

*A Study on the Effects of Golf Course Organophosphate and Carbamate
Pesticides on Endangered, Cave-Dwelling Arthropods
Kauai, Hawaii*



**U.S. Fish and Wildlife Service
Ecological Services
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Background

During the formation of the Hawaiian islands, lava flows created caves, cracks, gas pockets and smaller, interconnected subterranean spaces. While unique subterranean faunas have long been known from temperate continental cave systems, obligate cave inhabiting animals were long thought to be absent from tropical and island systems. However, in 1971, two remarkable, eyeless cave arthropods, a spider and amphipod, were discovered from caves in Kauai. The Kauai cave wolf spider (*Adelocosa anops*), and the Kauai cave amphipod (*Spelaeorchestia koloana*) are known only from a single exposed lava flow that covers approximately 4 square miles on the southern coast of Kauai (see accompanying map). In 1995, a third endemic species, an as yet unclassified species of isopod, was documented in the Kauai caves. These endemic species are critical components of the cave ecosystems and are possibly the last representatives of what was once a more diverse and populous cave community. The cave spider and the amphipod were listed as endangered species on January 14, 2000.

These endemics are restricted to the dark, moist areas of larger caverns and smaller subterranean spaces within the lava flow. The amphipod and isopod are detritivores and feed primarily on rotting tree roots that penetrate the cave roofs whereas the spider is a carnivore and preys upon the amphipod and alien arthropods that venture underground. The only source of water is rain or irrigation water that percolates in from above. The Kauai caves are characterized by low populations and limited species diversity. The extinction of any one of the remaining endemics could have disastrous effects on the cave ecosystem.

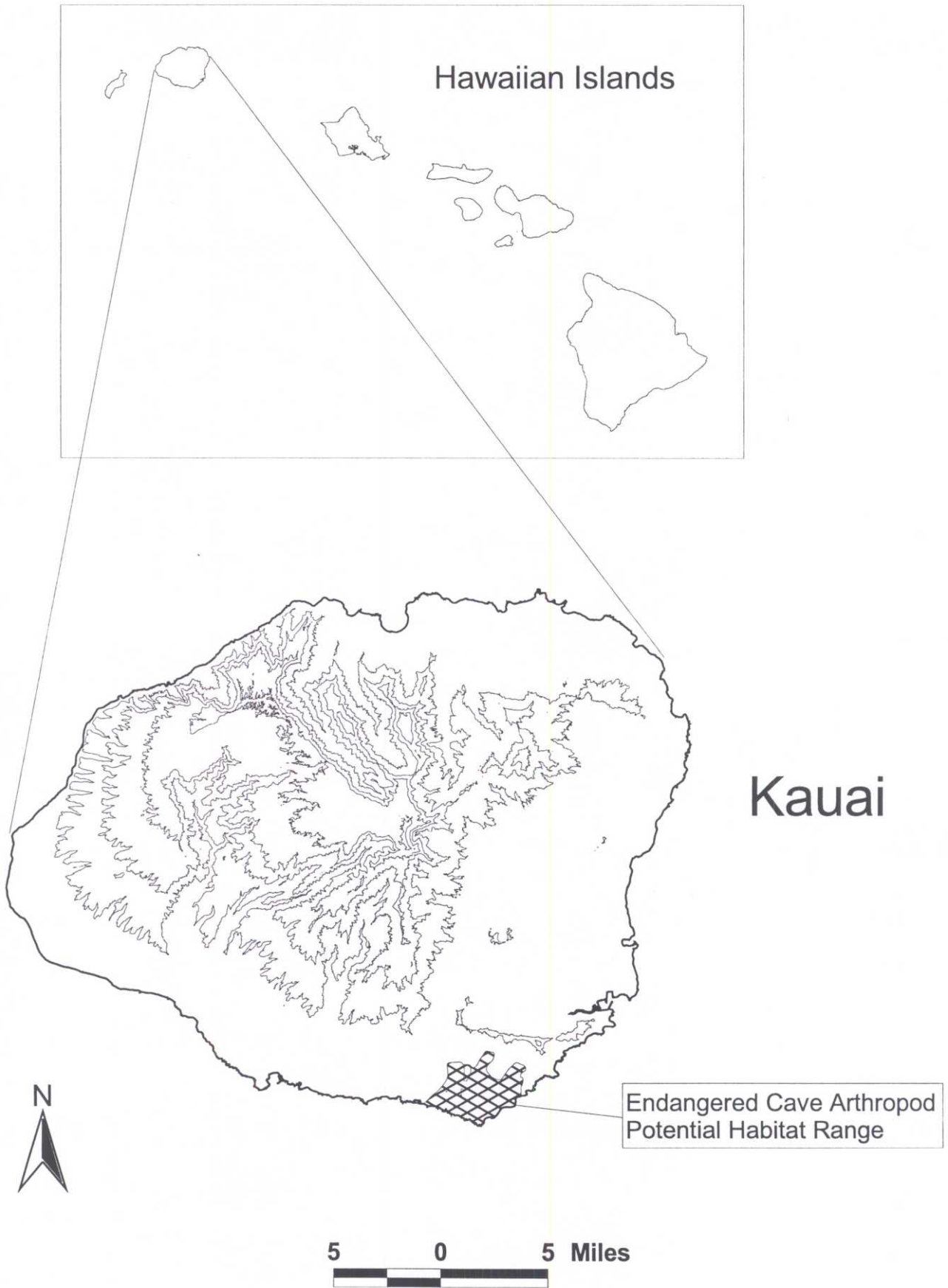
One of the most biologically diverse of the five known Kauai caves is located directly under the fairway of the Kiahuna Golf Course. While the golf course declined to specify the pesticides they use, over 30 different pesticides are commonly used on golf courses in Hawaii, including insecticides to control turf grass pests (Brennan et al., 1992). For example, most golf courses in Hawaii apply the insecticide chlorpyrifos at the rate of 3 pounds of active ingredients per acre, one to three times per year, but rates and frequency of applications are sometimes much higher. Organophosphates, carbamates, triazines, chlorophenoxy compounds, and organic arsenicals are all commonly used in large quantities on Hawaiian golf courses (Brennan et al., 1992).

Organophosphates and carbamates exert their toxicity by suppressing the production of the cholinesterase enzymes necessary for the breakdown of the neurotransmitter acetylcholine and the normal functioning of the peripheral and central nervous systems (Edwards and Fisher 1991). If acetylcholine is not broken down after its release into the neural synapses, the nervous system cannot function properly and lethal and a variety of sublethal effects can occur.

The endemic cave arthropods were feared to be at a high risk from golf course pesticide applications for the following reasons:

- ▶ Current population data indicate that the populations of endemic cave arthropods are very low to begin with and exist in a highly restricted habitat with low species diversity (Asquith, 1996).
- ▶ These animals are known only from a single lava flow in the "very rocky" to "extremely rocky" Waikomo soil series, which is largely unweathered, with very little erosional sediment (U.S. Department of Agriculture, Soil Conservation Service 1972). Therefore,

Kauai Cave Arthropod Habitat Range



the soils and caves are very porous and runoff or leachate from the golf course may have a direct route of entry into the caves, with relatively little opportunity for contaminants to be trapped in or sorbed to organic matter in soils.

- ▶ Some of the commonly used golf course pesticides, such as dicamba, trichlorfon, simazine, mecoprop, and metribuzin have a high potential for leaching, especially in soils with low organic content (Murdoch and Green, 1989).
- ▶ The absence of light in the caves will prolong the time required for the breakdown of pesticides, which are commonly subject to photodegradation in above-ground environments.
- ▶ Insecticides are designed to be highly toxic to arthropods (often in concentrations less than 1 part per million) and many herbicides and fungicides also have lethal and sublethal effects on many invertebrates (Brown, 1978).
- ▶ These cave animals are particularly susceptible to pesticides because of their tendency to seek water sources (Howarth 1983). Even if pesticides are not used directly above a lava tube, pesticides that leach into adjacent subterranean caverns are serious threats because the animals may be attracted to the water and come into contact with the chemicals. In addition, the exoskeleton of the endemic cave organisms is more permeable to water than that of surface dwelling, congeneric species and alien cave dwelling species (Hadley et al., 1981). This exposes them to additional risk via absorption of contaminants through their exoskeleton.
- ▶ Predators such as the Kauai cave spider are generally more susceptible to insecticides than the target pests (Croft 1990). Even if not killed outright, the sublethal effects of both insecticides and herbicides on the cave animals could include reduced fecundity, reduced life span, slowed development rate, and impaired mobility and feeding efficiency (Messing and Croft 1990).

Study Objectives

We chose to focus on organophosphates and carbamates because these classes of chemicals account for a large percentage of the pesticides used on golf courses and their immediate physiological effects on arthropods can be quantified. The objectives of the study were to:

- ▶ Determine the presence or absence of a contaminant pathway into the caves by comparing organophosphate and carbamate concentrations in cave soils and in the water entering the golf course and control caves;
- ▶ Extrapolate the presence or absence of exposure and physiological effects on endemic cave arthropods, caused by carbamate and organophosphate pesticides, by comparing cholinesterase inhibition levels in alien arthropods living in the golf course and control caves; and
- ▶ Use study data to determine the need for altering pest management practices at the golf course and specify pesticides of concern.

Study Methods and Results

Water, soil, and cockroach samples were collected from the caves on February 26, May 25, and June 18, 1998. Two water samples were collected by placing sterile glass jars on the cave floor under areas where water dripped from the cave ceiling. A pyrex funnel was placed on the top of each jar to catch the water. Upon retrieval, the lids were screwed onto the jars and they were cooled to approximately 4 degrees C. In some cases, water jars were retrieved up to three weeks after being placed out. The control cave was too dry to collect water, so both samples analyzed were taken from the cave under the golf course. Soil samples (3 from the golf course cave and 2 from the control) were collected from moist areas of the cave floor with a metal trowel and placed into large plastic baggies. They were also cooled to 4 degrees C. Cockroaches were caught by hand on the cave floor, placed in glass jars, and frozen on dry ice within 30 minutes of capture. Many of the roaches were very small, and we found only 3 of sufficient size in the golf course cave and 4 in the control cave.

Water and soil analysis was contracted to the University of Hawaii's Department of Environmental Biochemistry. A complete report of their laboratory methods and results is attached as Appendix 1. Cholinesterase inhibition analysis was conducted by the Hawaii Institute of Marine Biology. Their report on methods and results is attached as Appendix 2.

Discussion

Chemical analysis of soil and water samples taken in the cave did not indicate that carbamate or organophosphate pesticides were penetrating the cave. Likewise, the lack of cholinesterase inhibition in the cave cockroaches indicated that there was not a complete pathway for these pesticides from the golf course to the endemic cave arthropods. Other anthropogenic compounds, not tested in this study, could be present and having some effect.

Qualitative observations over time by several observers indicate that the highest population counts of spiders and amphipods occurred when water and organic matter (food) was recently introduced into the caves. Thus, the main limiting factors at this location may be the lack of sufficient food and water. The presence of alien predators in the caves, such as spiders and roaches, may also be detrimental.

Since this study was completed, Kiahuna Golf Course has agreed to plant deep-rooting vegetation over the entire length of the cave (replacing the golf course fairway) and will also maintain the currently posted signs prohibiting entrance to the cave. This will greatly decrease the risk of pesticides and fertilizers dripping into the cave, in addition to providing a much needed food source in the form of roots growing through the cave roof. The Service hopes to obtain similar conservation agreements for the area around the control caves, which the golf course intends to develop in the near future.

Recommendations for Future Studies

Lava tube ecosystems are present in several other coastal areas of the state, particularly on the islands of Hawaii and Maui. These ecosystems contain many rare, cave-adapted arthropods and are subject to non-point source pollution of the water that percolates down to the caves from residential areas, golf courses, and other sources. It is therefore quite likely that this type of study will need to be repeated in these areas.

Future studies should bear in mind the following lessons learned from this study:

- ▶ Monitor and test water and soils over a long period of time, including at least one rainy season;
- ▶ Design a long-term water collection device that doesn't require frequent servicing, using collecting materials that adsorb contaminants from the water (thus minimizing chemical breakdown in the water) as it passes through a funnel into a collecting jar;
- ▶ Add a toxicity testing component to the study design, examining lethal and sublethal effects in surrogate species placed in soil and water samples from the cave;
- ▶ Obtain soil cores from surface soil above caves to determine presence and mobility of pesticides;
- ▶ If locally abundant, use endemic arthropods to test cholinesterase inhibition;
- ▶ Test for the presence of additional contaminants such as fertilizers, metals, petroleum hydrocarbons and other pesticides; and
- ▶ Discuss and advocate multiple management strategies with the landowners, including maintaining deep-rooting vegetation over the entire length of the caves, restricting access to caves, maintaining high humidity in the caves, and avoiding use of pesticides and fertilizers in the area.

