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Journal of Chromatography A, 708 (1995) 71–81

JOURNAL OF CHROMATOGRAPHY A

Simultaneous determination of tebufenozide and five of its intact metabolites from forestry matrices by high-performance liquid chromatography

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First received 11 January 1995; revised manuscript received 13 March 1995; accepted 13 March 1995

Abstract

A simple, rapid and reliable high-performance liquid chromatographic (HPLC) method for the determination of tebufenozide insecticide and five of its intact metabolites in spruce foliage, litter, forest soil, sediment and natural water is described. The fortified litter, soil and sediment samples were extracted with acidic methanol in presence of Celite and the aqueous phase was partitioned with dichloromethane (DCM) after the alcohol was removed. The residues in the DCM phase, after evaporation to dryness, were dissolved in an acetone–hexane mixture and cleaned by Florisil column. The treatment of spruce foliage was similar except that the acidic methanol was also partitioned with hexane prior to DCM extraction. Natural water was extracted with DCM and the residues were dissolved in methanol for HPLC analysis without any column cleanup. The chromatographic system consisted of an ODS Hypersil (5 μm) 250 × 4 mm I.D. column linked to a UV detector set at 236 nm. The mobile phase consisted of methanol–water with 0.005 *M* PIC A as an ion-pairing agent. Mean recoveries of the analytes, depending on their structure, ranged from 69 to 102% with standard deviations ranging from 5 to 8%. Limits of detection for the solid matrices ranged from 0.01 to 0.03 μg/g; for water it was 1.5 μg/l. The method was applied successfully to the analysis of field samples sprayed with tebufenozide.

1. Introduction

Canadian forest insect pests have been effectively controlled for forty years by conventional broad-spectrum synthetic insecticides [1,2]. Although the judicious use of insecticides is beneficial in controlling pests, there is public concern about their use because of their potential toxicity to non-target organisms, including humans [3,4]. Researchers are now seeking for narrow-spectrum insecticides, preferably species-specific compounds, which would presumably cause minimum hazard to the environment.

Currently, a new moult-inducing insecticide, tebufenozide, also known as RH-5992 [N′-*tert.*-butyl-N′-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl)hydrazine] (trade name: MIMIC), discovered by Rohm and Haas (Spring House, PA, USA), is being field tested because of its specificity and low toxicity to non-target organisms [5], as a candidate narrow-spectrum insecticide to control various lepidopteran insect pests in Canadian forests [6]. As part of the evaluation process, field trials were conducted to study the

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SSDI 0021-9673(95)00353-3

persistence and metabolic fate of tebufenozide in different forestry matrices following spray application of the material over selected aquatic and terrestrial sites of a mixed forest in northern Ontario, Canada.

Analytical methods to quantify the intact tebufenozide in spray formulations [7] and in some forestry matrices [8] have been reported recently. Otherwise, very little is known about its degradation pathways, the type of metabolites formed in the environmental matrices and analytical methods available to identify and quantify them. The manufacturer's proprietary report [9] recorded the formation of three oxidative products as metabolites in agriculture soils with an additional two alcohol metabolites observed in

Table 1
Analytical standards of tebufenozide and its metabolites used in the study

Name	Functionality	Purity (%)	Lot No.	Structure
Tebufenozide (MIMIC or RH-5992)	Parent compound	99.64	AMB9-40B	
RH-2651	Short acid, metabolite in soil	97.4	DPL2093	
RH-2703	Long acid, metabolite in soil	97.6	DPL2090	
RH-6595	Ketone, metabolite in soil and plants	97.7	DPL2095	
RH-1788	A-ring, alcohol metabolite in plants	98.7	ZL6:32F	
RH-9886	B-ring, alcohol metabolite in plants	99.07	TRO5794	

the course of plant metabolism studies (Table 1). All of these products have the two benzoyl moieties and the hydrazine bridge intact except the conversion of the ethyl or methyl substituents on the two aryl rings. Analytical methods to identify and quantify the five metabolites from forestry matrices are not yet available in the open literature. It is important to analyze these residues from forestry materials to understand their persistence, environmental behavior and potential toxicity to insects and other non-target organisms. Therefore, this study was undertaken with the following objectives in mind: (i) to develop a sensitive, rapid and reliable high-performance liquid chromatographic (HPLC) method with UV detection to quantify simultaneously the parent material and five of its intact metabolites, as recorded in Table 1, from fortified forestry matrices, and (ii) to apply the method to analyze the field samples treated with tebufenozide to evaluate the suitability of the method and to examine the types of metabolites formed.

