AN ABSTRACT OF THE THESIS OF

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TITLE: Development of A Screening Test for Atrazine, Alachlor and Chlordane in Turtles by Gas Chromatography.

ABSTRACT APPROVED: David E. Schroeder

A gas chromatographic method for the simultaneous determination of alachlor, atrazine and chlordane residues in deformed turtles is described. The column used was SE-30, along with an flame ionization detector. The procedure was carried out by extraction with chloroform, followed by clean-up with Florisil. Recoveries varied for turtle tissues from 0 to 86%. Florisil cleanup interfered with analysis of atrazine and alachlor. The method was applied to the analysis of fat, liver and unidentified gland tissue of seven deformed turtles. Alachlor and atrazine were not detected among fat, liver and unidentified gland tissue. The different isomers of chlordane were found in the liver. The chlordane concentration varied from 0.14 to 52 mg/g of tissue. The considerable uncertainties exist in the concentration data due to interference from fat in the liver.

DEVELOPMENT OF A SCREENING TEST FOR ATRAZINE, ALACHLOR AND CHLORDANE IN TURTLES BY GAS CHROMATOGRAPHY

A Thesis

Presented to

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CONTENTS

THE LIST OF FIGURES

THE LIST OF TABLES

CHAPTER 1. INTRODUCTION

1. Literature Review

Wildlife biologists are expressing increasing concern for the future of aquatic wildlife in Kansas, because some species are declining in numbers while others, such as the ancient group, turtles, which are known to be relatively pollution-tolerant, exhibit increasing frequencies of anatomical deformities [1,2].

Since the first deformed turtle was found in the Arkansas River nine years ago, Marty Capron, a biologist from Oxford, has found deformities in about five of every ten turtles which he has examined in certain rivers of southeast Kansas [1]. In 1990 and 1991, two surveys of aquatic turtles were completed by David Edds [3]. In his research, numerous deformities such as missing legs, deformed carapace, plastron and jaws were found, as shown in Figure 1. Usually, as given in Figure 2, there are regular grains on the normal plastron of turtles. 5.1 % of turtles found in the relatively nonindustrial region drained by the Marais des Cygnes, Neosho, Verdigris and Spring rivers were deformed, while 11.2 % were deformed in the more industrialized lower Arkansas River. The specific cause of these deformities is unknown. Each of these drainage areas include farm fields and urban areas. For example, the lower Arkansas river basin is influenced by industrial run-off from Wichita and refineries near El Dorado and Augusta. Marty

Figure 1. The Deformed Plastron of Turtle

(Photograph Courtesy of Dr. David Edds in Division of Biological Science, Emporia State University, February, 1993)

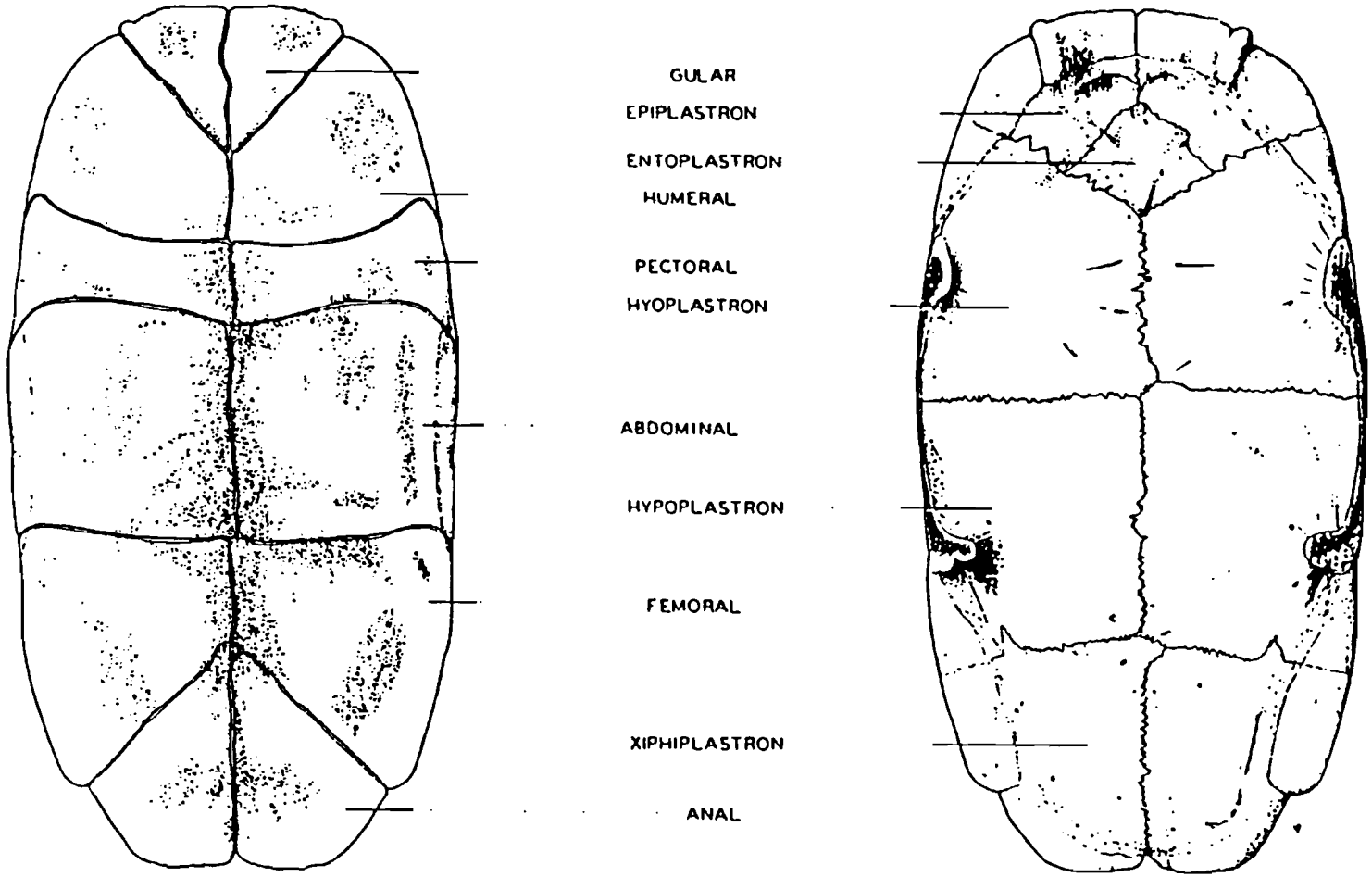
* The irregular grains are emphasized by the white correct pen

Figure 2. The Normal Plastron of Turtle

(From Ashley, L.M., "Laboratory Anatomy of The Turtle")

Capron carried out a study over nine years, which shows that there are more deformities of turtles in rivers which are near areas where more pesticides are used [1]. It is reasonable to speculate that industrial and agricultural pollution may cause these deformities, but there is no direct evidence that pesticides do produce them or which pesticides induce the deformities. Since the list of possible causes is a long one, development of a single screening procedure for selected pesticides in the tissues of aquatic animals will narrow down the list of possible substances and contribute to the successful management of non-game wildlife, which is the primary purpose of this project.

Chlordane [1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methano-1 H-indene] ($C_{10}H_6Cl_8$) with molecular weight of 409.80 [4], one of the organochlorine pesticides, is a viscous, amber-colored liquid. The boiling point and melting point are not available through some reference books, but viscosity is known to be reduced considerable by heating to 49-60°C. It is insoluble in water. Commercially it is a mixture containing 60 to 75% of the pure compound and 25 to 40% of related compounds. Chlordane, like other organochlorine pesticides, had been used in the control of pest populations and in combating the spread of infectious disease for many years. Unfortunately, it accumulates in the parts of environment, such as plants and aquatic animals, etc, which has resulted in restrictions on its use.


GULAR
EPIPLASTRON
ENTOPLASTRON
HUMERAL
PECTORAL
HYOPLASTRON
ABDOMINAL
HYPOPLASTRON
FEMORAL
XIPHIPLASTRON
ANAL

Alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl) acetamide] ($C_{14}H_{18}ClNO_2$) is a crystalline solid with a molecular weight of 269.77 [3]. Its melting point is 40-41°C and its solubility in water at 23°C is 140 mg/L. Alachlor is widely used as a herbicide for the control of weeds. Alachlor effectively kills grassy and broad leaf weeds. According to the EPA estimates, 90 to 95 million pounds are applied each year in the United States, primarily by corn and soybean farmers. But in 1984, alachlor was categorized by EPA as a potent carcinogen [5] because it was found to cause cancers in rats and mice. Additionally, the rats developed several different types of cancers, including a rare nasal tumor, and some cancers even occurred at relatively low doses. At the other hand, because alachlor is soluble in water, there were some reports [5] about alachlor detected in the surface and ground water of several states, including Kansas, Iowa, and Nebraska. There is insufficient information to determine if the level of alachlor in rivers is high enough to affect aquatic animals and human beings. However, tighter restrictions on alachlor use has been proposed by EPA.