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APPENDIX 1

*University of Hawaii, Department of Environmental Biochemistry, Final Report on
Carbamate and Organophosphate Pesticides in Water and Soil Samples
Taken From Kauai Caves*

Final Report to

U.S. Fish and Wildlife Service
Pacific Island Office, 300 Ala Moana Blvd., Room 3-122
Honolulu, HI 96850

on

**Analysis of Water Samples for Low Levels of Pesticides and
Analysis of Inhibition of Neurotransmitter Breakdown in Insects**

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Two additional soil samples were delivered to this laboratory on June 19, 1998. Three bags of each soil sample were sealed. The samples were logged by the analyst immediately upon arrival, and labeled as "USFWS-061898-01" and "USFWS-061898-02" for the Makai and the Mauka soils, respectively. Soils were then stored at 4 °C in the walk-in refrigerator of Henke 210. These two soils were analyzed by Dr. Fengmao Guo within a week after arrival.

Table 1. A list of samples

sample type	sample ID #	description	sample storage and moisture
leachate water	USFWS-022698-01	Kiahuna golf course (Mauka)	store at 4 °C
soil	USFWS-022698-02	Makai, reference	store at -20 °C, 6.1% H ₂ O
soil	USFWS-022698-03	Kiahuna golf course (Mauka)	store at -20 °C, 11.9% H ₂ O
soil	USFWS-022698-04	Kiahuna golf course (Mauka)	store at -20 °C, 17.3% H ₂ O
cockroach	USFWS-022698-05	Makai, reference	store at -20 °
cockroach	USFWS-022698-06	Kiahuna golf course (Mauka)	store at -20
leachate water	USFWS-052598-01	Kiahuna golf course (Mauka)	store at 4 °C
soil	USFWS-061898-01	Makai, reference	store at 4 °C, 24.2% H ₂ O
soil	USFWS-061898-02	Kiahuna golf course (Mauka)	store at 4 °C, 51.6% H ₂ O

Standard analytes and surrogates: All analytical references used in this study were recently certified standards. The standards were selected according to their possible uses on the golf courses and leaching potential. These standards are listed in Table 2. These standards are used to confirm the analytical recoveries and to serve as references.

Table 2. Standard analytes and surrogates

standard analyte	purity, %	comments
alachlor	99.0	
bromacil	99.0	
carbaryl	99.0	
chlorpyrifos	99.0	
chlorthalonil	99.7	
decachlorobiphenyl	99.9	CAS No: 2051-24-3, surrogate
hexazinone	99.0	
metalachlor	99.0	
metribuzin	99.9	
2-nitro-m-xylene*	99.9	CAS No: 81-20-9, surrogate

* 1,3- dimethyl-2-nitrobenzene, 2,6-dimethylnitrobenzene or 2-nitro m-xylene.

Standard Preparation: Standard stock solutions of about 1,000 ppm were prepared according to the following procedure. Standards were first checked and logged-out of the storage locker. Approximately 100 mg of each standard was massed in a glass weighing boat on a Mettler AJ100 analytical balance last certified on November 11, 1997. Each weighing boat containing the standard was washed with methyl-*t*-butylether (MTBE) several times, the washes were then collected and placed in a 10-mL volumetric flask. After complete transfer was assured, the flask was diluted to the volume. The solution was then transferred to an amber reagent bottle which was sealed with Teflon strips. All standards and surrogates were prepared in this fashion except for decachlorobiphenyl. The final concentration of this was smaller due to limited amounts of standard in stock. Spiking solutions were made by pipetting 50 μ L and diluting to a final volume of 10 mL of MTBE thus creating a 5 ppm spiking solution.

Leachate Extraction (USFWS-022698-01 and USFWS-052598-01): The Leachates were extracted following procedures outlined in the EPA methods 507 and 508. These procedures outline specific protocols to determine residual amounts of nitrogen and phosphorus-containing pesticides (EPA method 507) and chlorinated pesticides (EPA method 508) in water. Slight modifications of the methods were done to accommodate the small sample sizes available.

During the course of each analysis additional references of the Laboratory Reagent Blank (LRB), containing reagent water and surrogates, and the Laboratory Fortified Blank (LFB), containing reagent water, surrogates and spiked analytes, were extracted along with the leachate water samples.

Leachate water from the Kiahuna cave was decanted into a 1 liter separation funnel. Reagent water (200 mL) was placed into a separation funnel to serve as the LRB and LFB. Each sample was then spiked with 0.3 μ g of decachlorobiphenyl (DCBP) and 0.3 μ g of 2-nitro-*m*-xylene. DCBP and 2-nitro-*m*-xylene were surrogates used to quantify recoveries.

Prior to extraction 50 g of NaCl and 25 mL of phosphate buffer (pH 7.0) were added in the sample. Methylene chloride (30 mL) was then added to extract the analytes. The LFB and LRB samples were also extracted following the same procedure. Extraction was initiated by vigorous shaking and venting of the funnel for 2 min. After the organic layer was allowed to separate for 10 min, it was collected in a 250 mL round bottom flask. This procedure was repeated two more times to give 90 mL of final extracts. Extracts were then dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated according to the following procedures.

- a. Temperature of the circulating condenser was adjusted to $-10\text{ }^\circ\text{C}$
- b. Extract was then dried by filtering it through a solvent- rinsed funnel, containing approximately 20 g of anhydrous Na_2SO_4 , into a 250 mL round bottom flask. Rinsing the flask several times ensures complete transfer.
- c. Filtrate was then placed on a rotary evaporator. Methylene chloride was concentrated down to approximately 5 mL.
- d. MTBE (25 mL) was added to the round bottom flask and Step c was repeated.
- e. Step d was repeated two more times to ensure that methylene chloride had evaporated.
- f. Using a glass pipette the extract solution was transferred to a 10 mL volumetric vial. The flask was rinsed with MTBE (3x1 mL) to ensure complete transfer of the extract.

Soil Extraction (USFWS-022698-02, USFWS-022698-03 and USFWS-022698-04):

These soil samples were extracted by supercritical fluid extraction (SFE), following an established procedure in this laboratory. The SFE procedure utilizes supercritical carbon dioxide (CO_2), Na_2EDTA and solvent modifiers. The method is capable of recovering a large range of polar and non-polar chemicals from soils.

Soil preparation: After soils were air dried, the samples were ground to a uniform particle size with a ceramic pestle. A longer mash time was required the Kiahuna Green samples because of the large amounts of moisture, which formed large clumps. The moisture content was determined by placing approximately 1 g of the sample in an oven at 150 °C for 16 h. Percent moisture was calculated and is listed Table 1.

SFE: Air-dried soil (2.0 g) placed in a 10 mL solvent-rinsed beaker was spiked with distilled deionized water to adjust a final moisture content to 15%. Na_4EDTA (0.1 g) was also added and thoroughly mixed. The samples were packed in a 2.5-mL extraction vial. Methanol (1 mL) was used to rinse the beaker. Methanol (2 mL) is an extraction fluid modifier. An Isco SFX 2-10 extractor was used to extract the samples according to the following procedure.

- a. Samples in the extraction vial were equilibrated to the extraction temperature 60 °C.
- b. Static extraction of the samples was then done for 5 min at a pressure of 5,000 psi.
- c. After the 5 min of static extraction fluid was collected in a trap tube containing 15 mL of methanol as trapping solvent.
- d. Dynamic extraction using 45 mL of supercritical CO_2 was passed through the sample and collected in the trap tube.
- e. Following the dynamic extraction, samples were re-modified with the addition of 1.0 mL of methanol.
- f. Steps a through d were repeated.
- g. Sample extracts were dried over approximately 10 g of anhydrous Na_2SO_4 (dried overnight at 400 °C) and collected in a 250 mL round bottom flask.
- h. Solvents were evaporated with a rotary evaporator to approximately 5 mL.
- i. The extracts were transferred using a glass pipette to a filter syringe fitted with a Gelman Acrodisc having a pore size of 0.45 μm . The flask was washed with MTBE for three times to ensure complete transfer.
- J. Filtrate was collected in a 10-mL volumetric tube and reduced under a gentle nitrogen stream to a final volume of 5 mL.
- k. These tubes were capped and sealed with TFE strips. The extracts were stored at 4 °C until analysis.

Solvent Extraction: Because of the larger sample size of USFWS-022698-03 it was used for further qualitative analysis. The air-dried soil (90 g) from the Kiahuna Green site was placed in a 250-mL Teflon centrifuge tube and methylene chloride (75 mL) was added. The sample was shaken on a Burrell shaker for 30 min. The sample was centrifuged on a Sorvall centrifuge at 7,000 rpm until the suspension had clarified. The supernate was decanted and the extraction was repeated twice. All supernates were combined and dried over anhydrous Na_2SO_4 (about 25 g). The filtrate was placed in a 500-mL round bottom flask and concentrated with a rotary evaporator to 5 mL. MTBE (25 mL) was added to the flask. The MTBE was then evaporated

down to 5 mL, this step, including the addition of MTBE, was repeated two more times.

After rotary concentration the extract was filtered to remove particulates using a 0.45- μm Gelman Acrodisc. The filtrate was concentrated to 5 mL under a stream of nitrogen gas. The extract was stored at 4 °C for later analysis.

Soil Extraction (USFWS-061898-01 and USFWS-061898-02):

- a. Weigh 124.2 g of USFWS-061898-01 or 151.6 of USFWS-061898-02 (i.e., 100 g of dry basis) into a 1-L Mason jar. Add 175.8 and 148.4 mL of distilled deionized water to USFWS-061898-01 and -02, respectively, to make a suspension (water:soil = 2:1). The suspension was sonicated for 10 min to disperse soil aggregates. A LRB was prepared in the same manner as the two samples except there is no soil.
- b. Add 100 mL of acetonitrile and 100 mL of methylene chloride to the suspension. A Sorvall Omni-mixer with motorized steel blades was used to stir rigorously the soil suspension for 30 min. The suspension was then filtered through an acetone-washed Whatman No. 541 filter paper under vacuum. The liquid phase was collected in a 1-L separation funnel.
- c. The soil on the filter paper was re-suspended in 100 mL of water, and re-extracted according to step *b*. The liquid phase was combined with the first aliquot in a 1-L separation
- d. Repeat step *c*.
- e. Add 50 g of NaCl (dried at 400 °C overnight) to the extracts in each separation funnel. Add 50 mL of phosphate buffer (pH 7), and 50 μL of 1,3-dimethyl-2-nitrobenzene. Shake rigorously for 3 min. After 10 min, collect the liquid phase at the bottom of the separation funnel.
- f. Add 60 mL of methylene chloride, shake rigorously the separation funnel for 3 min. After 10 min, collect the liquid phase at the bottom of the separation funnel.
- g. Repeat step *f*.
- h. The extracts were evaporated to near dryness using a rotary evaporator with a water bath set at 60 °C. Two 25-mL aliquots of MTBE were consecutively added and evaporated to 1~2 mL.
- i. The final extracts were completely transferred to 5-mL volumetric centrifuge tubes and volume was adjusted to 5 mL.

Detection of Analytes: The extracts were analyzed on a Hewlett Packard (HP) 5890 Gas Chromatograph (GC) equipped with a Nitrogen Phosphorus Detector (NPD). The column was a 30 m x 0.252 mm, J&W DB-5 with a film thickness of 0.25 μm . A mixture of the standard pesticides at different concentrations was used as references for identification.

All samples were also analyzed on a HP 5890 Series II GC equipped with a HP 5989A Mass Spectrometer (MS) and a HP 5673 auto-sampler. The GC-MS was run with the HP 3365 Series II Chemstation Software. The GC column was J&W DB-5 (30 m x 0.25 mm, 0.25 μm film). The oven temperature was increased from 70 °C for 1.0 min to 230 °C at a constant ramp rate of 5 °C per min.