2. Experimental

2.1. Reagents

Solvents

Acetone, dichloromethane (DCM), hexane and methanol were LC (Optima) grade (Fisher Scientific, Unionville, Ont., Canada), filtered through a Nylaflo filter of 0.20 μm pore size. Water was deionized, purified by a Milli-Q water system, and filtered as described above. PIC A (low UV), the ion-pair reagent (tetrabutyl ammonium hydrogen sulphate) (Waters, Millipore Corporation, Milford, MA, USA) used in the study, was prepared as per the manufacturer's instructions and filtered as described above.

Chemicals

Florisil PR grade (Floridin Co., Pittsburgh, PA, USA) was purified and deactivated as described earlier [10]. Celite, hydrochloric acid and sodium chloride were ACS grade supplied by Fisher Scientific. Analytical grade tebufenozide and its major metabolite standards used during this study were supplied by the Rohm and Haas Company. The names, purities, lot numbers and structural formulas of the analytical standards are given in Table 1.

2.2. Stock and standard solutions

Individual stock solutions, containing 10 mg/100 ml of the analytes, were prepared in volumetric flasks using methanol as solvent. Individual and mixed standard solutions used for fortification of the matrices and instrument calibration purposes were prepared by serial dilution of these stock solutions. All volumetric flasks containing the solutions were covered with aluminum foil and stored at 0°C in darkness. The stock and standard solutions were stable for more than 10 weeks, nevertheless fresh solutions were prepared every eight weeks.

2.3. Forest matrices for fortification

Natural water (pH 6.1, alkalinity 37 mg $CaCO_3$/l, specific conductance 16.2 μmho/cm) was obtained by grab sampling from a small lake located about 80 km northwest (46°53'N, 84°03'W) of Sault Ste. Marie, Ont., Canada. The surface water (5 cm depth) was scooped by using wide-mouthed 1-l mason jars. Multiple samples were pooled into clean 2 × 4-l amber-coloured bottles and stored in darkness at 4°C until use. Sediment was collected from the lake by gently lowering a Wildco Stratification Core Sampler (Hoskin, Ont., Canada) to the lake bottom and allowing it to sink into the sediment to a depth of 15 cm and then pulling it out carefully without disturbing the surroundings. The samples were transferred to clean 1-l mason jars and stored at 4°C for further processing. Forest litter (organic matter 90%, sand 57%, silt 27%, clay 16%, pH 5.40), soil (organic matter 3.9%, sand 51%, silt 42%, clay 7%, pH 5.85) and white spruce [*Picea glauca* (Moench) Voss] needles were collected from a typical mixed-wood boreal forest located around the lake. Detailed procedures used during the collection, transportation, storage, and

processing of the various forestry matrices before analysis are described elsewhere [11].

2.4. *Tebufenozide-treated field samples*

Water samples were collected from 4.0-m deep aquatic enclosures [12] treated with formulated tebufenozide at 500 μg/l using a hand-held, battery-powered FLAK spinning disc sprayer (Micron Corp., Houston, TX, USA). Four subsurface water samples (0, 21, 49 and 92 days postspray) were collected from a 3.0-m deep water column by using an integrated sampler [12]. Postspray sediment samples were also collected at the same four intervals of time using the Wildco core sampler.

Four spruce trees (2.3 to 2.5 m in height and 7 to 8 cm in stem diameter) enclosed in polyethylene shelters were sprayed individually using the FLAK sprayer at the dosage of 140 g AI (active ingredient)/ha. One-year-old needles were sampled at 0, 64, 107 and 169 days postspray from the four quadrants of each tree at the mid-crown level. Each postspray sample was pooled, packed in plastic bags, labelled and stored at −20°C in a freezer until analysis.

Litter and soil (litter layer removed) plots (4.5 m^2 each) were sprayed with 140 g AI/ha using the FLAK sprayer. Both sample types were collected as 5-cm cores using metal augers at the same intervals of time as for foliage. All samples were sieved (2-mm opening) to remove debris, stones etc., packed and stored at −20°C as described for foliage.

2.5. *Apparatus*

HPLC instrumentation

The high-performance liquid chromatograph (HPLC) used in the study was a Hewlett-Packard (HP) (Analytical Division, Palo Alto, CA, USA) 1090 M Model incorporating an HP diode-array detector (DAD) (λ range, 190 to 600 nm); an HP 9000 Series 300 (Model 310) computer work station (HP 79988A operating software, Rev. 5.3); an automated sampler; and a variable volume auto-injector fitted with a 250-μl syringe. The instrument also had a binary solvent delivery system with a helium-purge degassing system and two dual-syringe metering pumps that gave stable and reproducible flows. The parameters used during the course of this study were as follows: DAD wavelengths, sample 236 ± 4 nm, reference 430 ± 50 nm; analytical column, ODS Hypersil, 5 μm, 250 × 4 mm I.D. (HP); guard column, LiChrospher 100 RP-18, 5 μm, 4 × 4 mm I.D. (HP); mobile phase, solvent A: methanol–water (1:4) containing 0.005 *M* PIC A, solvent B: methanol; flow-rate, 1.0 ml/min; oven temperature, 40°C; injection volume, 25 μl. Gradient elution was performed as follows: $t = 0$ min, 100% solvent A; $t = 35$ min, solvent A–solvent B (12.5:87.5, v/v) (held for 5 min for column cleanup); total run time, 40 min.

