 2356-TCA; 3. alpha HCH; 4. 2356-TCTA; 5. beta HCH; 6. gamma HCH.

confirmation of residues. The method, as presented here, provides adequate resolution between Tecnazene, its metabolites, and alpha-, beta- and gamma-HCH (Figure 4). This highlights the need for environmental chemists to investigate their analytical protocols thoroughly and to ensure that sufficient confirmation of residues is undertaken, either by use of GC-MS, chemical treatment and re-analysis, or repeated analyses using columns of differing selectivity as appropriate. Analytical protocols should also be rigorously tested before use to establish their applicability and performance.

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