Results and Discussion

Leachate Water Samples (USFWS-022698-01 and USFWS-052598-01):

The analytical procedure for the leachate samples was followed with the EPA methods for the analysis of nitrogen and phosphorus-containing pesticides (EPA method 507) and chlorinated pesticides (EPA method 508) in water. Recoveries of surrogates and standard pesticides ranged from 80 to 135%. 2-Nitro-m-xylene and decachlorobiphenyl were surrogates and were spiked in the laboratory reagent blank (LFB). 2-Nitro-m-xylene, chlorthalonil, chlorpyrifos, and decachlorobiphenyl were spiked in the laboratory fortified blank (LFB).

The two leachate samples contained numerous chemicals as shown in the total ion chromatograms (TIC) in Figures 1 and 2. These chromatograms showed very different peak patterns, particularly in the region of 4-15 min. The spectra of these peaks were compared with those in the MS spectral libraries. The comparison showed that many peaks are straight chain alkanes, especially after 20 min of retention time, in both leachate water extracts. No spectra of these peaks bore any resemblance to organophosphorus and carbamate pesticides as well as other common pesticides. The different peak patterns in Figures 1 and 2 suggest that the chemicals in the leachates vary with plant growth seasons. Many of the chemicals identified are probably of plant-origin. It would further suggest that the presence of pesticides in the leachates relate to the pesticide application, sampling date and rain fall. Therefore, continuous monitoring of possible pesticide leaching into the caves is recommended.

The major peaks in Figure 1 are tabulated and listed in Appendix A along with the spectra and their matching spectra from the MS spectral library. The major peaks in Figure 2 are tabulated and listed in Appendix B along with the spectra and their matching spectra from the MS spectral library. Appendix B also includes the spectra of the LRB and LFB for the major peaks in Figures 3 and 4.

Soil Samples from Makai (USFWS-022698-02 as reference) and Kiahuna golf course (USFWS-022698-03 and USFWS-022698-04):

These samples were determined for their nitrogen, organic carbon (OC), and moisture content (Table 3). These soil samples were extracted with solvent and supercritical fluid extraction methods. The extracts were analyzed with GC-NPD and GC-MS. The results of the GC-NPD showed that no peak in all the extracts matches with these of the pesticide standards atrazine, carbaryl, chlorpyrifos, chlorthalonil and diazinon. The MS spectra of all the peaks in the TIC indicated that no pesticides were present in the extracts.

Table 3. Characterization of soil samples

soil sample	N, %	OC, %	H ₂ O, %
USFWS-022698-02	0.14	1.63	6.1
USFWS-022698-03	0.24	2.59	11.9
USFWS-022698-04	0.17	1.77	17.3

SFE: We have recently developed a Na₄EDTA-assisted SFE procedure for a quantitative extraction of various chemicals from soil. This method utilizes supercritical carbon dioxide fluid, Na₄EDTA and small amount of solvent modifiers. Table 4 shows the recoveries for some polar herbicides and the two polycyclic aromatic hydrocarbons (PAH) naphthalene and

anthracene in a clayey soil. This procedure was applied to the extraction of possible pesticides in the soil samples collected from Kiahuna golf course. The SFE extracts were then analyzed by GC-MS. There were only a few peaks shown in the total ion chromatograms (Figures 5-7). MS spectra data indicated three consistent peaks in all SFE extracts and the only difference in the spectra was that two additional peaks at retention times 14.5 and 17.6 min appeared in the Makai reference soil. However, these peaks were not identified by matching with the spectra in the Wiley Chemical Library. None of the visible peaks showed any patterns of molecular (M^+) and $M^+ + 2$ ions suggesting the possibility of chlorinated compounds. No nitrogen and phosphorus-containing pesticides were identified in the SFE extracts.

The major peaks in Figures 5-7 are tabulated and listed in Appendix C along with the spectra and their matching spectra from the MS spectral library.

Solvent extraction: We have also extracted the soil sample USFWS-022698-03 with organic solvents. However, we did not have enough samples of USFWS-022698-02 and -04 for solvent extraction. A large amount of the soil (90 g) was extracted with methylene chloride. This sample extract yielded more peaks (Figure 8) than that of the SFE extract (Figure 6). However, no spectrum of any peaks were matched with the spectra of pesticides in the MS spectral library. Appendix D lists all major peaks in the TIC and their corresponding spectra.

Soil Samples from Makai (USFWS-061898-01 as reference) and Kiahuna golf course (USFWS-061898-02):

Additional soil samples were collected from a Makai reference site and Kiahuna golf course. These two samples were analyzed by Dr. Fengmao Guo. One hundred grams of soil were extracted with a mixture of methylene and acetonitrile (1:1) twice (2x200 mL). The extracts were analyzed by GC-NPD. Comparison of GC chromatograms of a standard mixture (Figure 9), the samples (Figures 10-11) and the LRB (Figure 12) showed that none of the peaks fell within the retention time window ($t \pm 3$ standard deviation) of the reference pesticides alachlor, bromacil, hexazinone, metalachlor and metribuzin. Other peaks occurred and indicated the presence of some nitrogen- and phosphorus-containing compounds. However, the concentrations of these solutes are very low. Extracts of 100 g soils were concentrated to 5 mL.

The extracts were further analyzed with GC-MS to identify both the nitrogen- and phosphorus-containing compounds and others that were not detected by the GC-NPD. Results in

Table 4. Extraction efficiency of Na_4EDTA -assisted SFE¹ for some polar and nonpolar chemicals in a clayey soil[†]

compounds	recoveries (%) ²	
	no Na_4EDTA	add Na_4EDTA
2,4-D	36 ± 4	98 ± 2
2,4-DB	63 ± 6	100 ± 5
2,4,5-T	50 ± 6	102 ± 1
dicamba	62 ± 7	106 ± 8
naphthalene	86 ± 24	93 ± 1
anthracene	67 ± 12	87 ± 16

¹ Extraction temperature was 60 °C. Soil moisture was 15%. Na_4EDTA and Methanol were added at 50 mg and 0.5 mL g^{-1} soil, respectively.

² Spike concentration was 25 $\mu\text{g g}^{-1}$ soil. Data were mean of 4 replicates except for the 2,4-D sample, which was that of 12 replicates.

Figures 13-15 indicated that no common pesticides were present at sub-ppb levels. Notable peaks are non-pesticides, phthalates and chemicals from solvents used.

Some low-molecular-weight soil organic materials and/or compounds from solvents and reagent water may contain nitrogen and phosphorus and thus correspond to some peaks on the GC-NPD chromatograms. However, the concentrations of most of these compounds are very low, and were not detected by the GC-MS. These two samples may not contain any nitrogen and/or phosphorus containing pesticides (i.e., phosphates and carbamates), if any, the levels should be ultra low.

Acetylcholinesterase Inhibition and Recommendation:

Above much effort has been made to determine the presence of organophosphorus and carbamate insecticides (i.e., acetylcholinesterase inhibitor) in the leachate and soil. The results suggest that GC-able pesticides were not present in the samples analyzed. Therefore, there would be little need to run the assay for acetylcholinesterase inhibition. However, the results can not exclude the possibility of pesticide pollution in the leachate and soil. For example, pesticides may degrade and thus were not been detected. We propose to run Microtox assays for the samples. Microtox assay is more sensitive and generates information on general toxicity of the samples. We also propose to determine the levels of ATP, ADP and AMP in the endemic species collected from the caves. We have preliminarily established a capillary electrophoretic method to measure ATP, ADP and AMP. Such an experiment can define adverse effects of possible pollutants on the respiratory system.

We also recommend proper timing and strategic sampling. Continuous monitoring may provide more information and detailed profile of possible pollutants in the leachate. Knowing pesticide application information is also helpful.

Appendices A-D are archived in the Department of Environmental Biochemistry, University of Hawaii and are available upon requests.

Appendix A. A list of major peaks and spectra of chemicals in the extract of the leachate USFWS-022698-01 (Kiahuna) on a GC-MS-TIC chromatogram.

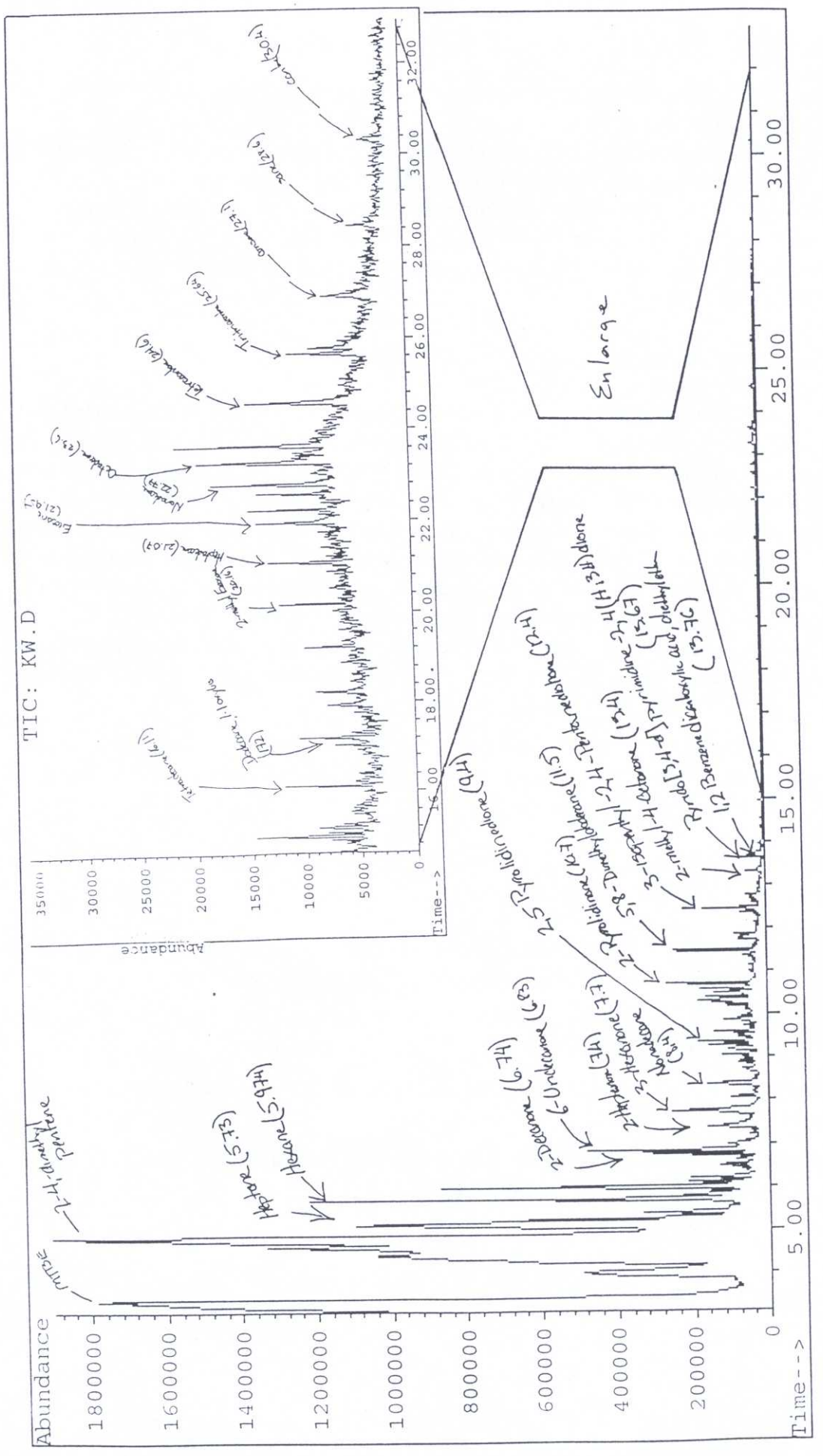
Appendix B. A list of major peaks and spectra of chemicals in the extract of the leachate USFWS-052598-01 (Kiahuna) on GC-MS-TIC chromatograms.

Appendix C. A list of peaks and spectra of chemicals in the SFE extracts of the soil USFWS-022698-02-4 on a GC-MS-TIC chromatogram.

Appendix D. A list of peaks and spectra of chemicals in the solvent extracts of the soil USFWS-022698-03 on a GC-MS-TIC chromatogram.

Figure 1. Total ion chromatogram of an extract of the leachate USFWS-022698-01.