The chemical name for atrazine is 6-chloro-N-ethyl-N′-(1-methylethyl)-1,3,5-triazine-2,4-diamine ($C_8H_{14}ClN_5$), with a molecular weight of 215.7. Commercially it is available as 50% and 80% wettable powder, in granular and in liquid form. Atrazine is an odorless, white crystalline solid. Its melting point is 175-177°C and it is slightly soluble in water (28

mg/L at 20°C) [4]. Atrazine has been employed for a long time as a widely used selective herbicide. It is used to control broad leaf and grassy weeds in corn, sorghum and other crops. Atrazine has been in use in Kansas since 1959. In Kansas approximately 6 million lbs of atrazine were applied to corn and sorghum on 3.5 million acres for weed control in 1978 [6]. According to the EPA, atrazine is linked to liver and heart damage and has been listed as a possible cancer-causing agent. For this reason, the EPA's drinking-water standard for atrazine has been reduced from 150 ppb to 3 ppb [7]. Some reports state that, in the several lakes of northeast Kansas, the Cottonwood River near Emporia and the Neosho River near Americus, atrazine levels are above the new standard of 3 ppb [9]. The use of atrazine or herbicides blended with atrazine will be limited by a third to a half in 1993 [8, 9], i.e. its recommended use is reduced from a maximum of 3 pounds per acre to 1.6 or 2 pounds per acre, depending on how much soil-holding crop residue a farmer has on his field.

For all the reasons mentioned above, all three pesticides (alachlor, atrazine and chlordane) are selected as suspected organic contaminants in turtles in this project. Although some authors have published analytical methods for organochlorine [10], alachlor and atrazine [11, 12, 13] residue analysis, no analytical procedure providing for the simultaneous residue determination of three pesticides has been previously reported either in water or the tissue of

aquatic animals.

As a first step in determining the cause of deformities in aquatic turtles, it was necessary to develop a screening method that could determinate the concentrations of alachlor, atrazine and chlordane in turtle tissues by a single procedure, which was also another purpose of this project. The methodology of this project was gas chromatography.

2. General Introduction to Gas Chromatography

Chromatography is a physical method for separating components in a mixture. The basis of the method lies in that different substances have different partition ratios within two mutually immiscible phases; one phase is stationary and the other mobile. Species in the sample undergo repeated interactions between the mobile phase and the stationary phase. This makes it possible to separate molecules that differ only slightly in their physical and chemical properties. If the mobile phase is a gas, the technique is called gas chromatography (GC)

Since its appearance in 1952, gas chromatography has become one of the most widely used modern analytical technique and is part of the equipment used by virtually all industrial, academic and government laboratories. It offers rapid qualitative and quantitative analysis of complex mixtures with precision and sensitivity, yet the equipment is also relatively inexpensive to operate.

GC is probably the best available method for the

separation of organic compounds. The separation of a mixture containing many volatile components may be achieved by introducing it as a single "plug" into a continuously flow of carrier gas, which passes through a column of material whose properties may be chosen to bring about this separation. The solutes are adsorbed at the head of the column by the stationary phase and then desorbed by fresh carrier gas. This partitioning process occurs repeatedly as the sample is moved toward the outlet by the carrier gas. Each solute will travel at its own rate through the column, which is determined by its partition coefficient. The larger the partition coefficients are, the later the solute comes out. In time, the individual components are separated and emerge from the column for evaluation. For the purpose of analysis, the separated components are detected and electronically displayed by a recorder in the form of peaks of approximately Gaussian shape. The time of emergence of each component, referred to as its retention time, is characteristic of that component. If the output of the detector - recorder system is linear with concentration, and the flow rate of the carrier gas is constant, the height, width and area of these peaks on the chromatogram can be measured to yield quantitative analytical data.

The heart of chromatography is the column (packed or capillary). In this project, a capillary column was employed because of its high separation efficiency in contrast to

standard packed columns. For this reason, better separations are obtainable and can be achieved at lower temperatures and in a short of times.

Although there are several operationally important factors, such as flow rate and length of the column, that affect the separation of components, column temperature in GC analysis is the single most important factor for obtaining separation. In attempting to establish the optimum conditions for any particular analysis, isothermal operation and linear or nonlinear temperature programming can be chosen. Since problems such as baseline shift, retention variations and poor precision are associated with temperature programming, isothermal procedures are preferred, especially for quantitative work. Unfortunately, the organic mixtures usually have a wide boiling range, which makes temperature programming still widely used a technique. In this thesis, temperature programming was applied.

CHAPTER 2. EXPERIMENTAL

2.1 Selection of Conditions for the Analysis of Atrazine, Alachlor and Chlordane by Gas Chromatography

2.1.1 Apparatus

A. Gas Chromatograph:

A Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector (FID) was employed. All outputs were recorded by an HP 3900A integrator. The following three columns were evaluated:

Column I: 30 m x 0.25 mm capillary column packed with 0.25 μm SE-30 (Alltech Associates, Inc.).

Column II: 10 m x 0.53 mm capillary column packed with 2.0 μm OV-17 (Quadrex Co.).

Column III: 30 m x 0.25 mm capillary column packed with 0.25 μm Carbowax (Alltech Associates, Inc.).

B. Glass Apparatus:

A 10 μL GC syringe (Hamilton Co.) was used to inject samples. Various micropipets, (e.g, 100 μL, 250 μL, 500 μL, and 1000 μL) manufactured by Eppendorf were used for solution preparation. 25-mL and 10-mL volumetric flasks were used to prepare solutions.

2.1.2 Reagents:

A. Standard Samples: Atrazine, alachlor and chlordane (mixture of isomers) at 99% purity were purchased from CHEM

SERVICE Co.

B. Solvents: The following reagents, all ACS-grade, were used in this project: chloroform, hexane and methanol. Acetone was used to rinse glassware and syringes.

C. Internal Standard: Catechol (Practical grade) was used.

D. Stock Solutions: 5000 ppm atrazine, alachlor, catechol, and 10,000 ppm chlordane stock solutions were prepared in separate 25-mL volumetric flasks in chloroform. All stock solutions were stored in the refrigerator to prevent solvent evaporation.

2.1.3. Procedure

To prevent contamination, all glassware was washed with concentrated KOH solution, then with concentrated sulfuric acid, then rinsed with distilled water, and finally with acetone.

Methanol, hexane and chloroform were tried as a solvent for atrazine, alachlor and chlordane.

To prepare the calibration curve, two series of atrazine and alachlor standards of 20, 40, 60, 80 and 100 ppm were prepared by diluting a 5,000 ppm stock solution to the appropriate concentrations. Meanwhile, chlordane standards of 200, 300, 400, 500 and 600 ppm were prepared by diluting the 10,000 ppm stock solution to the desired concentration. 500 µL of 5000 ppm catechol solution was added to all standard solutions as an internal standard, making the final concentration 100 ppm. The calibration curves for each of the

three pesticides were prepared by plotting the ratio of areas produced by the analyte peaks with the catechol peak vs. the concentration.

All analyses were carried out on the GC. Duplicate 1 μL injections were made for each sample. The GC temperature program was set at 160°C for 1 min, then increased by 10 °C/min to 260°C, and held for 1 min. The injector temperature was set at 200°C and the detector temperature was set at 290°C. The volume flow rate of the carrier gas (helium) was 0.86 - 0.90 mL/min. Three gas-chromatographic columns, i.e., column I, II and III, were evaluated.

2.2. Analysis of Turtle Tissues

2.2.1. Apparatus

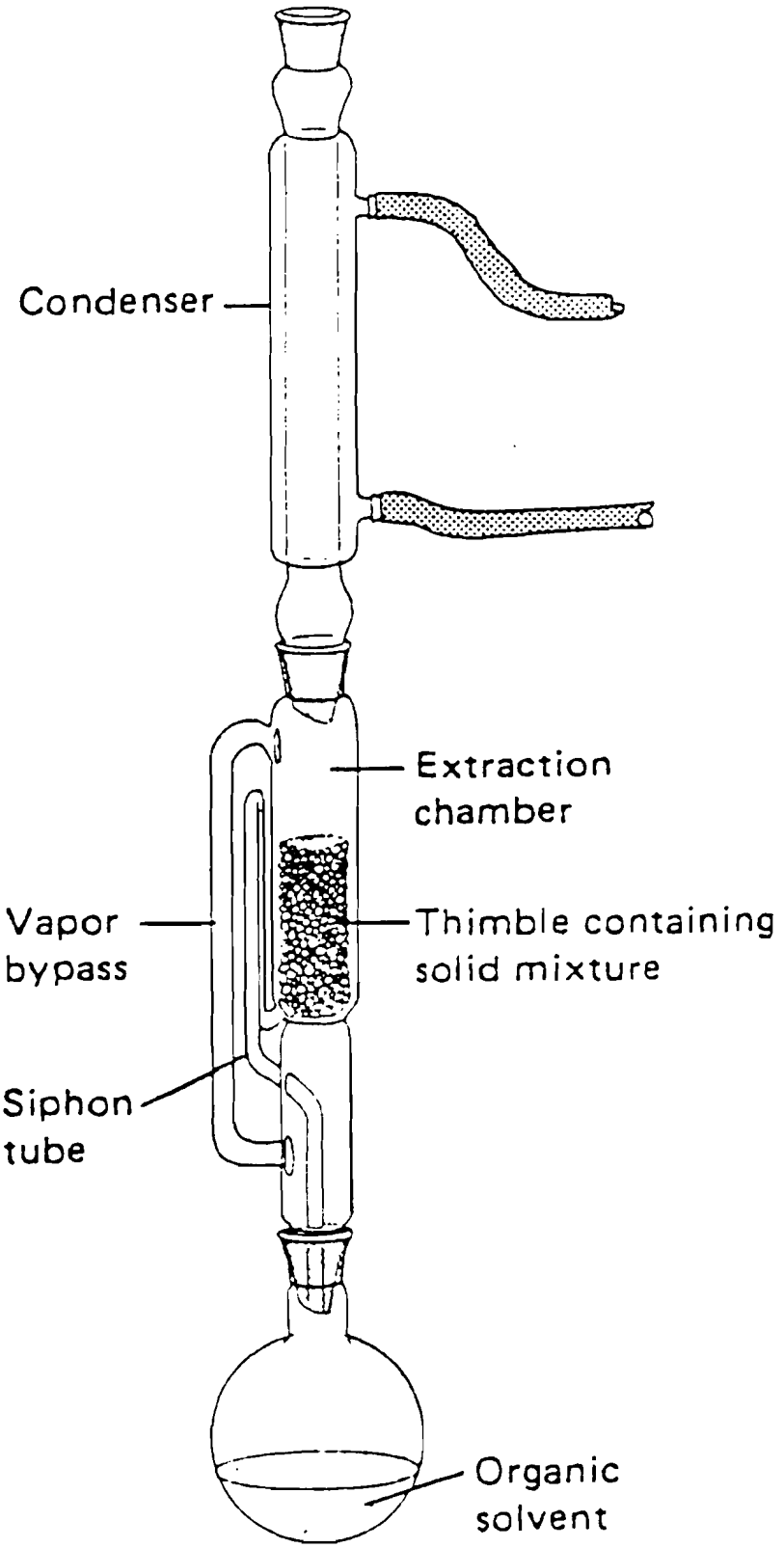
A. A triple beam balance was employed to weigh the frozen turtles.