Equipment

The following instrumentation was used: shaker, Magni-Whirl (Blue M Electric Company, Blue Island, IL, USA); homogenizer, Sorvall Omni-mixer (Ivan Sorvall, Norwalk, CT, USA); nitrogen evaporator, Meyer N-Evap analytical evaporator, Model No. 111 (Organomation Associates, South Berlin, MA, USA); sonicator, ultrasonic bath, Model No. B-92H (Branson Ultrasonics Corp., Danbury, CT, USA); Rotary evaporator, Buchler Model No. PTFE-IGN (Buchler Instruments, Fort Lee, NJ, USA); filters, Acrodisc LC13 PVDF 0.2 μm (Gelman Sciences, Rexdale, Ont., Canada); Nylaflo nylon membrane filter, 0.2 μm (Gelman Sciences); Whatman No. 1 (Fisher Scientific); syringe, disposable, 3 ml capacity with Luer Lok fitting (Becton Dickinson and Co., Rutherford, NJ, USA); water purification system, Milli-Q water system (Millipore, Mississauga, Ont., Canada); pasteur pipets, 15 cm × 8 mm I.D. (Fisher Scientific).

2.6. *Sample preparation*

Aliquots (50 g) of spruce needles, litter, soil and sediment samples, in triplicate, were weighed and placed in separate 250-ml Erlenmeyer flasks fitted with ground-glass stoppers. Each sample was fortified separately with 1.0, 0.50 and 0.10 μg/g of the mixed analyte stan-

dard. Similarly, 500-ml aliquots of water, filtered (5-μm PTFE membrane) under suction, were fortified with the mixed analyte standard at 100, 50, and 10 μg/l. All samples were shaken well for 30 min in a Magni-Whirl shaker at 200 excitations/min and allowed to equilibrate for one hour before extraction, cleanup and analysis.

2.7. Extraction and cleanup procedures

Extraction of the analytes from litter, soil and sediment

Ten grams of each sample were weighed into a 250-ml stainless steel Sorvall homogenizer cup. Five grams of Celite and 50 ml of acidic methanol (0.5 *M* $HCl–CH_3OH$, 3:7, v/v) were added to each sample and blended in a Sorvall Omnimixer for 3 min. The supernatant was filtered under suction through a pre-rinsed (20 ml of extraction solvent) Büchner funnel containing Whatman No. 1 filter paper into a 250-ml suction flask. The residue was re-extracted using 25 ml of the extraction solvent and transferred quantitatively to the Büchner funnel using 3 × 10 ml of acidic methanol rinses. After filtration under suction, the filter cake was rinsed with 20 ml of the acidic methanol. The crude extract was quantitatively transferred to a 500-ml round bottom flask using 3 × 5 ml rinses of methanol. The extract was flash evaporated (Buchler rotary evaporator) with extreme care to avoid bumping and the methanol was removed. We found that all the methanol must be removed to achieve optimum quantitation of the analytes. The aqueous extract was transferred quantitatively to a 125-ml Teflon separatory funnel using 50 ml of 10% NaCl solution and the pH was adjusted to 1–2 using 2 *M* HCl. The aqueous extract was partitioned thrice, each time using 25 ml of DCM. Caution was necessary to break up the emulsion either by stirring the aqueous phase with a clean glass rod or by submerging the separatory funnel in an ultrasonic bath (Branson sonicator). Excessive emulsion formation was minimized to improve the recovery of the analytes. The technique of using the ultrasonic bath was found to be quite effective in breaking up the emulsion. The DCM layers were pooled and the aqueous layer was discarded. Attempts to dry the DCM layer with anhydrous sodium sulfate resulted in the loss of acidic metabolites, RH-2651 and RH-2703. The DCM layer was carefully flash evaporated to dryness and the residue was transferred to a 15-ml centrifuge tube with 5 × 2 ml of acetone. The solution was then evaporated (Meyer N-Evap) to 0.5 ml under a gentle stream of dry nitrogen and brought up to the 5.0 ml mark with hexane for Florisil column cleanup.