File : C:\HPCHEM\3\DATA\KS\KW.D
 Operator : mdd
 Acquired : 13 Jun 98 6:13 am using AcqMethod KS
 Instrument : 5989x - I
 Sample Name: Kiahua 5.10
 Misc Info :
 Vial Number: 16



File : C:\HPCHEM\3\DATA\KS\68-5.D

Operator : mdd

Acquired : 9 Jun 98 1:19 pm using AcqMethod KS

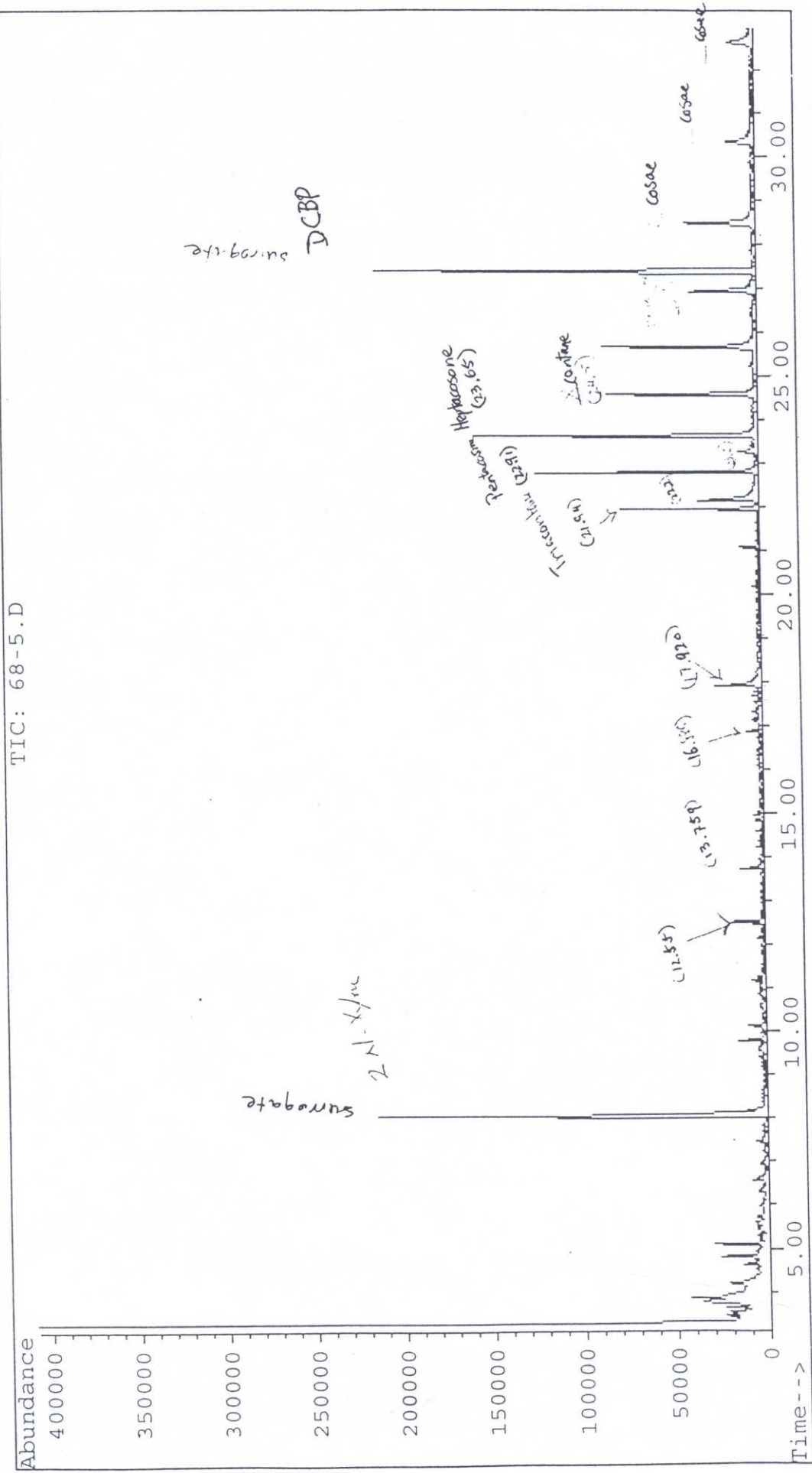
Instrument : 5989X - I

Sample Name: KW

Misc Info :

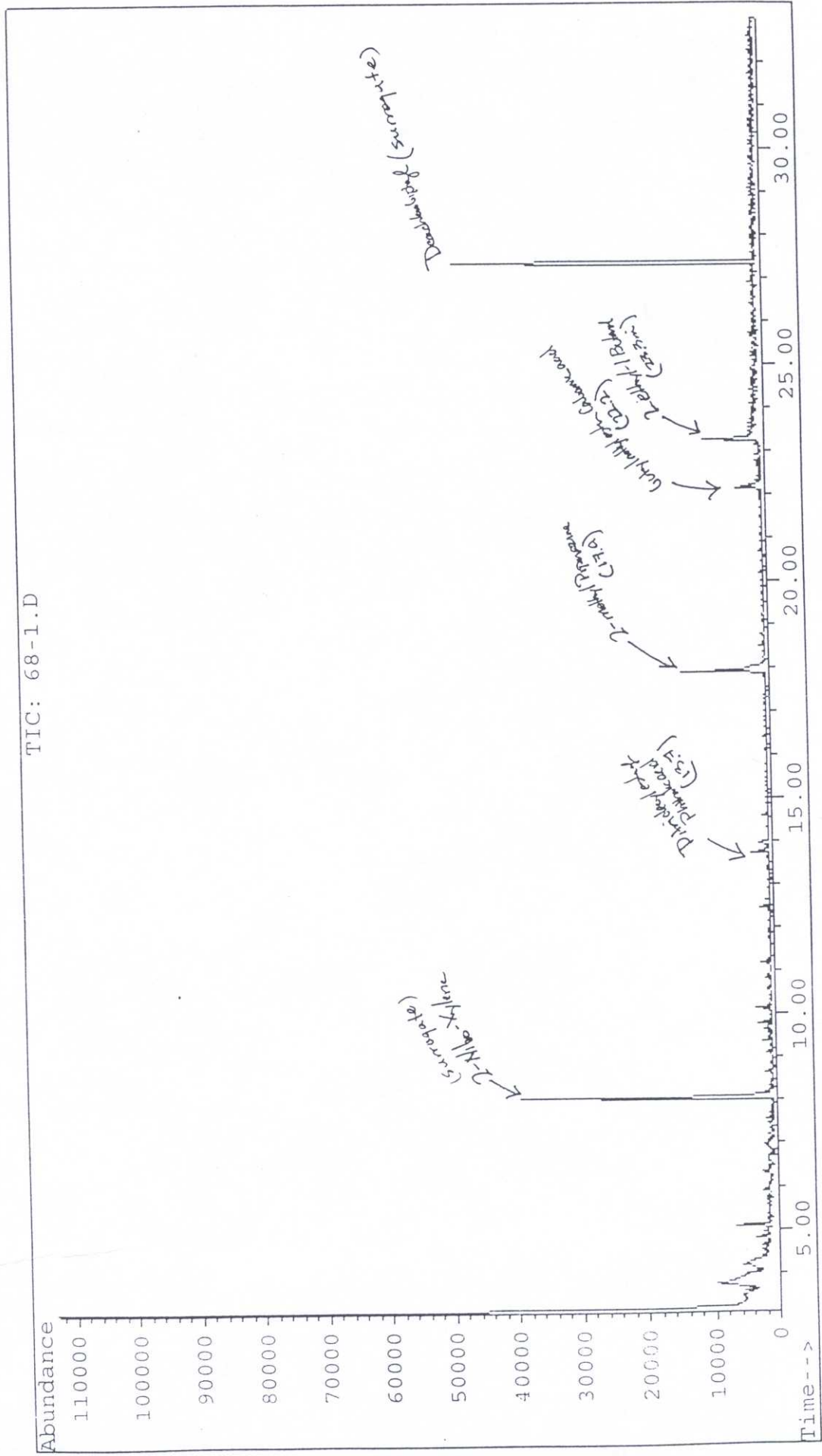
Vial Number: 5

Figure 2. Total ion chromatogram of an extract of the leachate USFWS-052598-01.



File : C:\HPCHEM\3\DATA\KS\68-1.D
 Operator : mdd
 Acquired : 9 Jun 98 10:46 am using AcqMethod KS
 Instrument : 5989x - I
 Sample Name : LD
 Misc Info :
 Vial Number : 1

Figure 3. Total ion chromatogram of a laboratory reagent blank (reagent water and surrogates).



File : C:\HPCHEM\3\DATA\KS\68-3.D

Operator : mdd

Acquired : 9 Jun 98 12:03 pm using AcqMethod KS

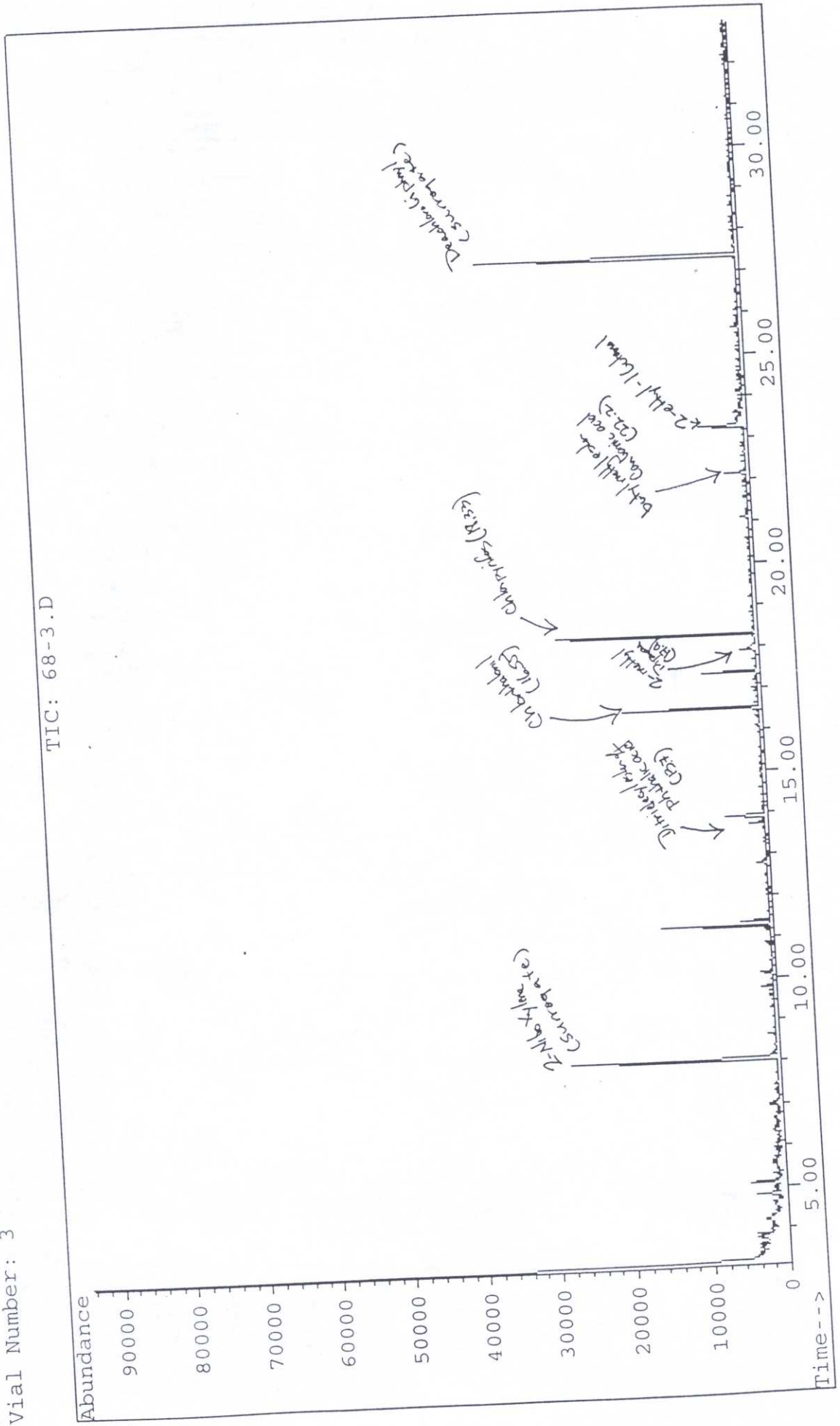
Instrument : 5989x - I

Sample Name: LF

Misc Info :

Vial Number: 3

Figure 4. Total ion chromatogram of a laboratory fortified blank (reagent water spiked with surrogates and reference standard).