B. Turtle's shells were opened with a hack saw and surgeon's knife.

C. A 250 mL 45/50 Soxhlet extractor was utilized to extract turtle tissue. Figure 3 shows a Soxhlet extraction apparatus. The turtle tissue was placed in the extraction thimble (made of heavy filter paper - type materials), and the extraction solvent (chloroform) was placed in the flask. When brought to reflux, the solvent was condensed onto the material in the thimble. When the solvent level in the thimble reached the same level as the top of the siphon arm, the entire liquid

Figure 3. A Soxhlet Extraction Apparatus

(From Solomon Marmor, " Laboratory Methods of Organic Chemistry", Pg 113)


Condenser
Extraction chamber
Vapor bypass
Thimble containing solid mixture
Siphon tube
Organic solvent

contents (consisting of a dilute solution of the extracted material) of the thimble was siphoned into the flask. The Soxhlet extractor was heated on a 500 mL 330-watt heating mantle filled with sand. The temperature of extraction was controlled by a powerstat.

D. Extracts were concentrated in 500 mL 19/22 Kuderna-Danish evaporators in a water bath maintained at 70 °C .

E. Each Florisil column was prepared by transferring Florisil into a 5.75 inch disposable pipet plugged with glass wool, filled about 2/3 full.

2.2.2. Materials and Reagents

A. Turtles: All turtles used in this project were provided by Dr. David Edds from Division of Biological Science at Emporia State University. The species of turtles were all red-eared sliders (*Trachemys scripta elegans*). For the detailed information about the weight, sex and the collection locations where turtles were obtained see Table 1.

B. Florisil: PR 60/100, 227G (Alltech Association Inc.)

C. 1000 ml 50% Methylene Chloride / 1.5% Acetonitrile / 48.5% Hexane (v/v/v) Cleanup Solvent [14]: solvent solutions were made by pipeting 15 mL acetonitrile (HPLC grade, Fisher) into 500 mL methylene chloride (HPLC grade, Fisher), then diluting with hexane (HPLC grade, Fisher) to the mark in a 1000 mL volumetric flask.

2.2.3 Procedure

After a frozen turtle had been weighed, both sides of

TABLE 1. INFORMATION ON TURTLES USED IN THIS PROJECT

Collection Number	Legal Location	Locality	Date	Sex	Deformity	Frozen Weight (g)
DET-116	T34S/R17W Sec. 16	Salt Fork Arkansas River 9 mile east of Buttermilk Comanche County	7/16/91	not determined	Extra scutes on carapace	528
DET-116-5	T34S/R17W Sec. 16	Salt Fork Arkansas River, 1.5 mile south, 1 mile of Butter-milk, Comanche County	7/2/91	Male	Extra scutes on carapace	245.5
DET-116-7	"	"	7/2/91	Female	Extra scutes on carapace	283.5
DET-116-9	"	"	7/2/91	Female	Irregular plastron scutes	531.7
DET 116	"	"	7/2/91	Female	Extra scutes on carapace and irregular scutes on plastron	over 610 *
DET-165-1	T25S/R3W Sec.8&9	Arkansas River, 2 mile north of Mount Hope at bridge on highway 230, Sedgwick County	8/13/91	not determined	Extra scutes on carapace and plastron	338
DET-173-1	T28S/R1E Sec. 15	Arkansas River, bridge on 47th street in Wichita east of I-35 (North side of bridge), Sedgwick County	8/15/91	"	Irregular plastron scutes	304
DET-173-2	"	"	8/15/91	"	Deformed upper jaw	430.5

* The weight of this turtle is beyond the maximum scale of the balance

the turtle shell were cut with the hack saw. Then the turtle was placed in a ziploc plastic bag to defrost in tap water. The plastron was then totally removed by the use of a surgeon's knife. The liver, fat and an unidentified gland (a kind of green tissue) were collected from each turtle. The selected tissues were then placed in a clean beaker, frozen and preserved.

The turtle tissues were extracted in a Soxhlet extractor with 150 mL chloroform for 12 hours at a siphoning rate of 4 - 5 times per hour according to PAM [16], controlled by powerstat with voltage set at 70 V. Then, extracts were concentrated in the Kuderna-Danish evaporative concentrator to a volume of 3 - 4 mL. Subsequently, the 3 - 4 mL concentrated solution was transferred into the Florisil column to remove the fat. 10 - 15 mL cleanup solvent was applied to wash the Florisil column to recover the analytes. The eluant was placed in the hood about 10 hours to evaporate the solvent, 200 µL of 5,000 ppm internal standard was added, and then diluted to 10 mL prior to injection in the gas chromatograph. Two 1 µL volumes of the unknown were injected, and the concentration was determined from the calibration curves.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Selection of Conditions for Analysis of Alachlor, Atrazine and Chlordane by Gas Chromatography

Table II demonstrates that the best solvent for alachlor, atrazine and chlordane mixtures is chloroform. Atrazine was dissolved in methanol only after heating in a warm water bath, but some white powder was formed on the mouth of the

TABLE II
THE SOLUBILITIES OF ALACHLOR, ATRAZINE AND CHLORDANE IN DIFFERENT SOLVENTS

Compounds	Solvents		
	Methanol	Hexane	Chloroform
Alachlor	++	++	++
Atrazine	+	-	++
Chlordane	-	++	++

1. ++ means that sample is very soluble.
2. + means that sample dissolves only at higher temperature.
3. - means that sample is not very soluble.

volumetric flask after the stock solution had been kept for few weeks. In methanol, chlordane was observed to stick on the wall of the beaker after stirring was stopped. After adding 3 drops 0.1 M HCl and heating, the atrazine solid disappeared, but liquid drops of atrazine were still observed in hexane.

Because of the shortage of a chlordane standard at the beginning of the project, only alachlor and atrazine were used

to select the condition for analysis. Although the boiling points for the three compounds were not listed in the usual reference books, it was known that there is a large range of melting point among alachlor, atrazine and chlordane, from 40°C to 177°C (3). Therefore, a large range of boiling points were assumed to exist. For this reason, temperature programming was considered. Based on the information provided by Alltech, three columns were selected for evaluation: SE-30 (Column I), OV-17 (Column II) and Carbowax (Column III). According to the retention times listed in Table III, no analyte peaks come out of the Carbowax column, which may be caused by the strong London dispersion forces existing between polar stationary phase and the polar solutes (alachlor, atrazine and chlordane), so that those compounds were retarded by the stationary phase. The peak of alachlor obtained with the OV-17 column may overlap with the solvent peak.