Extraction of analytes from spruce needles

Most of the extraction steps and precautions used for the spruce needles were similar to those described for litter soil and sediment in the previous section, except that 10-g aliquots of the matrix were shaken (Magni-Whirl) for 30 min using 75 ml of acidic methanol first and 50 ml for the second time. After necessary rinsings with the solvent, the pooled filtrate was partitioned twice with 50-ml portions of hexane, using a 250-ml Teflon separatory funnel. After discarding the hexane layer, the aqueous layer was flash evaporated carefully using a 500-ml round bottom flask, adhering to the precautions described above, until all the methanol was removed. The aqueous layer was transferred to a clean 250-ml Teflon separatory funnel, 100 ml of 10% aqueous NaCl added and partitioned thrice using 40 ml DCM for each step. Emulsion formation was severe and was minimized by submerging the separatory funnel in an ultrasonic bath. The organic layer was flash evaporated to dryness, reconstituted in acetone, evaporated to 0.5 ml (Meyer N-evap) and the volume was adjusted to 5.0 ml using hexane for Florisil column cleanup.

Extraction of analytes from natural water

One hundred (100) ml of fortified water was transferred to a 250-ml Teflon separatory funnel, acidified with 2 *M* HCl to pH 1.5 and extracted thrice using 40 ml of DCM for each extraction step. The pooled organic layer was transferred to a 250-ml round bottom flask and gently flash evaporated to dryness. The residue was taken up in 5 × 2 ml methanol, transferred to a 15-ml

graduated centrifuge tube and concentrated under a stream of dry nitrogen (Meyer N-Evap) to an exact volume depending upon the fortification level. The methanolic solution was passed through a 0.45-μm syringe filter for HPLC analysis. No Florisil column cleanup was necessary for the water samples.

2.8. Florisil column cleanup

Florisil column adsorption chromatography was used to clean the crude extracts of litter, soil, sediment and spruce needles. A micro-column was prepared by packing a pasteur pipet (15 cm $\times$ 8 mm I.D.) from bottom to top with a small wad of glass wool, 1.5 g of purified [10] deactivated Florisil (10% H_2O) and topped off with a glass wool plug. The column was prewashed first with 10 ml acetone and then with 10 ml hexane. One ml of the crude extract, corresponding to 2.0 g of matrix, was added to the column, eluted with 7 ml of 10% acetone in hexane and the eluate was discarded. The non-acidic metabolites, RH-1788, RH-6595 and RH-9886, and the parent material (RH-5992), were eluted first from the column using 8 ml of acetone–hexane (50:50, v/v) and the eluate was collected in a centrifuge tube. The polar impurities adsorbed onto the column were removed by eluting the column with 10 ml of acetone and the eluate was discarded. The acid metabolites, RH-2651 and RH-2703, were then eluted using 8 ml of methanol and the eluate was collected in a separate centrifuge tube. The eluates in the two centrifuge tubes were evaporated to dryness using the Meyer N-Evap, reconstituted each with 1 ml of methanol and passed through 0.45-μm syringe filters for HPLC analysis.

3. Results and discussion

3.1. HPLC conditions

Considering the structure and the functional groups of each molecule listed in Table 1, it is apparent that the use of HPLC to separate and quantitate the analytes would be simpler and easier compared to gas–liquid chromatography (GLC), where additional derivatization of the acidic metabolites to form volatile species has to be performed with associated cleanup problems. Various HPLC conditions (column type, mobile phase composition, flow-rate, etc.) were tested on a trial basis to separate the parent material and individual metabolites from each other. The analytical columns tested varied in length, particle size and the nature of stationary phase. The columns tested included ODS Hypersil, LiChrosorb RP-8, LiChrosorb RP-18, Partisil ODS-2, Spherisorb ODS-2 and Ultracarb RP-18. Most of these columns showed good separation for the parent and its metabolites, however the best resolution and symmetrical peak shapes were obtained using the Hewlett-Packard ODS Hypersil, 5 μm (250 $\times$ 4 mm I.D.) column.

Many mobile phases combining two or more solvents (acetone, acetonitrile, diethyl ether, dioxane, phosphate buffers of different pH and molarities, 2-propanol and tetrahydrofuran) without and with modifiers (50 mM phosphate buffer + 1% of acetic acid, pH $\sim$3) to minimize tailing and peak broadening, were tried to optimize the separation for all the six analytes. Many different isocratic and gradient elution modes were tried using different columns, oven temperatures and flow-rates. The major problems encountered were the inconsistency in peak resolution (especially in the case of the two acid metabolites, RH-2651 and RH-2703) and shifting of peaks, which made the separation of target peaks impossible from the mixed standard and from the fortified samples. The use of water–acetonitrile with 14 μM phosphate buffer (pH 5.0) alleviated the peak shifting and enhanced peak resolution, however the problems became more acute as the column aged. To circumvent these difficulties, 0.005 M PIC A (tetrabutylammonium hydrogen sulfate from Waters) reagent was added as an ion-pairing agent to the methanol–water mobile phase (solvent A in the section on HPLC instrumentation). Consequently, the additive formed neutral ion pairs with the acid analytes causing them to behave like nonpolar solutes, thus improving considerably the symmetry, retention and resolution of each peak in the

mixed standard. Under these optimized mobile phase parameters, using the ODS Hypersil column, all six target compounds were successfully separated within 40 min.