File : C:\HPCHEM\3\DATA\KS\G2F5.D

Operator : mdd

Acquired : 13 Jun 98 9:23 am using AcqMethod KS

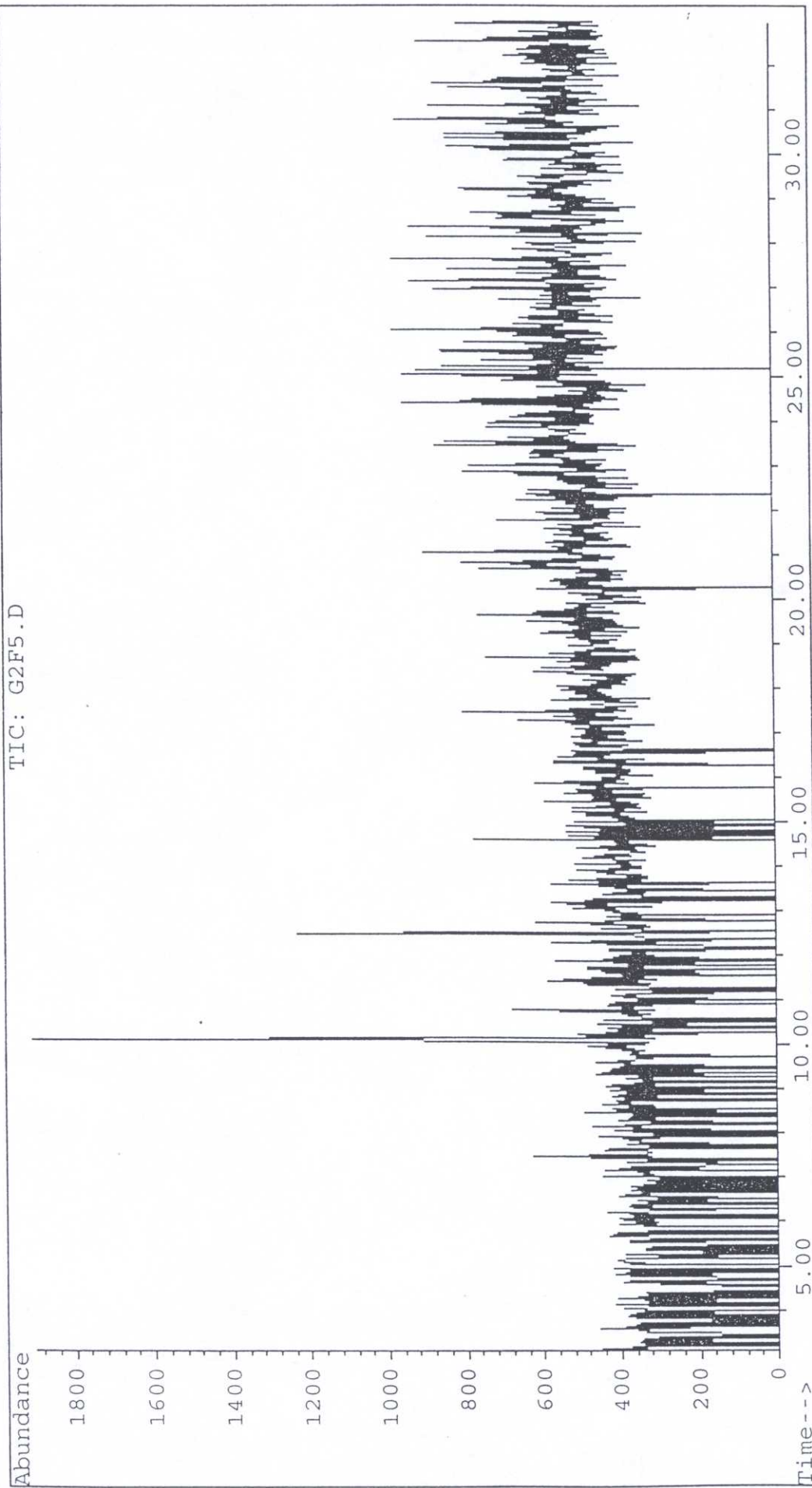
Instrument : 5989x - I

Sample Name: SFE Kiahua Soil G2F5

Misc Info :

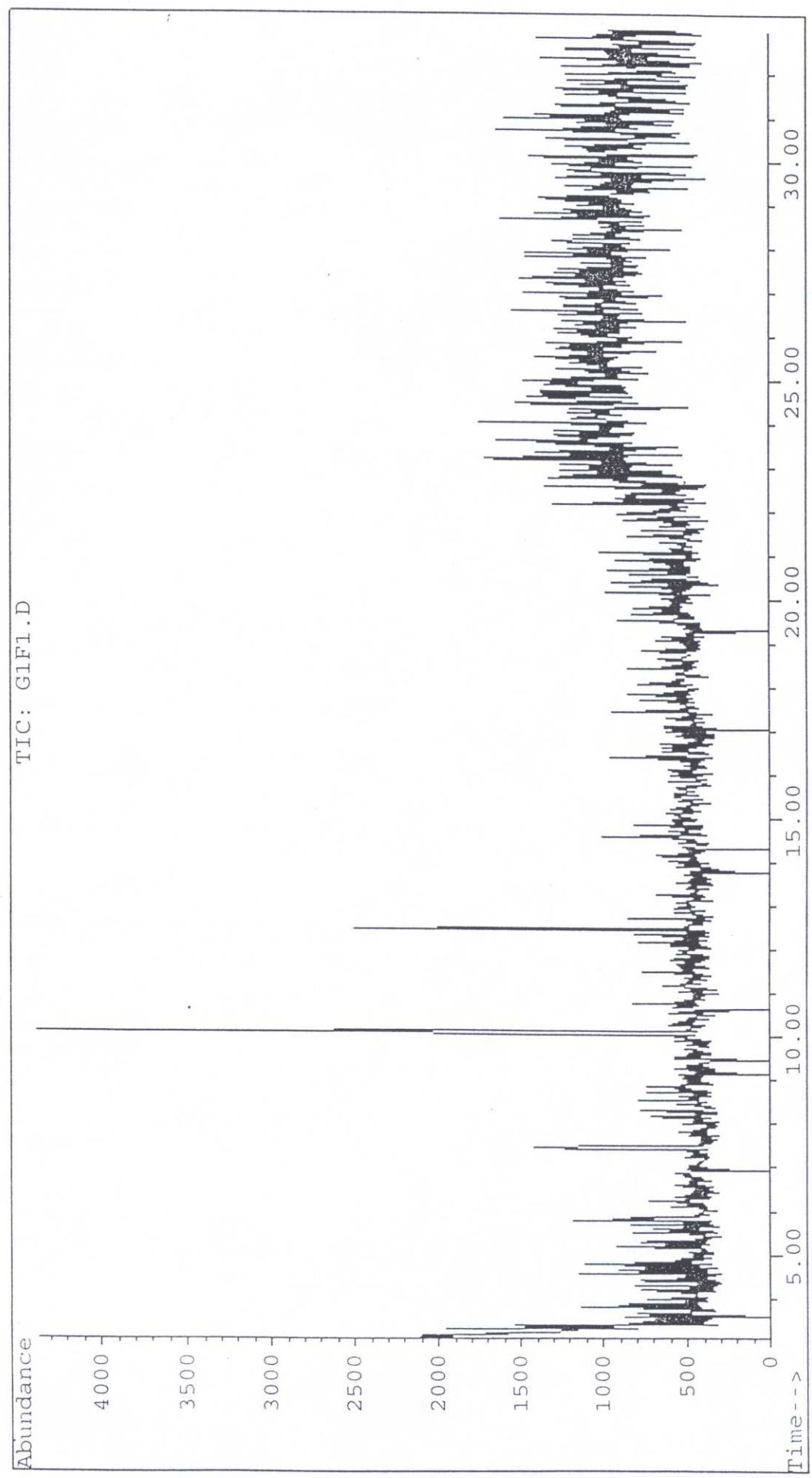
Vial Number: 21

Figure 5. Total ion chromatogram of a SFE extract of the soil USFWS-022698-02.



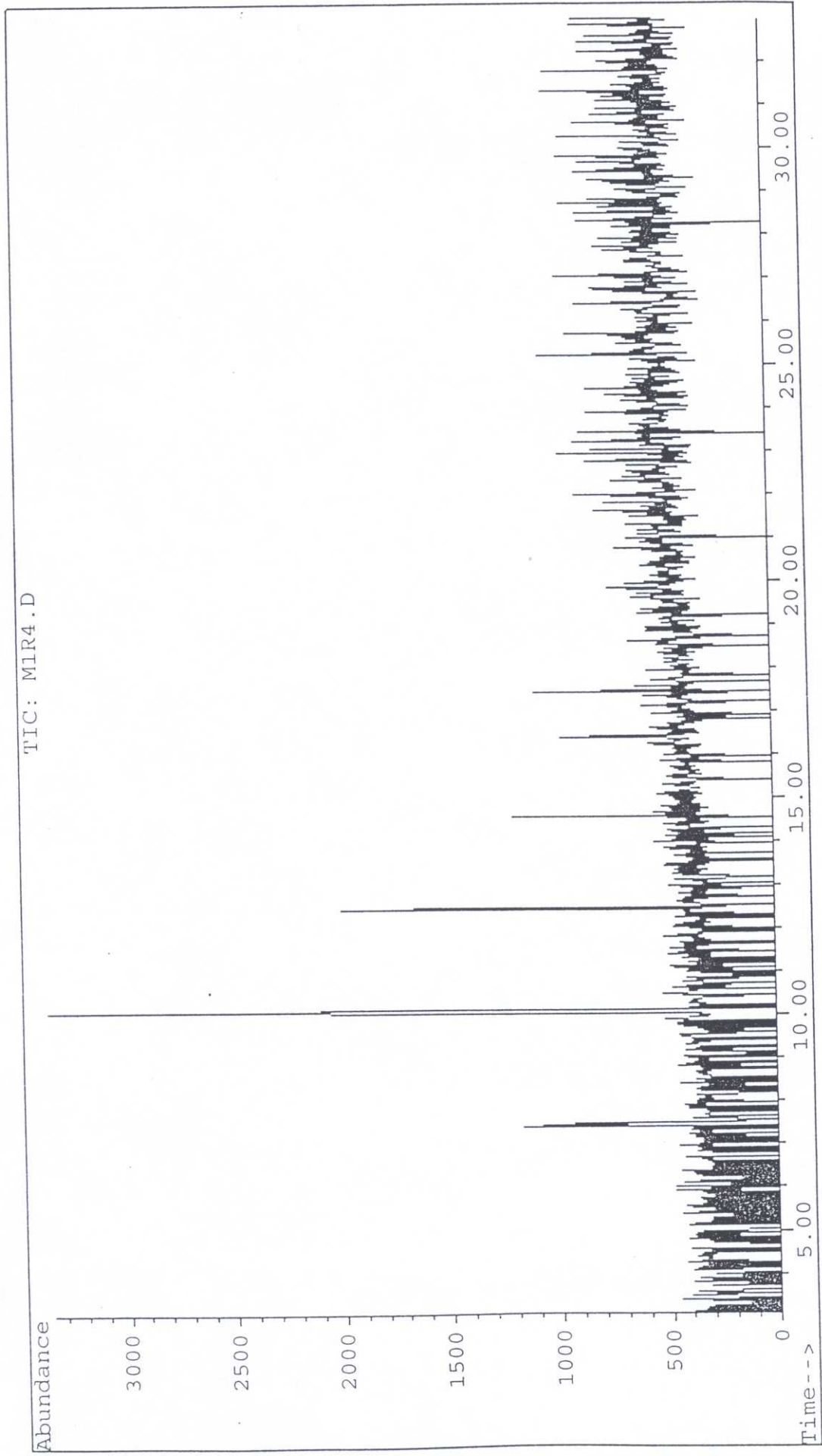
File : C:\HPCHEM\3\DATA\KS\G1F1.D
Operator : mdd
Acquired : 13 Jun 98 6:51 am using AcqMethod KS
Instrument : 5989x - I
Sample Name: SFE Kiahua Soil G1F1
Misc Info :
Vial Number: 17

Figure 6. Total ion chromatogram of a SFE extract of the soil USFWS-022698-03.