TABLE III

RETENTION TIMES FOR ALACHLOR AND ATRAZINE IN THREE COLUMNS

Compounds	Retention Time (min)		
	Column I	Column II	Column III
Alachlor	8.68	1.69	xxx
Atrazine	7.01	2.38	xxx

The retention times shown in Figures 4, 5 and 6 show that the separation of alachlor, atrazine and chlordane can be achieved using SE-30 column under the operating condition below:

TABLE IV
THE OPERATING CONDITIONS OF GAS CHROMATOGRAPHY

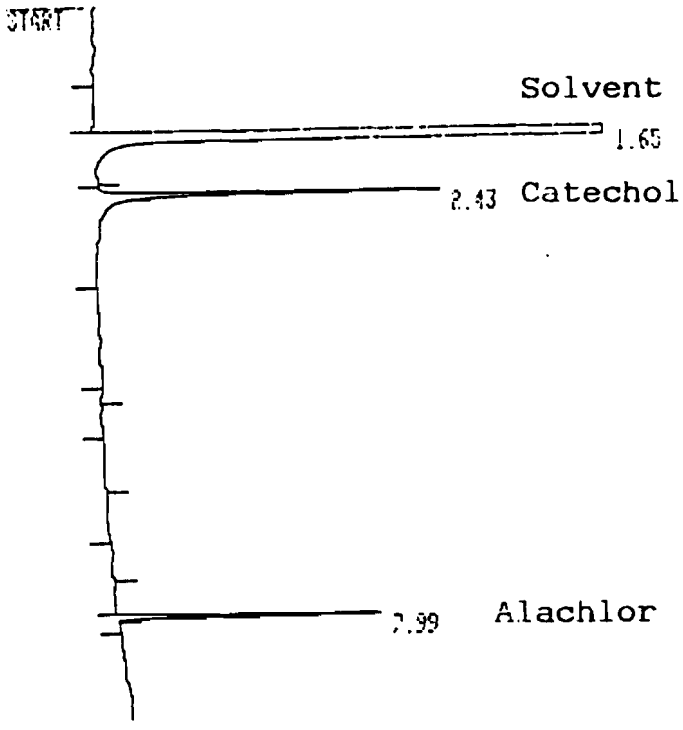
Initial Temperature (°C)	160
Initial Time (min)	1
Temperature Rate (°C/min)	10
Final Temperature (°C)	260
Final Time (min)	1
Injector Temperature (°C)	200
Detector	FID
Detector Temperature (°C)	290
Flowrate of Helium (mL/min)	0.86 - 0.90

Figures 7, 8 and 9 show the calibration curves for alachlor, atrazine and chlordane isomer standards obtained by plotting the ratio of areas against the concentration. The relative standard deviations of the slopes of the three calibration curves are within 4.1 percent. The data for the calibration curves are given in Table V - VII in which the corrected area of the internal standard was calculated by the following equation:

$$\textit{The Corrected Area} = \frac{\textit{Weight of Blank}}{\textit{Weight of Sample}} \times \textit{Area}$$

then,

Figure 4. Gas Chromatogram of Alachlor Standard



STOP

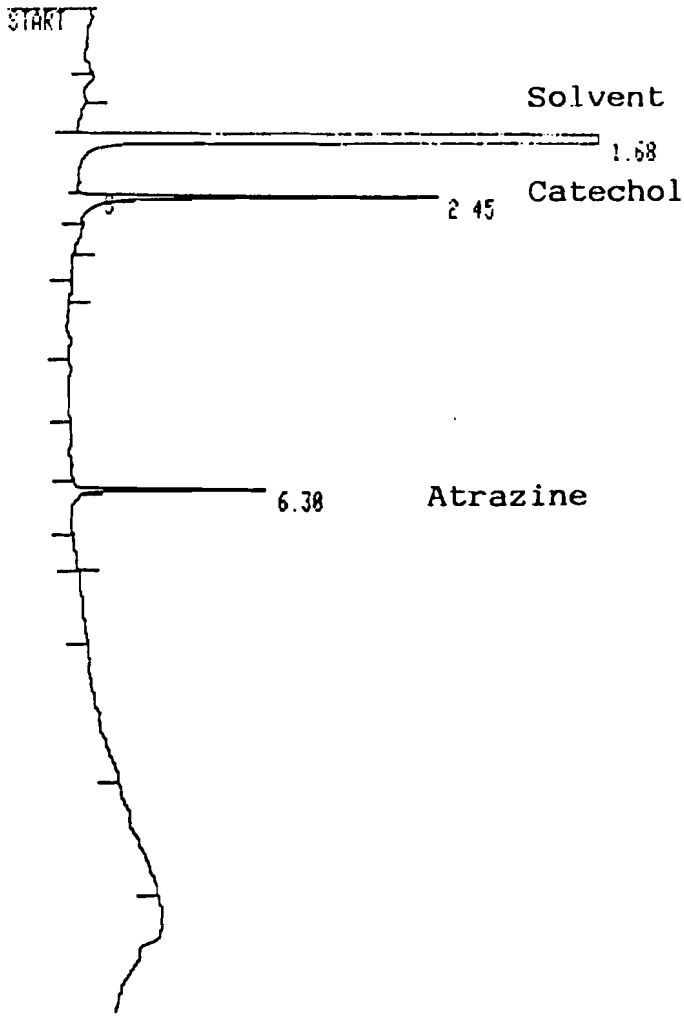
RUN # 16

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.65	2.9666E+07	↑SBB	0.060	99.890
2.43	17668	PB	0.032	0.060
7.99	14919	PB	0.033	0.050

TOTAL AREA= 2.9699E+07
MUL FACTOR= 1.0000E+00

Figure 5. Gas Chromatogram of Atrazine Standard



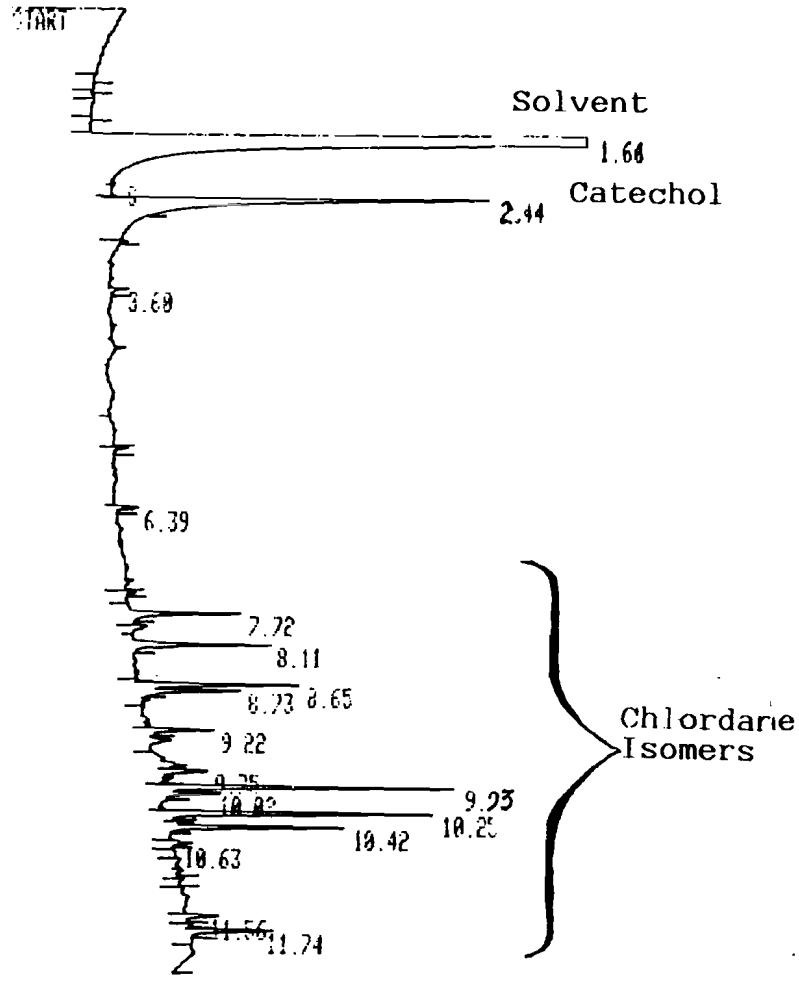
RUN # 46 OCT/02/92 10:53:57

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.68	2.8969E+07	↑SPB	0.059	99.901
2.45	18414	BB	0.031	0.064
6.30	10438	PB	0.032	0.036

TOTAL AREA= 2.8998E+07
MUL FACTOR= 1.0000E+00

Figure 6. Gas Chromatogram of Chlordane Isomers Standard



STOP

RUN # 56 FEB/08/9- 11:42:43

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.64	1483200	SBH	0.014	6.301
1.66	2.1981E+07	†SHB	0.043	93.380
2.44	22905	PB	0.070	0.097
3.60	489	BB	0.025	0.002
6.39	761	PB	0.035	0.003
7.72	3854	BB	0.039	0.016
8.11	4958	PB	0.041	0.021
8.65	5177	BV	0.037	0.022
8.73	3291	VB	0.038	0.014
9.22	2139	BB	0.037	0.009
9.25	1568	BP	0.045	0.007
9.93	9305	BV	0.036	0.040
10.02	1817	VB	0.038	0.008
10.25	8263	BB	0.035	0.035
10.42	5424	VB	0.036	0.023
10.63	787	BB	0.043	0.003
11.56	1231	BB	0.042	0.005
11.74	3092	BB	0.043	0.013

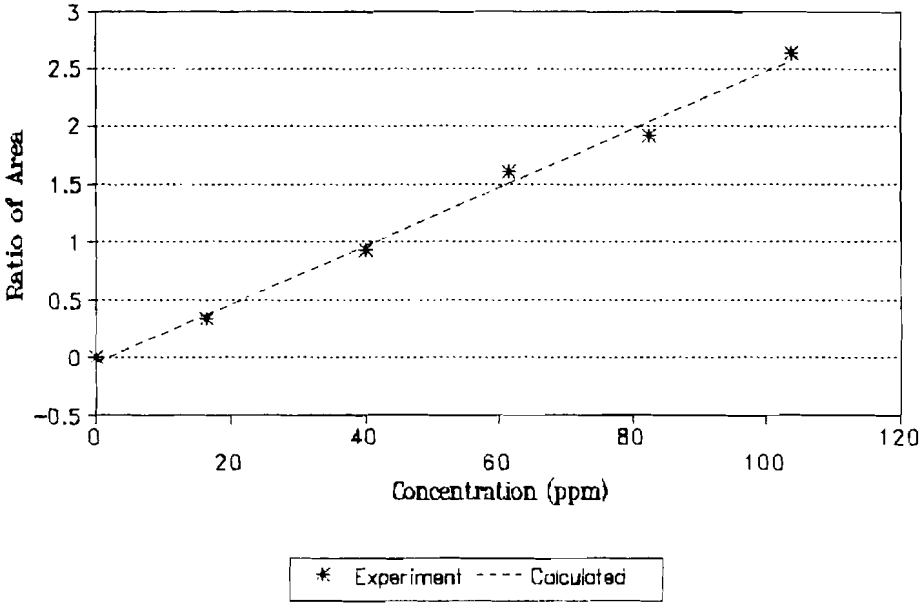
TOTAL AREA= 2.3540E+07
MUL FACTOR= 1.0000E+00

Figure 7. Calibration Curve of Alachlor Standard

* Rel S of Slope (Relative Standard Deviation of Slope) is not from Regression Output.