3.2. Calibration graph, detection limits and chromatograms of standards

The calibration graphs for the analytes in Table 1 were linear between 2 and 1000 ng and fitted the following equations, where y is the peak area (mAU s) and x is the amount of analyte injected (ng): RH-5992 (tebufenozide), $y = 1.8520x - 3.1732$ ($r = 0.9999$); RH-2651, $y = 1.8118x - 3.1794$ ($r = 0.9999$); RH-2703, $y = 1.5666x - 3.5064$ ($r = 0.9999$); RH-1788, $y = 1.5957x - 3.1092$ ($r = 0.9999$); RH-6595, $y = 1.4069x - 2.2708$ ($r = 0.9999$); RH-9886, $y = 1.8308x - 3.5365$ ($r = 0.9999$).

All the correlation coefficients (r) were highly significant ($p < 0.01$) and all the calibration lines were linear. The limit of detection for each analyte, calculated as three times the S.D. of the blank response [13], varied according to the type of matrix and are recorded in Table 2. The limit of quantitation was arbitrarily fixed at twice the value of the limit of detection.

A typical chromatogram obtained by injecting 25 μl of a mixed standard of tebufenozide and its five metabolites (2.0 μg/ml of each analyte) is shown in Fig. 1. All the peaks are symmetrical with baseline separation and well removed from any extraneous peaks. The retention time (min) and the peak area [milliabsorbance units × seconds (mAU s)] of each analyte were, respectively, 17.7 and 89 for RH-2651, 17.8 and 76 for RH-2703, 20.9 and 78 for RH-1788, 22.6 and 70 for RH-6595, 24.1 and 90 for RH-9886, and 28.2 and 91 for tebufenozide. Deviation in retention time for each injection was negligible and the relative standard deviation (2σ) for the peak area of each analyte in five replicate injections was less than 6%.

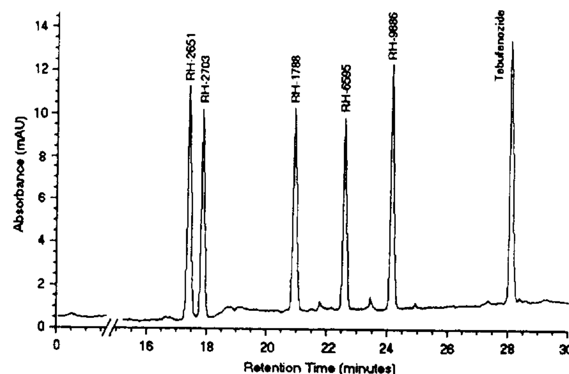

Fig. 1. Typical chromatogram obtained by a 25-μl injection of a mixed standard containing tebufenozide and its metabolites all at a concentration of 2.0 μg/ml under the following HPLC conditions: Instrument, HP 1090M with diode-array detector (DAD); data handling, HP 9000 Series 300 (Model 310) computer work station; DAD wavelengths, sample 236 ± 4 nm, reference 430 ± 50 nm; analytical column, ODS Hypersil, 5 μm, 250 × 4 mm I.D.; guard column, LiChrospher 100 RP-18, 5 μm, 4 × 4 mm I.D.; mobile phase, solvent A: methanol–water (1:4) containing 0.005 M PIC A, solvent B: methanol; flow-rate, 1.0 ml/min; oven temperature, 40°C; injection volume, 25 μl; gradient elution: $t = 0$ min, 100% solvent A; $t = 35$ min, solvent A–solvent B (12.5:87.5, v/v); Run time, 40 min.

Table 2
Detection limits of tebufenozide and its metabolites in fortified forestry substrates

Substrate	LOD[a] [μg/g (μg/l for water)]					
	Tebufenozide	RH-2651	RH-2703	RH-1788	RH-6595	RH-9886
Spruce foliage	0.02	0.03	0.03	0.01	0.01	0.03
Litter	0.02	0.02	0.02	0.01	0.01	0.02
Soil	0.02	0.02	0.03	0.02	0.02	0.02
Sediment	0.02	0.02	0.01	0.03	0.03	0.03
Water	1.0	1.5	2.5	2.5	2.0	2.0

[a] LOD = limit of detection; values below this level are reported as non-detectable (N.D.).