File : C:\HPCHEM\3\DATA\KS\M1R4.D
Operator : mdd
Acquired : 13 Jun 98 8:45 am using AcqMethod KS
Instrument : 5989x - I
Sample Name: SFE Kiahua Soil M1R4
Misc Info :
Vial Number: 20

Figure 7. Total ion chromatogram of a SFE extract of the soil USFWS-022698-04.



File : C:\HPCHEM\3\DATA\KS\KS-G1B.D
Operator : mdd
Acquired : 27 May 98 3:58 pm using AcqMethod KS
Instrument : 5989x - I
Sample Name: Kevin's Soil Sample
Misc Info :
Vial Number: 1

Figure 8. Total ion chromatogram of a solvent extract of the soil USFWS-022698-03.

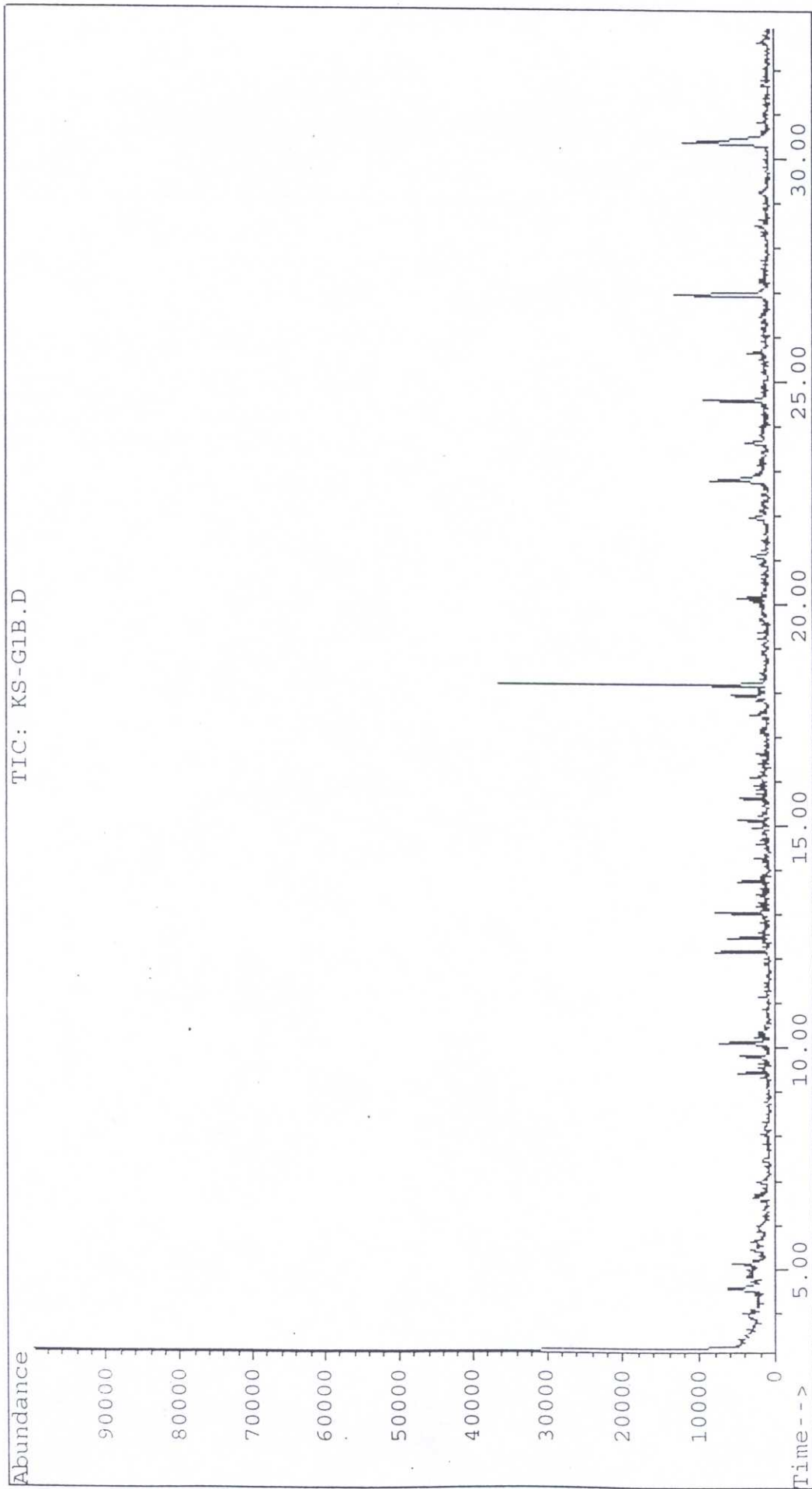


Figure 9. Gas chromatogram of a standard mixture.

Standard mixture @ 20 times estimated detection limits

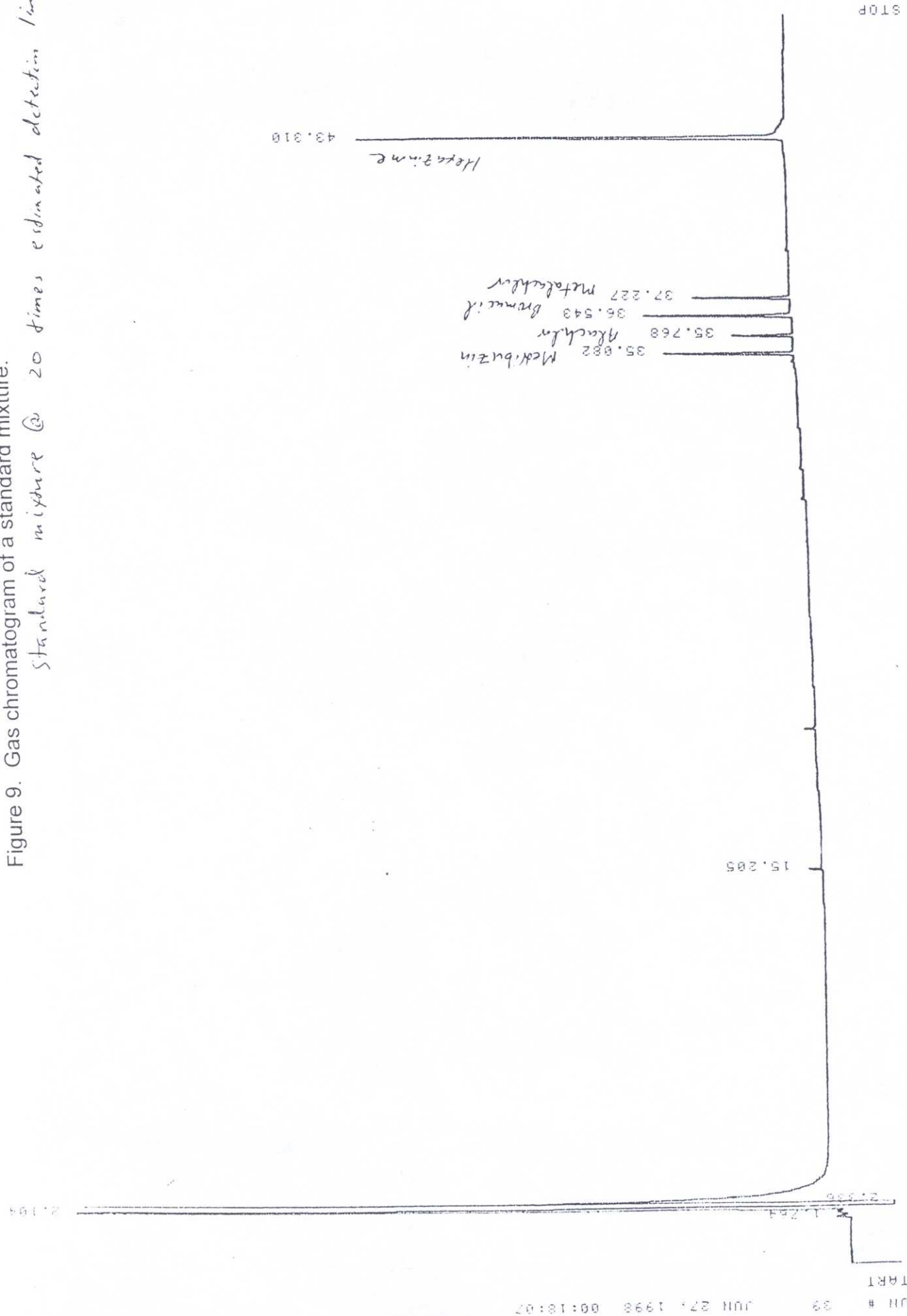
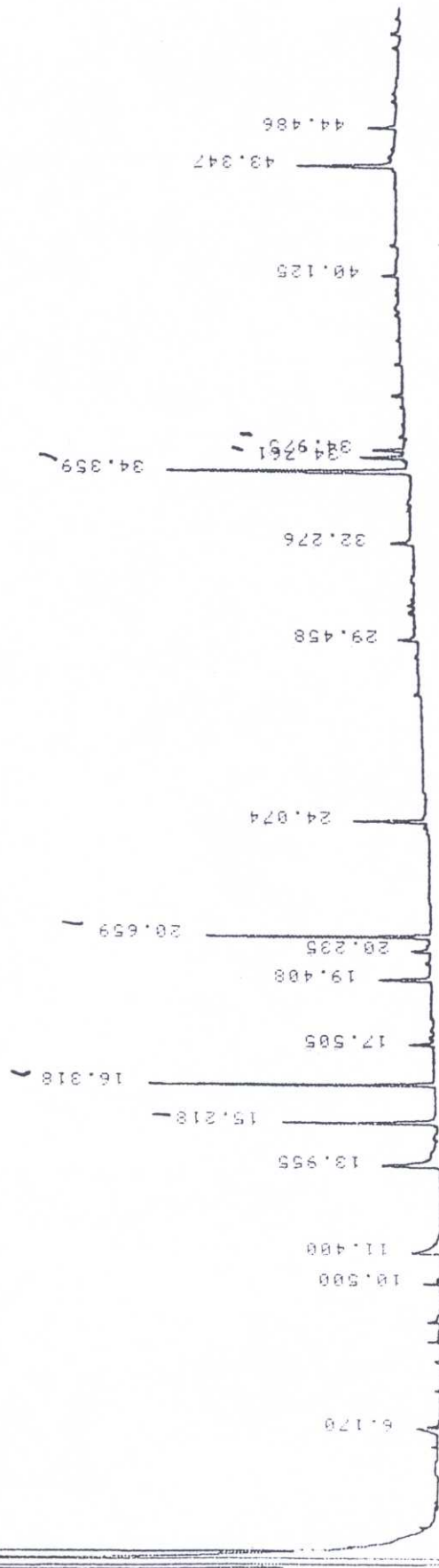


Figure 10. Gas chromatogram of a solvent extract of the soil USFWS-061898-01.

*USFWS-061898-01
(Matka, Reference)*



STOP

Figure 12. Gas chromatogram of an extract of a laboratory reagent blank.