Calibration Curve of Alachlor Standard

9-28-92

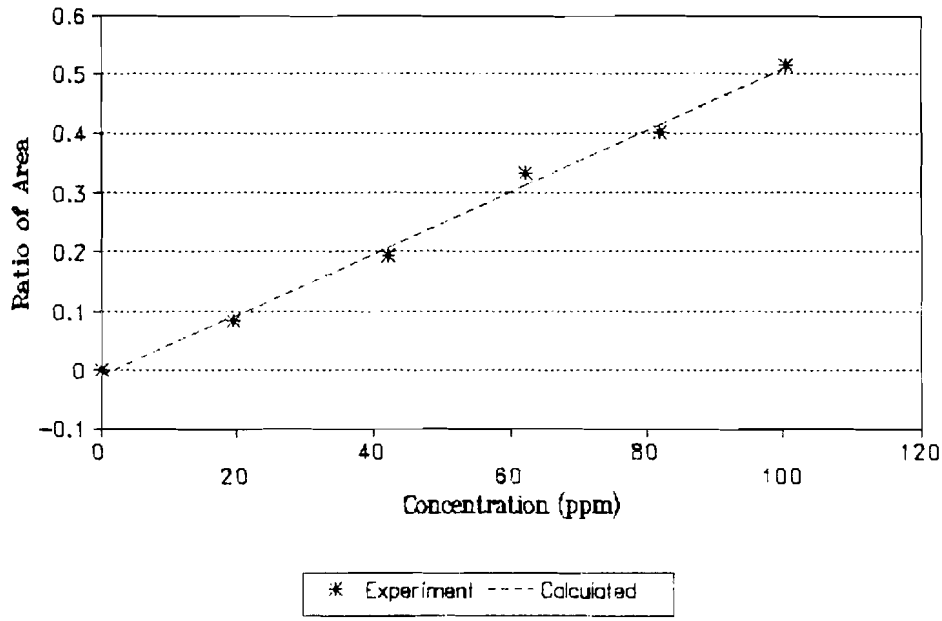


Regression Output:

Constant	-0.0426
Std Err of Y Est	0.0915974
R Squared	0.9932843
No. of Observations	6
Degrees of Freedom	4
X Coefficient(s)	0.0251936
Std Err of Coef.	0.0010358
Rel S of Slope	4.1 %

Calibration Curve of Atrazine Standard

2-17-93



Regression Output:

Constant	-0.009041
Std Err of Y Est	0.0154911
R Squared	0.9950357
No. of Observations	6
Degrees of Freedom	4
X Coefficient(s)	0.00516933
Std Err of Coef.	0.00018256
Rel S of Slope	3.5 %

Figure 8. Calibration Curve of Atrazine Standard

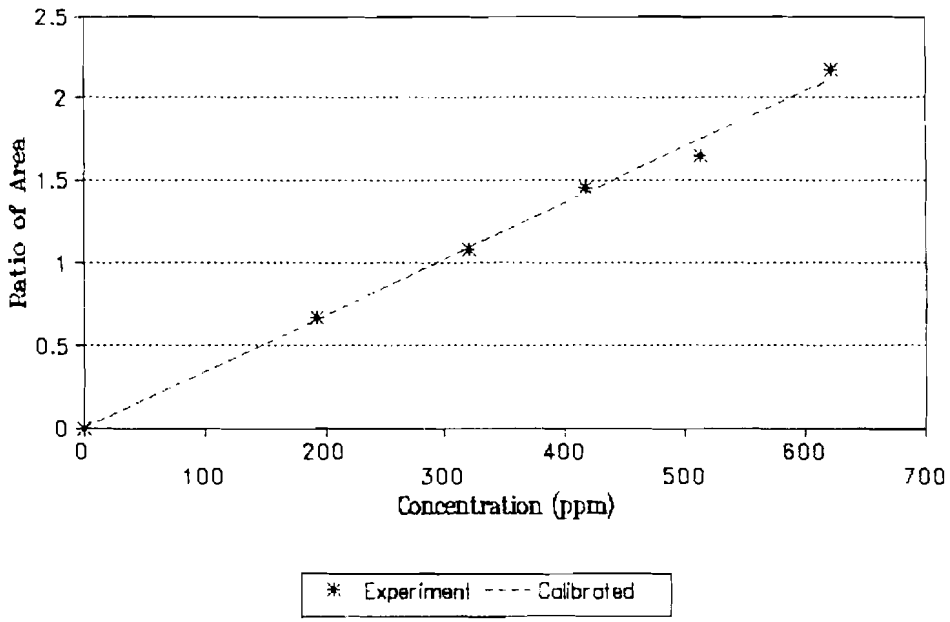
* Rel S of Slope (Relative Standard Deviation of Slope) is not from Regression Output.

Figure 9. Calibration Curve of Chlordane Isomers

* Rel S of Slope (Relative Standard Deviation of Slope) is not from Regression Output. (See Table VII for explanation)

Calibration Curve of Chlordane Isomers

2-17-93



Regression Output:

Constant		0.0017951
Std Err of Y Est		0.0586554
R Squared		0.9953045
No. of Observations		6
Degrees of Freedom		4
X Coefficient(s)	0.00339108	
Std Err of Coef.	0.00011646	
Rel S of Slope	3.4	%

TABLE V
THE CALIBRATION CURVE DATA FOR ALACHLOR SOLUTION

Vol. (μL)	xxx	100	200	300	400	500
Weight (g)	xxx	0.120	0.294	0.452	0.607	0.763
Wt.(IS) (g)	0.709	0.754	0.774	0.703	0.765	0.785
Density ($CHCl_3$)	1.47 (g/mL)					
Conc. (ppm)	0	16.3	40.0	61.5	82.6	103.8
Conc.(IS) (ppm)	96.5	102.6	105.3	95.7	104.0	106.8
Area 1	0	6768	15692	24128	29959	43018
Area 2	0	6859	14919	22819	33516	38649
A(IS) 1	11751	21281	18203	14186	16190	18292
A(IS) 2	17378	23011	17688	14923	19792	16055
Cr.A(IS) 1	11751	20011	16674	14307	15005	16521
Cr.A(IS) 2	17378	21638	16184	15050	18343	14501
Ratio 1	0	0.338	0.941	1.69	2.00	2.61
Ratio 2	0	0.317	0.922	1.52	1.83	2.67
Mean	0	0.328	0.931	1.60	1.91	2.64
Range	0	0.021	0.019	0.17	0.17	0.06

1. IS refers to the internal standard.
2. A(IS) refers to the area of internal standard.
3. Cr.A(IS) refers to the corrected area of internal standard.
4. The concentration of alachlor stock solution was 5,000 ppm.

TABLE VI
THE CALIBRATION CURVE DATA FOR ATRAZINE SOLUTION

Vol. (μL)	xxx	100	200	300	400	500
Weight (g)	xxx	0.154	0.332	0.491	0.648	0.796
Wt.(IS) (g)	0.782	0.752	0.738	0.818	0.725	0.768
Density ($CHCl_3$)	1.47 (g/mL)					
Conc. (ppm)	0	19.4	41.9	62.0	81.8	100.5
Conc.(IS) (ppm)	106.4	102.3	100.4	111.3	98.6	104.5
Area 1	0	2696	5597	10310	10756	13817
Area 2	0	2516	5716	10009	11094	16169
A(IS) 1	21106	30258	28029	32869	24681	27223
A(IS) 2	22343	30326	27099	30997	25670	29979
Cr.A(IS) 1	21106	31465	29700	31422	26621	27719
Cr.A(IS) 2	22343	31536	28715	29633	27688	30525
Ratio 1	0	0.0857	0.188	0.328	0.404	0.498
Ratio 2	0	0.0798	0.199	0.338	0.401	0.530
Mean	0	0.0827	0.194	0.333	0.402	0.514
Range	0	0.0059	0.011	0.010	0.003	0.032

1. IS refers to the internal standard.
2. A(IS) refers to the area of internal standard.
3. Cr.A(IS) refers to the corrected area of internal standard.
4. The concentration of atrazine stock solution was 4,640 ppm.