Table 3
Average recoveries of tebufenozide and its metabolites from forestry substrates fortified at 0.1, 0.5 and 1.0 μg/g levels (10, 50 and 100 μg/l for water)

Substrate	Percent recovery (mean ± S.D.) ($n \geq 12$)					
	Tebufenozide	RH-2651	RH-2703	RH-1788	RH-6595	RH-9886
Spruce foliage	97 ± 5	77 ± 8	72 ± 6	85 ± 5	93 ± 5	94 ± 8
Litter	97 ± 5	71 ± 7	69 ± 6	87 ± 6	94 ± 5	86 ± 5
Soil	95 ± 5	73 ± 5	69 ± 6	96 ± 7	96 ± 6	94 ± 7
Sediment	94 ± 6	78 ± 5	76 ± 5	92 ± 7	96 ± 6	94 ± 5
Water	99 ± 6	98 ± 6	84 ± 6	102 ± 6	99 ± 5	97 ± 7

3.3. Extraction, cleanup and recoveries of analytes

Forestry matrices such as conifer foliage, litter and sediment are complex in composition and usually introduce numerous endogenous materials as impurities during solvent extraction. The acidic methanol extraction used for terrestrial matrices and sediment fortified at 0.1 to 1.0 μg/g followed by Florisil column cleanup, and DCM extraction of water fortified at 10 to 100 ng/l, gave good recovery levels of the target analytes. The mean percent recoveries of the analytes with their S.D. from the fortified forest matrices are

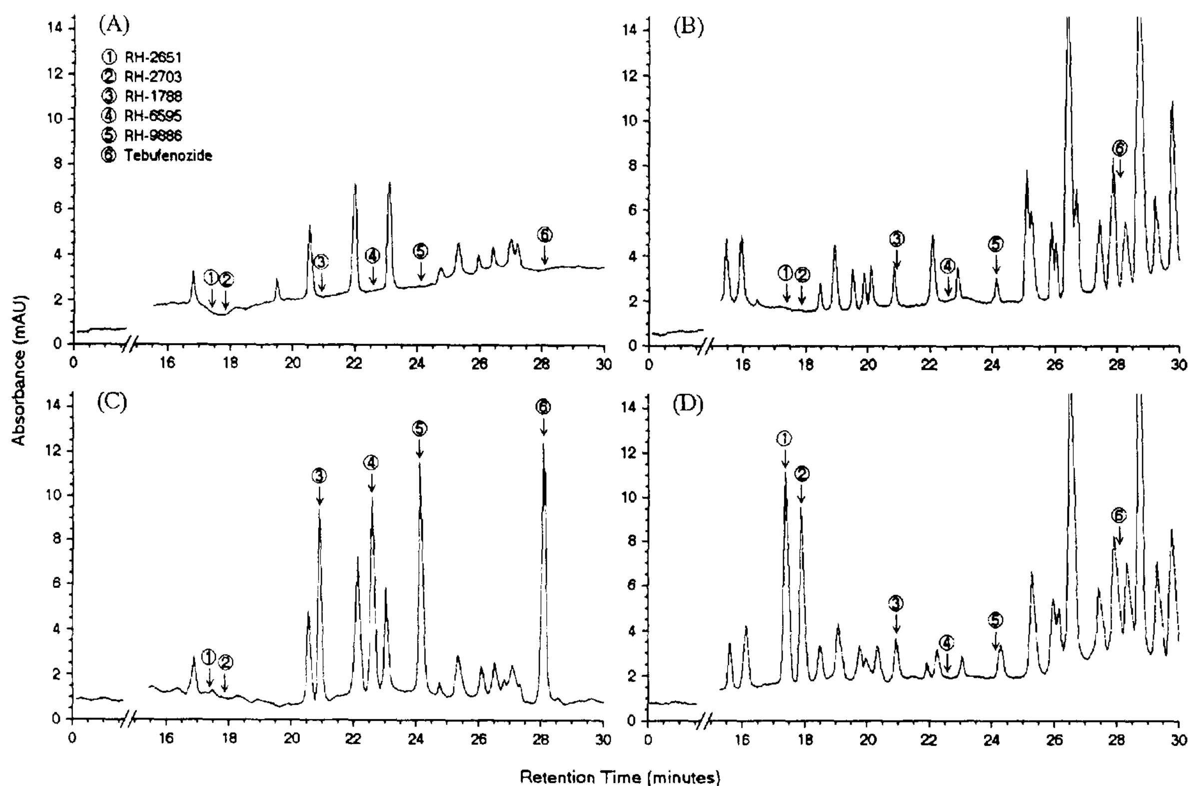

Fig. 2. Chromatograms obtained from HPLC analyses of tebufenozide and its metabolites in (A) blank spruce foliage, non-acid fraction, (B) blank spruce foliage, acid fraction, (C) spruce foliage fortified at 1.0 μg/g, non-acid fraction, and (D) spruce foliage fortified at 1.0 μg/g, acid fraction (see Fig. 1 for HPLC parameters).