USFWS-066898-03
Laboratory Reagent blank

MS
GC
SIR

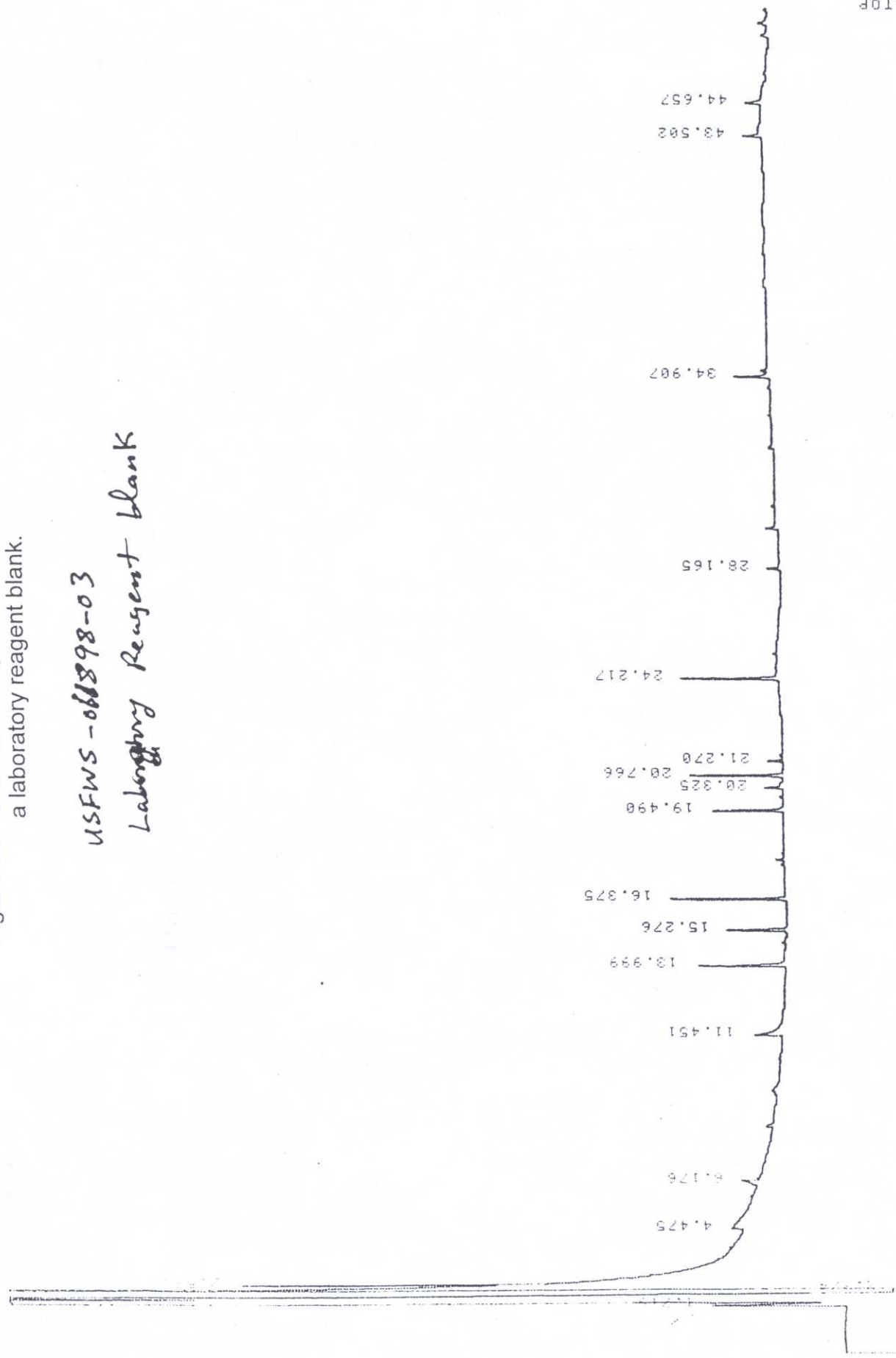
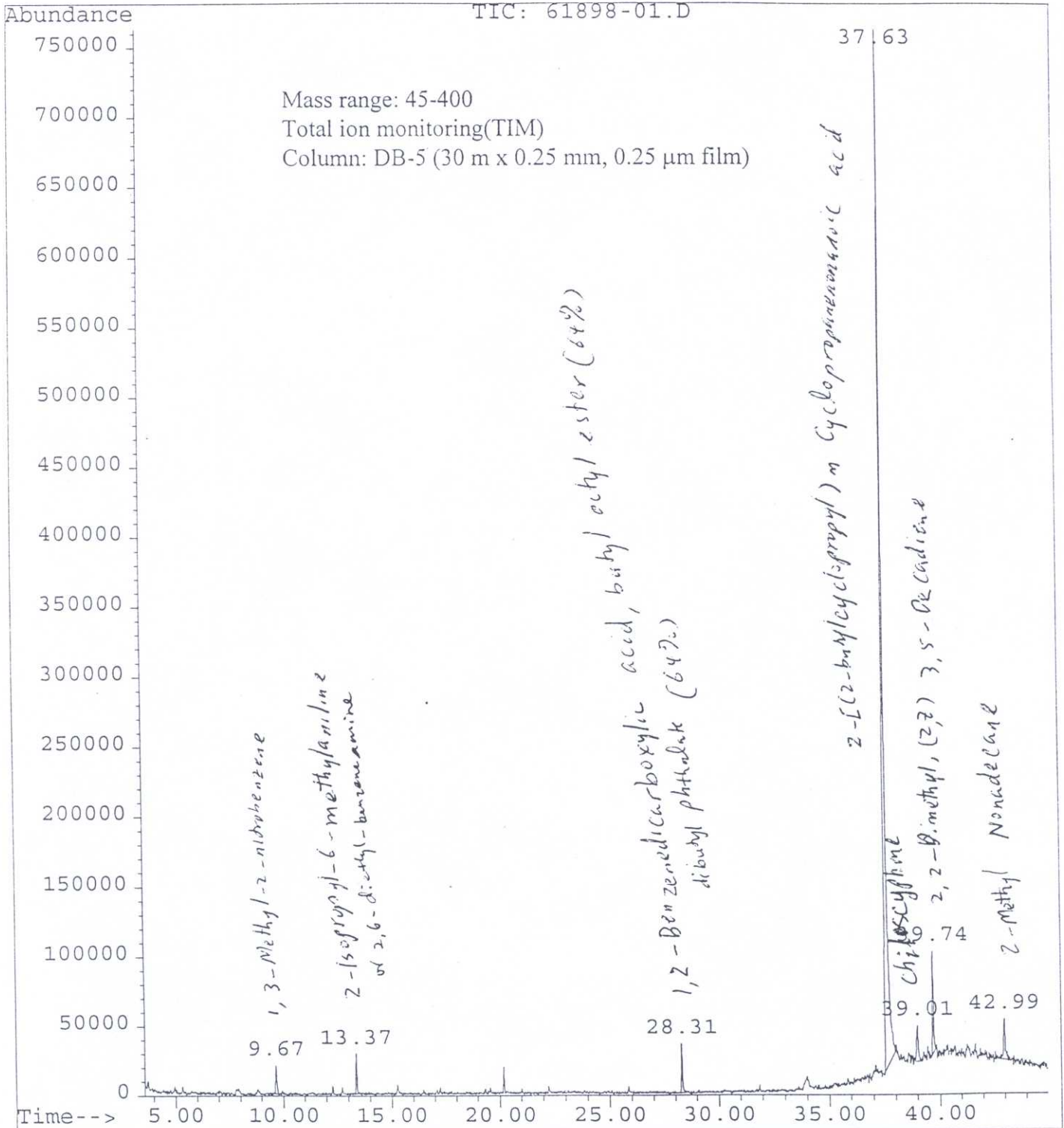


Figure 13. Total ion chromatogram of a solvent extract of the soil USFWS-061898-01.

File : A:\61898-01.D
 Operator : mdd
 Acquired : 26 Jun 98 3:34 pm using AcqMethod KS
 Instrument : 5989x - I
 Sample Name: makai-ref
 Misc Info :
 Vial Number: 1



File : A:\61898-02.D
Operator : mdd
Acquired : 26 Jun 98 4:25 pm using AcqMethod KS
Instrument : 5989x - I
Sample Name: mauka-gf
Misc Info :
Vial Number: 2

Figure 14. Total ion chromatogram of a solvent extract of the soil USFWS-061898-02.

Mauka, Golf Course

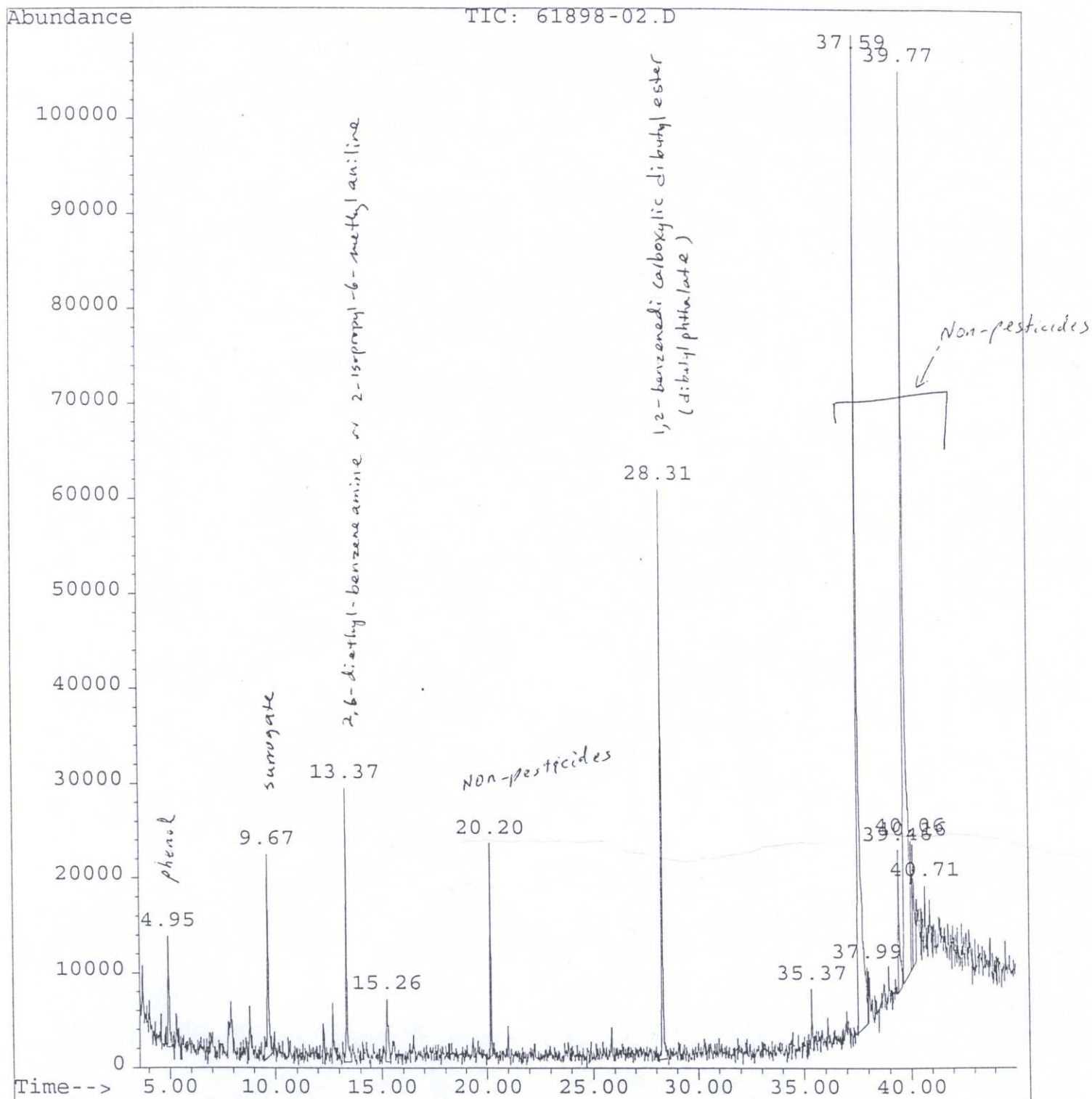
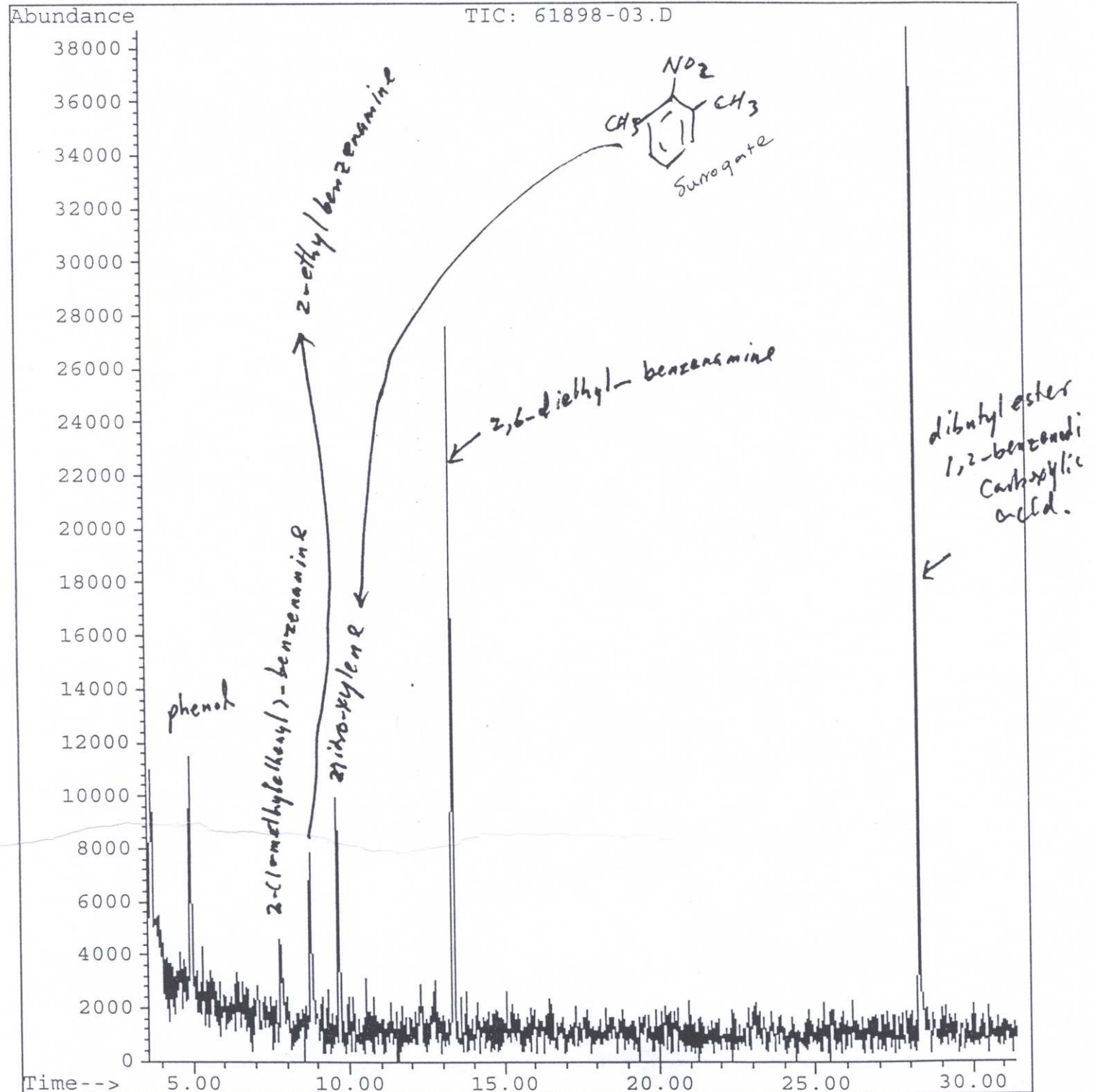