TABLE VII
THE CALIBRATION CURVE DATA FOR CHLORDANE ISOMERS SOLUTION

Vol. (μL)	xxx	500	750	1000	1250	1500
Weight (g)	xxx	0.704	1.175	1.529	1.883	2.277
Wt.(IS) (g)	0.782	0.795	0.788	0.791	0.823	0.786
Density ($CHCl_3$)	1.47 (g/mL)					
Conc. (ppm)	0	192.3	321.0	417.7	514.4	622.0
Conc.(IS) (ppm)	106.4	108.2	107.2	107.6	112.0	106.9
Area 1	0	17875	27014	33737	38243	43428
Area 2	0	13874	19237	32531	34183	48968
A(IS) 1	21106	26940	26331	26586	26282	25028
A(IS) 2	22343	20974	20993	24487	24557	25030
Cr.A(IS) 1	21106	26499	23691	23830	22641	22576
Cr.A(IS) 2	22343	20631	18888	21949	21155	22578
Ratio 1	0	0.675	1.14	1.42	1.69	1.92
Ratio 2	0	0.672	1.02	1.48	1.61	2.17
Mean	0	0.674	1.08	1.45	1.65	2.17
Range	0	0.003	0.12	0.06	0.08	0.25

1. IS refers to the internal standard.
2. A(IS) refers to the area of internal standard.
3. Cr.A(IS) refers to the corrected area of internal standard.
4. The concentration of chlordane isomers solution was 10,040 ppm.
5. The calculation of chlordane is based on the sum of area of the peaks followed: 7.77, 8.16, 8.70, 8.78, 9.96, 10.28, 10.46 and 11.79 min.

TABLE VIII
LINEAR EQUATIONS FOR EACH CHLORDANE ISOMERS

t_r (min)	Linear Equation	Rel. Std.(%) (Slope)
7.77	y = 0.000243x + 0.001568	2.3
8.16	y = 0.000333x + 0.005744	3.7
8.70	y = 0.000439x + 0.006644	2.9
8.78	y = 0.000203x + 0.004094	4.2
9.26	y = 0.000179x - 0.00056	4.3
10.28	y = 0.00061x + 0.014055	4.6
10.46	y = 0.000407x + 0.013649	7.8
11.79	y = 0.000262x + 0.003916	3.8

$$\textit{The Ratio of Areas} = \frac{\textit{Area of Analytes}}{\textit{The Corrected Area of Internal Standard}}$$

Because chlordane isomers have different retention time, and not all chlordane isomers may be found in tissues at the same time, the linear equations for different chlordane isomers listed in Table VIII are used to estimate the concentration of particular chlordane that existed in the tissues.

3.2. Analysis of Turtle Tissues

A challenging aspect of this work was the development of a clean-up method which would provide satisfactory resolution of analytes from the fat coextractives of the complicated sample matrix. A Florisil column was considered to remove fat from extractive. The recovery values in Table IX show that catechol and alachlor were also taken off by Florisil. In order to know the recovery of alachlor, atrazine

and chlordane after using clean-up solvent, duplicate 1 μL injections of 5 mL 20 ppm alachlor, 100 ppm atrazine and 300 ppm chlordane were made separately before going through the Florisil column. 1 μL eluant of each sample was then injected each time after going through the Florisil column and washed by the clean-up solvent. In order to compare samples, the volume was maintained constant at 5 mL. This means that when necessary additional chloroform was added to make up a total volume of 5 mL.

The recovery of atrazine was 4.1%, but chlordane was affected very little by Florisil, with recovery of 95%. After the Florisil column was washed by the clean-up solvent (50% methylene chloride / 1.5% acetonitrile / 48.5% hexane (v/v/v)), the greatest recovery was for alachlor, i.e. 91%. The recovery of atrazine increases to 50% while that of chlordane increases to 99%. The gas chromatogram of clean-up solvent shown in Figure 10 demonstrates there are no impurity peaks interfering with analyte peaks. Figures 11, 12 and 13 illustrate the comparison of the gas chromatograms of alachlor, atrazine and chlordane isomers before and after washing with the clean-up solvent.

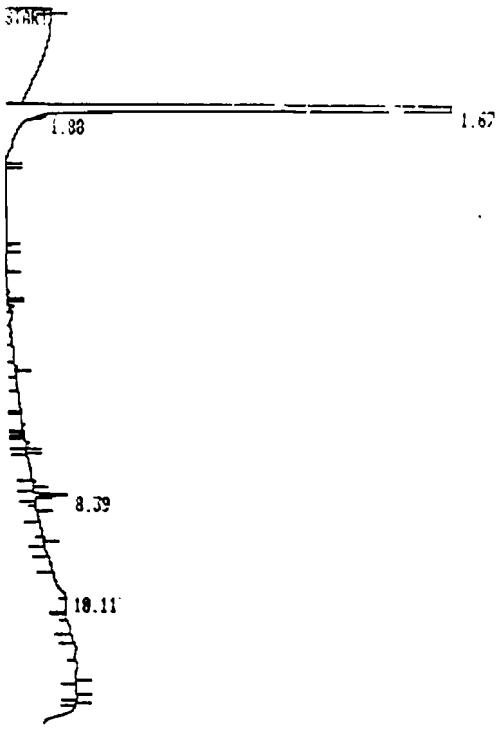
To insure that analytes were not lost or decomposed during the extraction, evaporation and cleanup procedure, a portion (0.816 g) of gland tissue which had no alachlor, atrazine and chlordane residue content as given in Figure 14 was spiked with 0.70 mg alachlor, 0.79 mg atrazine and 5.4 mg

TABLE IX
THE RECOVERY OF ALACHLOR, ATRAZINE AND CHLORDANE WASHED BY CLEAN-UP MIXTURE SOLVENT

Before going through Florisil column					
	Area 1	Area 2	Mean	Range	
20 ppm Alachlor	5243	5012	5128	231	
100ppm Atrazine	18446	20519	19482	2073	
300 ppm Chlordane	38125	36905	37515	1220	
After going through Florisil column					
	Area 1	Area 2	Mean	Range	Recovery
20 ppm Alachlor	0	0	0	0	0
100ppm Atrazine	800	792	796	8	4.1 %
300 ppm Chlordane	34288	36737	35513	2449	95 %
After the column washed by clean-up solvent					
	Area 1	Area 2	Mean	Range	Recovery
20 ppm Alachlor	4719	4570	4645	149	91 %
100ppm Atrazine	9866	9760	9813	106	50 %
300 ppm Chlordane	36903	37436	37170	533	99 %

chlordane as well as 2.0 mg catechol prepared by diluting the stock standard solution of each analytes to 10 mL before extraction. The 1 μL mixture solution was loaded before and after the extraction, evaporation and cleanup procedure. According to the results in Table X and contrasting the gas chromatograms in Figures 15 and 16, only alachlor and chlordane can be recovered by the overall procedure. The recoveries of alachlor and chlordane were 34% and 86%, respectively.

Figure 10. Gas Chromatogram of The Clean-up Solvent



RUN # 216 FEB/19/93 22:44:41

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.67	3.3521E+07	†SPB	0.065	99.992
1.88	399	TBB	0.018	0.001
8.39	2440	PB	0.031	0.007

TOTAL AREA= 3.3524E+07
MUL FACTOR= 1.0000E+00

Figure 11. A Comparison of Gas Chromatograms of 20 ppm Alachlor Before and After Washing by Cleanup Solvent

a refers to the chromatogram of 20 ppm alachlor before Florisil was used.

b refers to the chromatogram of 20 ppm alachlor after Florisil was used.

c refers to the chromatogram of 20 ppm alachlor after Florisil column was washed with the clean-up solvent.

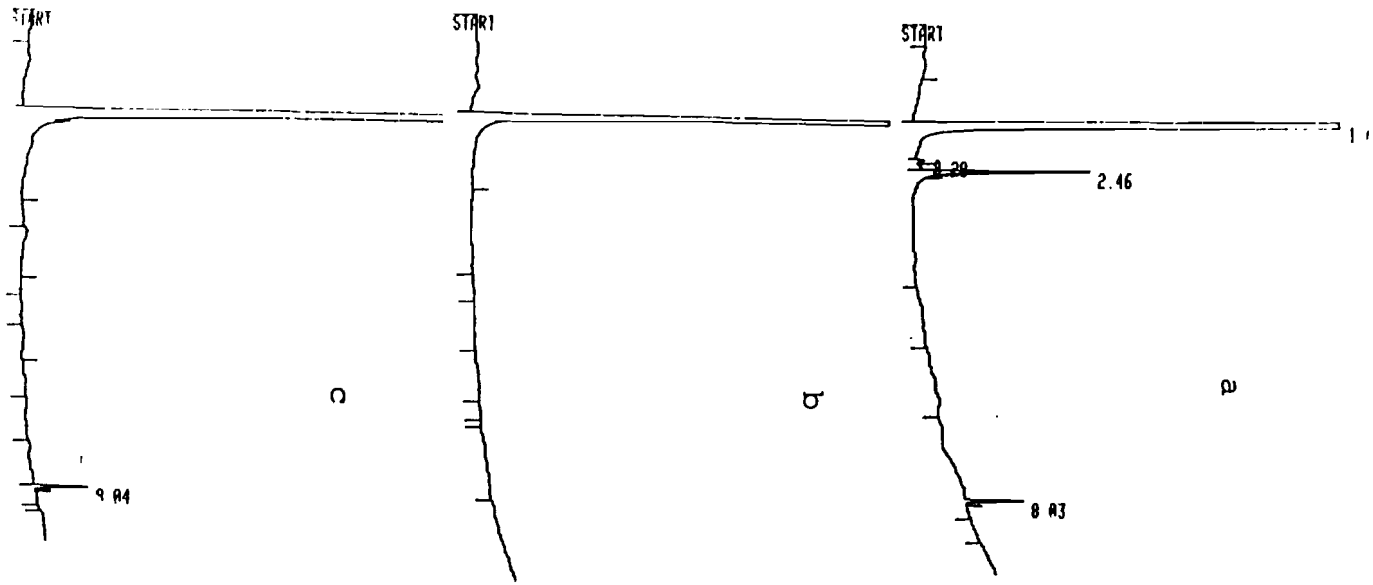

START
START
START
2.46
c
d
e

Figure 12. A Comparison of Gas Chromatograms of 100 ppm Atrazine Before and After Washing by Cleanup Solvent

a refers to the chromatogram of 100 ppm atrazine before Florisil was used.

b refers to the chromatogram of 100 ppm atrazine after Florisil was used.

c refers to the chromatogram of 100 ppm atrazine after Florisil column was washed with the clean-up solvent.