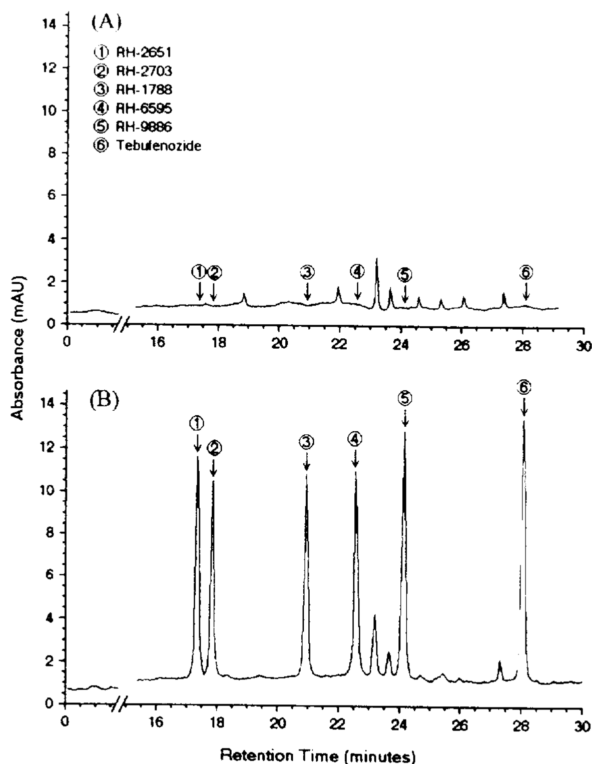

Fig. 3. Chromatograms obtained from HPLC analyses of tebufenozide and its metabolites in (A) blank natural water, and (B) natural water fortified at 50 μg/l (see Fig. 1 for HPLC parameters).

given in Table 3. Generally, the mean recoveries for the parent and its alcohol (RH-1788 and RH-9886) and ketone (RH-6595) metabolites (Table 1) were nearly quantitative and above 85% with low overall variability (<8% S.D.). However, for the two acid metabolites (RH-2651 and RH-2703), the recoveries were somewhat less quantitative and ranged from 69 to 78%. The recoveries of all the analytes from fortified natural water were consistently above 84% with low overall variability (<7% S.D.). The good recoveries and low S.D. together with the low limits of detection indicate that the method reported has good precision and would be accurate.

Representative chromatograms for the analyses of tebufenozide and its metabolites are shown in Figs. 2 and 3. Fig. 2 shows separate HPLC traces obtained for the non-acid and acid fractions of blank and fortified conifer foliage. The chromatographic peak shapes are excellent for the parent compound and the three non-acid metabolites and they are well resolved from any co-extractive impurities in the non-acid fractions. Similar chromatograms, but with fewer extraneous peaks, were obtained from blank and fortified samples of forest litter, forest soil and sediment. The use of methanol during column cleanup to elute the two acids brought down along with the target analytes, coextractive impurities producing numerous ghost peaks. Additional solvent partitioning and column chromatographic steps were not successful in eliminating these interferences. As can be seen in Fig. 2B, some of the extraneous peaks in the blanks eluted at retention times similar to those of the non-acid metabolites requiring a need to analyze separately the non-acid and acid fractions. However, a comparison of the blank water sample chromatogram (Fig. 3A) with the chromatogram of the blank conifer foliage (Fig. 2B) clearly shows that the extraneous peaks observed with retention times similar to those of the non-acid metabolites are absent in the water. Therefore, the fortified water sample was analyzed simultaneously for the parent material and five of its metabolites, as evidenced in Fig. 3.