Figure 15. Total ion chromatogram of an extract of a laboratory reagent blank.

File : C:\HPCHEM\3\DATA\61898-03.D
Operator : mdd
Acquired : 26 Jun 98 5:15 pm using AcqMethod KS
Instrument : 5989x - I
Sample Name: lrb
Misc Info :
Vial Number: 3



APPENDIX 2

*Hawaii Institute of Marine Biology Final Report on Cholinesterase Inhibition
in Non-Native Arthropod Samples Taken From Kauai Caves*

Organophosphate pesticides inhibit cholinesterase enzymes and may, therefore, be detected by measuring acetylcholinesterase activity of insects living in an environment treated with these pesticides (Mineau, 1991). We have used this approach to examine for the presence of organophosphate in the environment of two caves on Kauai. We measured the cholinesterase activity in the ventral nerve cord of the American cockroach, *Periplaneta americana*, from these sites. A lower level of cholinesterase activity in the ventral nerve cord of cockroaches from Kiahuna cave compared with those from Makai would suggest that organophosphate pesticides used on the golf course surrounding this site are entering the cave through the ground water.

The Rappaport and the Ellman assays are two *in vitro* methods for measuring cholinesterase activity with the later more widely used (Ellman *et al.*, 1961; Heppner *et al.*, 1987; Detra and Collins, 1986; Zhu and Clark, 1994). Both assays use a colorimetric measure of an end product of the cholinesterase reaction *in vitro*. The Rappaport method measures the production of acetic acid from the substrate acetylcholine, and the Ellman method measures the production of thiocholine from the substrate acetylthiocholine. The colorimetric reaction is detected by a spectrophotometer in the visible light range of 405-420 nm. The product of each reaction causes a change in the intensity of the color indicator which is proportional to the concentration of the product.

Each assay method was tested and validated. Direct comparison between the two methods is difficult because the units of enzyme activity are different. For the Rappaport method, the units (Rappaport units/ml) are a direct measure of acetic acid production in μ moles over a 30 minute incubation period. The units of activity of the Ellman method are a direct measure of the thiocholine production in μ moles/ml/min. Since cholinesterase is a membrane-bound enzyme, the ability to measure cholinesterase activity is dependent on extracting the

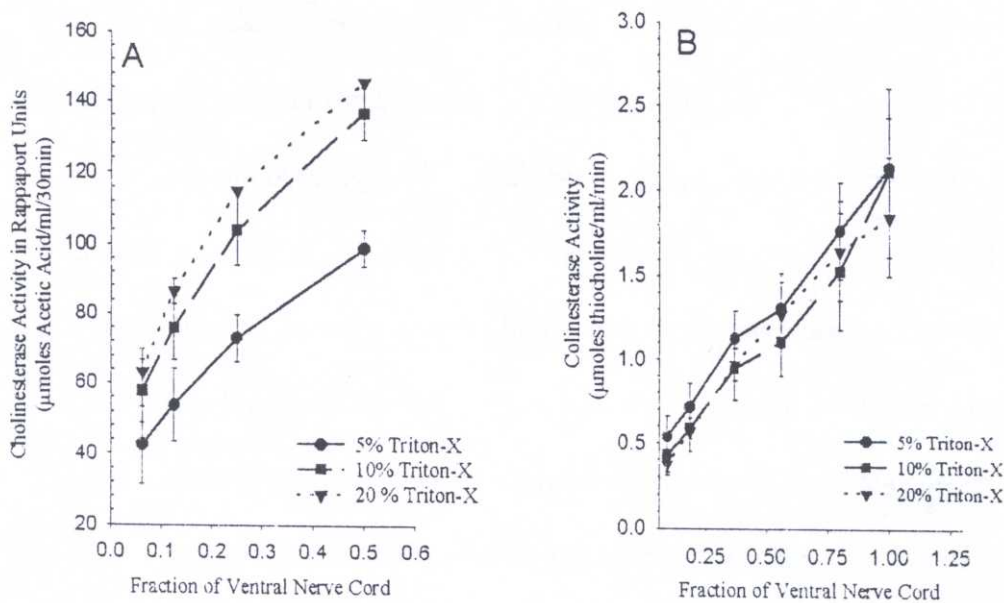


Figure 1. This figure illustrates the linear increase in cholinesterase activity with increasing amounts of ventral nerve cord for the Rappaport method (Panel A) and the Ellman method (Panel B). Ventral nerve cord tissue was homogenized as described in the text with 5, 10 or 20% Triton-X. Portions of the homogenate ranging from 0.06 to 1.25 fractions of ventral nerve cords were assayed for cholinesterase activity.

enzyme from the membrane using Triton-X, a chemical detergent. Validation was accomplished by demonstrating a linear increase in cholinesterase activity with increasing amounts of cockroach ventral nerve cord tissue. This was done at 5, 10, and 20% Triton-X solution to establish the best concentration for each assay (Figure 1).

Both methods produced a linear increase in cholinesterase activity with increasing amounts of ventral nerve cord. The Rappaport method was clearly affected by the concentration of Triton-X; cholinesterase activity in homogenates containing 5% Triton-X was lower than that obtained in homogenates with 10% or 20% Triton-X. The linear detection range of cholinesterase activity for the Rappaport method was 0.06 to 0.5 ventral nerve cord units of the cockroach/ml, and that of the Ellman method was 0.03 to 1 ventral nerve cord units of the cockroach/ml. We selected the Ellman method to assay cholinesterase activity in the experimental samples because this method displayed a linear sensitivity over twice the range of

the Rappaport method, was relatively insensitive to Triton-X concentration, and appears to be the preferred method in the literature.

Cholinesterase activity was determined in the ventral nerve cord of cockroaches collected at two test sites, Makai and Kiahuna, on Kauai. Samples were collected and quickly frozen on dry ice. To evaluate potential effects of freezing on cholinesterase activity, one group of cockroaches from Coconut Island (Oahu, Hawai'i) was caught and frozen on the same day that samples on Kauai were collected, and a second group of cockroaches from Coconut Island was caught the day before the assay and held live until just before removal of the ventral nerve cord. All frozen samples were held at -80°C for one week before being assayed, and all samples were assayed on the same day. For the assay, the

ventral nerve cords of fresh and frozen cockroaches were rapidly dissected and homogenized in 1 ml of phosphate-buffered saline containing 10% Triton-X. The samples were then centrifuged and the supernatant collected and assayed using the Ellman method. Since the cockroaches vary in size, cholinesterase activity was normalized to the amount of protein in the ventral nerve cord samples by dividing cholinesterase activity by protein concentration.

The results of the study are shown in figure 2. Although the frozen samples from

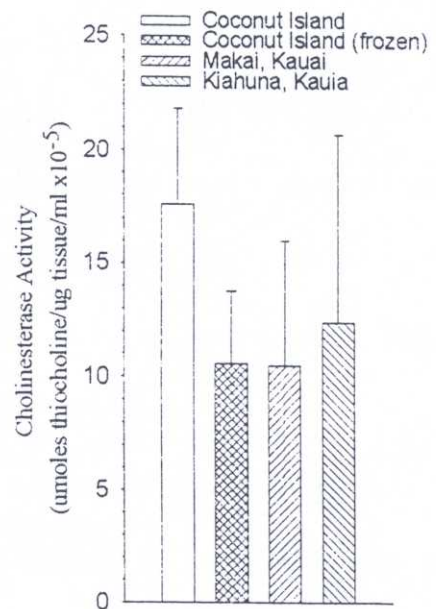


Figure 2. Cholinesterase activity (mean \pm S.E.) in ventral nerve cord tissue of cockroaches collected on Coconut Island fresh and frozen and of cockroaches collected from the two sites on Kauai. Significant differences were not observed. The number of tissues in each group was 4 except for those from Kiahuna which was 3.

Coconut Island showed a lower mean value of cholinesterase activity than the fresh samples, the levels were not statistically different ($P > 0.05$). The cholinesterase activity in the samples from both sites on Kauai were not significantly different ($P > 0.05$) from each other, nor were they significantly different ($P > 0.05$) from the cholinesterase activity of either fresh or frozen Coconut Island samples.

DISCUSSION

There are no significant differences in the cholinesterase activity among the three sites (Coconut Island, Makai and Kiahuna) nor among fresh and frozen samples. This result may indicate that organophosphate pesticides are not present at the Kiahuna site. Alternatively, these pesticides may be present at all three sites. The latter seems unlikely since pesticides are rarely, if at all, used on Coconut Island. The lower mean values of cholinesterase activity measured in the frozen samples, however, suggest that the effects of freezing might become significant if freezing were prolonged or if the sample size were increased. It is also possible, but unlikely, that an increase in sample size may also result in statistical differences between Makai and Kiahuna.

Little information is available on the effect of freezing on cholinesterase activity. The similarity of cholinesterase activity in the ventral nerve cord of fresh and frozen cockroaches from Coconut Island suggests that freezing the samples had little effect on cholinesterase activity. This is similar to the results found in a number of animal species. Cholinesterase activity in human serum was unaffected by storage temperatures ranging from a -20°C to 20°C or by repeated freezing and thawing (Huizenga *et al.*, 1985; Balland *et al.*, 1992). Similar results were found in the serum of organophosphate insecticide-treated horses (Plumlee *et al.*, 1994).

Storing the serum of the Japanese quail at temperatures from -25°C to 4°C had no effect on cholinesterase activity in males, while in females cholinesterase activity increased gradually over storage time (Hill, 1989).

Stress may also have an effect on cholinesterase activity. In our study, the principal stress on the cockroaches was handling. We attempted to minimize this by rapidly freezing the cockroaches after they were caught, and those that were not frozen were housed in a cool dark container with water and food. Other types of stress inducers have been shown to have various effects on cholinesterase activity. Thermal stress increased cholinesterase activity in the pons, cerebellum, amygdala, hippocampus, hypothalamus, mid-brain, and medulla oblongata of the pig. By contrast, acute water deprivation significantly depressed activity in these areas (Adejumo and Egbunike, 1988). Stress from immobilization stimulated the cholinesterase from the brain and intestine of laboratory rats (Romero-Vecchione *et al.*, 1987; Saunders *et al.*, 1997). It is difficult to determine from our data or from information found in the literature whether stress can affect the cholinesterase activity in the ventral nerve cord of the cockroach.

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