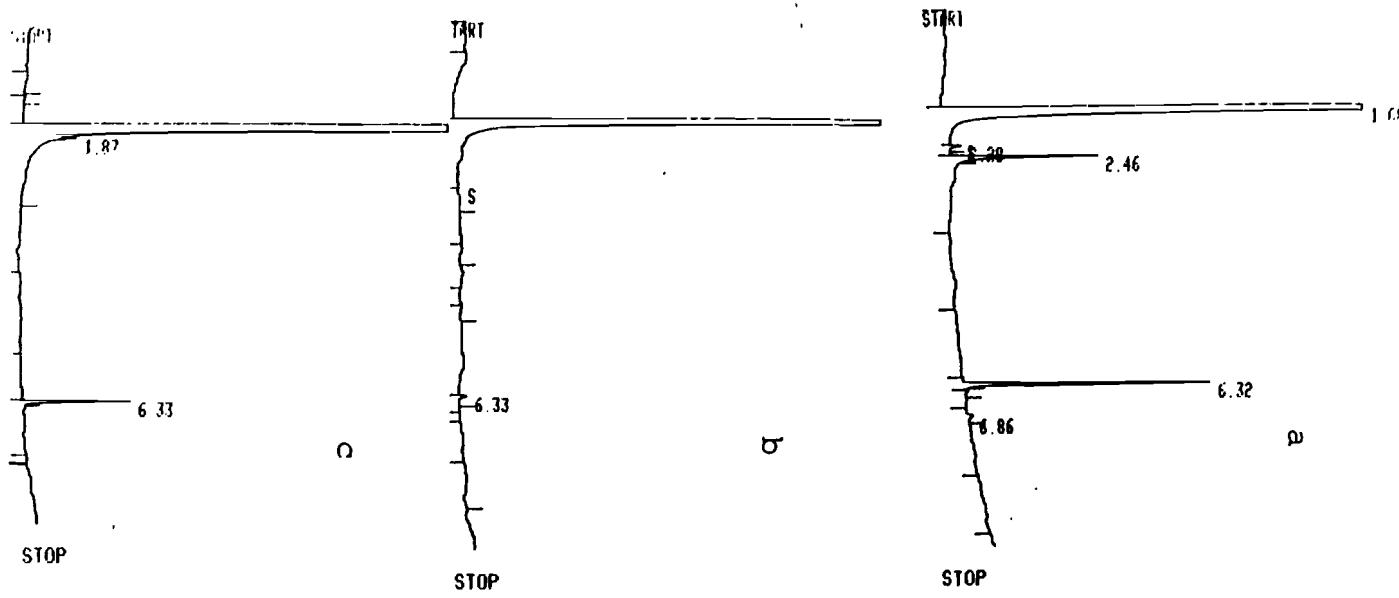
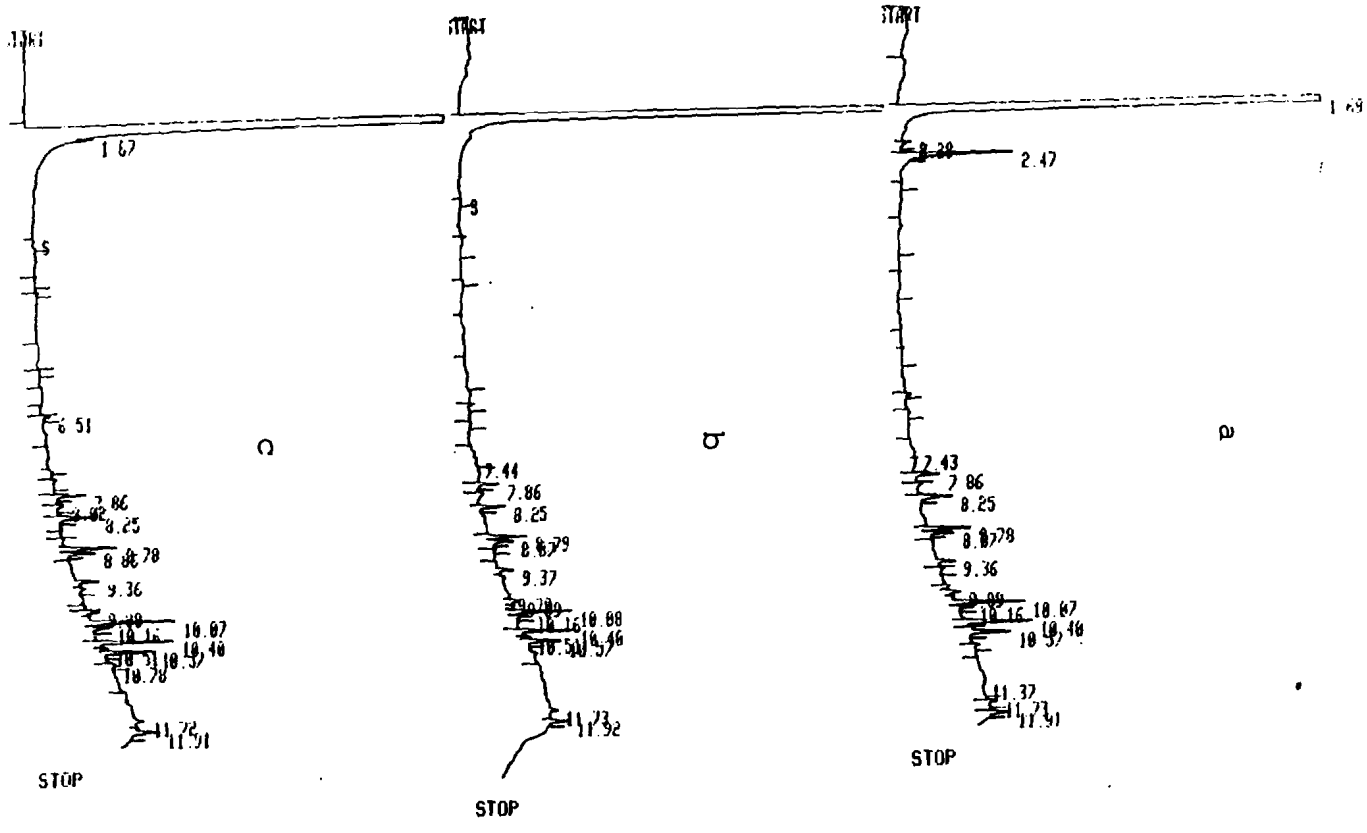

1.87
6.33
STOP
S
6.33
STOP
2.46
6.32
6.86
STOP

Figure 13. A Comparison of Gas Chromatograms of 300 ppm Chlordane Before and After Washing by Cleanup Solvent

a refers to the chromatogram of 300 ppm chlordane before Florisil was used.

b refers to the chromatogram of 300 ppm chlordane after Florisil was used.

c refers to the chromatogram of 300 ppm chlordane after Florisil column was washed with the clean-up solvent.


START
1.67
6.51
7.86
8.25
9.36
10.07
10.78
11.72
11.91
STOP
START
7.44
7.86
8.25
9.37
10.08
11.73
11.92
STOP
START
2.47
7.43
7.86
8.25
9.36
10.07
11.37
11.73
11.91
STOP

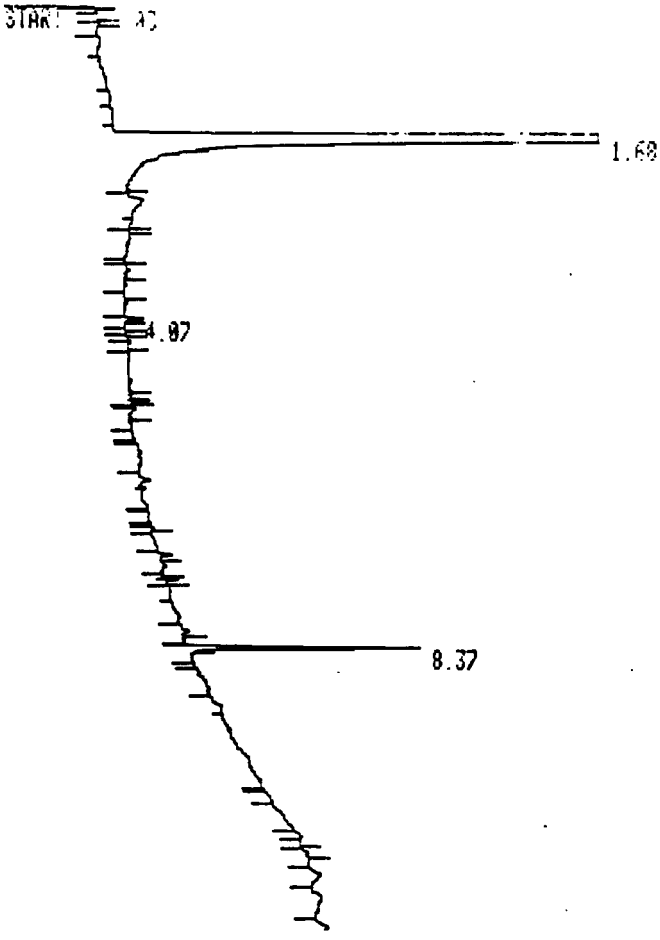
In order to make sure that there were no contaminants from other sources, a blank was prepared by carrying out all procedures, such as extraction, evaporation and cleanup, without any analytes contained in thimble. The gas chromatogram of this blank is given in Figure 17. There is no impurity that can produce the interference.