The use of acidic acetonitrile, acetone, ethyl acetate and DCM in the extraction process of solid matrices, in place of acidic methanol, resulted in either low recoveries or interferences due to coextractives requiring time-consuming multiple extractions and or rigorous column cleanup procedures. Florisil column cleanup was optimized after examining the parameters such as percent deactivation, bed size, solvent or solvent mixture for rinsing and elution, and elution pattern necessary to desorb the analytes from the column matrix. Attempts to substitute Florisil with silica, alumina or charcoal columns, either home-made or prepacked, were not successful either due to non-reversible adsorption of the analytes onto the column matrix during solvent elution or to coelution of impurities, causing interferences and often producing ghost peaks in the chromatograms. Nevertheless, pre-

Table 4
Mean recoveries of tebufenozide and its metabolites from postspray spruce field samples of spruce foliage, litter, soil, sediment and water collected at intervals of time ($n = 6$)

Matrix	Dosage applied	Sampling time (d)	Concentration (μg/g as sampled) (μg/l for water)					
			Tebufenozide	RH-2651	RH-2703	RH-1788	RH-6595	RH-9886
Spruce foliage	140 g AI/ha	0	8.54	N.D.	N.D.	N.D.	N.D.	N.D.
		64	3.55	N.D.	N.D.	0.18	0.04	0.05
		107	2.32	N.D.	N.D.	0.06	0.11	0.09
		169	1.18	N.D.	N.D.	0.07	0.07	0.12
Litter	140 g AI/ha	0	0.64	N.D.	N.D.	N.D.	N.D.	N.D.
		64	0.45	0.12	0.08	0.04	N.D.	N.D.
		107	0.34	0.13	0.11	N.D.	0.05	N.D.
		169	0.34	0.06	0.07	0.08	0.06	N.D.
Soil	140 g AI/ha	0	0.70	N.D.	N.D.	N.D.	N.D.	N.D.
		64	0.38	0.08	0.07	0.06	N.D.	N.D.
		107	0.10	0.06	0.08	0.09	N.D.	N.D.
		169	0.07	0.04	0.08	0.04	N.D.	N.D.
Water	500 μg/l	0	659	N.D.	N.D.	N.D.	N.D.	N.D.
		21	323	N.D.	N.D.	4.2	4.2	N.D.
		49	298	N.D.	5.2	5.1	6.4	4.4
		92	293	N.D.	5.1	N.D.	5.3	4.1
Sediment	500 μg/l applied to water surface	0	8.99	N.D.	N.D.	N.D.	N.D.	N.D.
		21	19.30	N.D.	0.33	1.2	1.7	0.9
		49	23.40	N.D.	0.08	2.1	2.4	0.7
		92	22.16	N.D.	0.06	1.7	2.1	0.6

N.D. = not detectable (< LOD) (see Table 2).

packed Florisil columns were found to be suitable, but they were expensive for our needs.

3.4. Analysis of field samples

Field treated samples collected at several time intervals were analyzed using the present method and the results are shown in Table 4. Aside from the parent material, the concentration levels of the intact metabolites found in most of the postspray matrices were rather low and in some cases near or below the limits of quantitation.

None of the acid metabolites were found in spruce foliage but low levels of the alcohol and ketone metabolites were detected in all the postspray spruce samples. It appears that the enzymatic and photolytic conversion of $-CH_2CH_3$ to $-CH_2COOH$ and then to $-COOH$ is not a favoured degradative pathway for the chemical in spruce foliage.

The formation of acid metabolites was common in the soil and litter, but the presence of ketone (RH-6595) and the secondary alcohol (RH-9886) were almost negligible. The presence of primary alcohol (RH-1788) was sporadic and just above the quantitation limits.

Water, in spite of the high concentration of tebufenozide present, had low and uncertain levels of the four metabolites except for RH-2651. Similar trends were also observed in the sediment. None of the RH-2651 metabolite was found in water or sediment.

Tebufenozide dissipated from the foliage, litter, soil and water with time. However it appears that sediment acted as a sink because of the build up of concentration of the material with time. The present study shows that the dissipa-

tion of tebufenozide from forestry matrices did not proceed readily through the formation of intact metabolites; very likely, other mechanisms are involved which require further investigation.

4. Conclusions

The liquid chromatographic method presented here would be suitable for the simultaneous assay of tebufenozide and five of its metabolites from forestry matrices. It can be used to determine the metabolic pathway of the parent material in laboratory microcosm studies where higher doses or ^{14}C-labelled materials are used. The advantages of the method are that it is rapid, reasonably sensitive, reproducible and economical. With suitable modification, it could be extended to the determination of intact metabolites in various environmental matrices. If necessary, the limit of detection can be lowered by altering the cleanup procedures and concentration of the column eluates.

Acknowledgements

This research was financed jointly by the Rohm and Haas Company and by the Forest Pest Management Institute, Canadian Forest Service. The authors are thankful to Drs. D.R. Hawkins and R.O. Deakyne of Rohm and Haas for supplying the analytical grade tebufenozide and its five metabolites used in the study and for the valuable advice and support provided during the course of the work. The excellent technical assistance of Jiusheng Zhu, Bobbi-Jo Rainbird, Johanna Curry, Linda Sloane, Roya Mogharrabi and Dr. J. Ma is greatly appreciated.

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