After analyzing liver, fat and the unidentified gland tissue, alachlor and atrazine were not found in any of these tissues. There are two possible reasons: one is the loss or decomposition of analytes during the process, because the recovery of alachlor was very low, and atrazine was lost completely. Another possibility is that both alachlor and atrazine are metabolized in the liver. Some chlordane isomers were detected in the extractive of liver, but not in fat and the unidentified gland tissue. The concentrations of these isomers are listed in Table XI, but there is a large uncertainty in these results, since the fat in liver can not be removed completely by Florisil column, even when several Florisil columns were applied repeatedly to one sample. Therefore, the interference from fat may make the apparent concentration of chlordane isomers much higher than the true value.

3.3. Suggestions for Further work

The procedure that has been developed allows the simultaneous determination of alachlor, atrazine and chlordane. The efficiency of the analytical method is

Figure 14. A Gas Chromatogram of Extract From the Gland Tissue Without Spiking by Alachlor, Atrazine and Chlordane



STOP

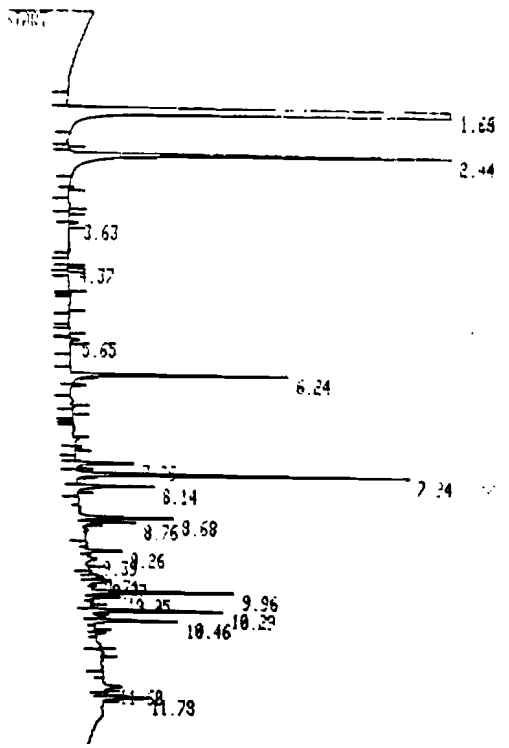
RUN # 16 MAR/24/93 14:16:38

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.68	2.8261E+07	TSHB	0.055	99.977
4.07	425	PF	0.026	0.002
8.37	6163	BV	0.021	0.022

TOTAL AREA= 2.8268E+07
MUL FACTOR= 1.0000E+00

Figure 15. A Gas Chromatogram of Alachlor, Atrazine and Chlordane Mixture Before Spiking the Gland Tissue



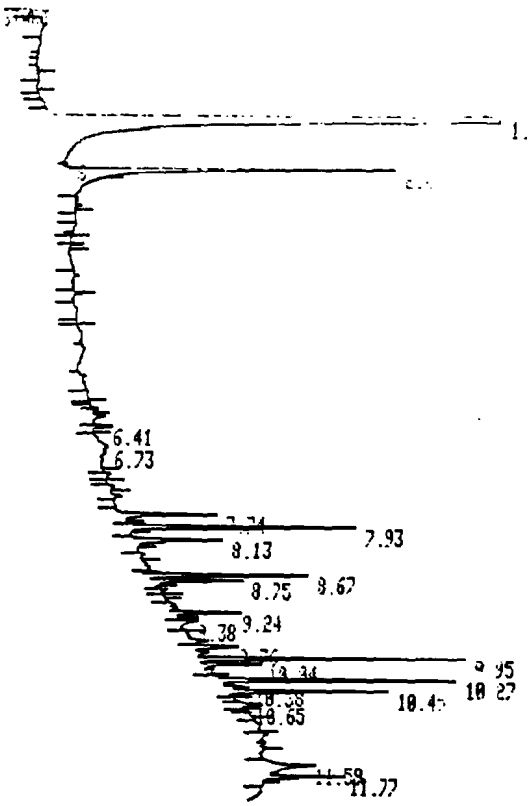
RUN # 177 FEB/19/93 11:05:46

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.66	1703700	SBH	0.014	35.063
1.68	3015300	†SHB	0.009	62.057
2.44	39158	PB	0.026	0.806
3.63	516	BV	0.022	0.011
4.37	415	BB	0.023	0.009	
5.65	575	PB	0.027	0.012
6.24	13725	PB	0.028	0.283
7.76	4759	BE	0.036	0.098
7.94	22832	PE	0.030	0.470
8.14	6288	Bb	0.036	0.129
8.68	6989	PP	0.033	0.144
8.76	3493	PS	0.029	0.072
9.26	3007	PV	0.035	0.062
9.39	1398	V5	0.054	0.029
9.71	798	Rb	0.035	0.016
9.77	1887	Br	0.044	0.039
9.96	11300	P%	0.035	0.233
10.05	2235	V?	0.032	0.046
10.29	9410	BB	0.032	0.194
10.46	5950	BP	0.032	0.123
11.60	1748	PS	0.042	0.036
11.78	3495	B?	0.036	0.072

TOTAL AREA= 4859000
MUL FACTOR= 1.000RE+00

Figure 16. Gas Chromatogram of Alachlor, Atrazine and Chlordane Mixture After Spiking the Gland Tissue by the Overall Procedure



STOP

RUN # 11 MAR/24/93 12:52:10

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.67	2.7945E+07	†SBB	0.054	99.730
2.45	13242	BB	0.042	0.047
6.41	692	BB	0.034	0.003
6.73	2108	BB	0.175	0.008
7.74	3274	PB	0.034	0.012
7.93	7992	BB	0.035	0.029
8.13	3757	BB	0.041	0.013
8.67	5193	BP	0.033	0.019
8.75	2796	PB	0.031	0.010
9.24	2074	PB	0.031	0.007
9.38	647	BB	0.036	0.002
9.76	1840	PP	0.041	0.007
9.95	8905	PV	0.033	0.032
10.04	1870	VV	0.032	0.007
10.27	8628	PV	0.035	0.031
10.38	1024	VV	0.041	0.004
10.45	5553	VB	0.034	0.020
10.65	990	BB	0.041	0.004
11.59	2129	BV	0.057	0.008
11.77	2373	VV	0.037	0.011

TABLE X
THE RECOVERY OF ALACHLOR, ATRAZINE AND CHLORDANE FROM THE SPIKED GLAND TISSUE SAMPLE BY THE OVERALL PROCEDURE

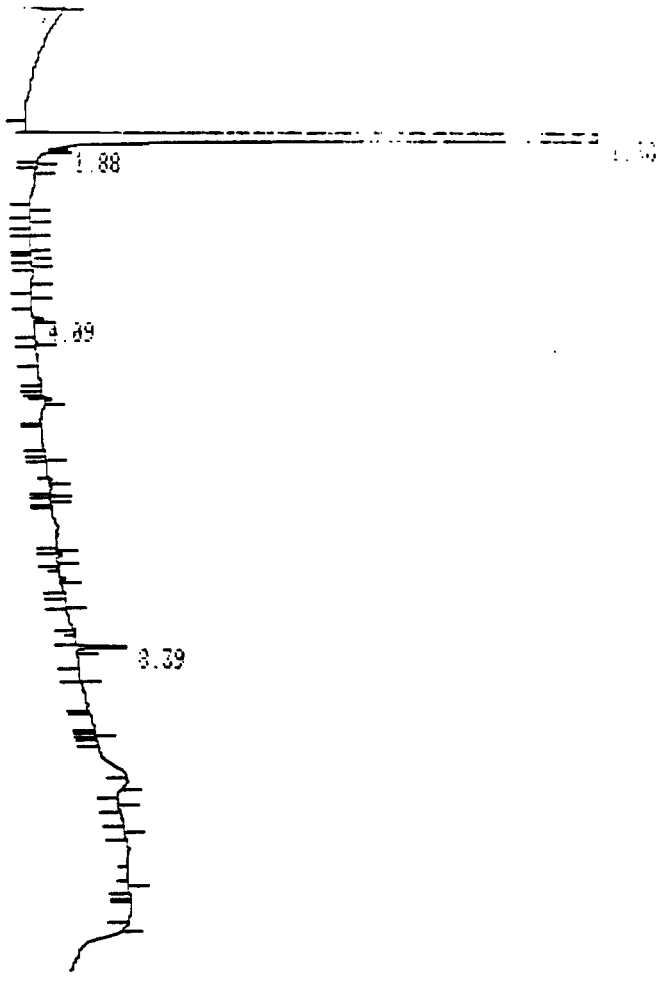
	Before Treatment		After Treatment		Recovery (%)
	Area	Mean	Area	Mean	
0.86 mg/g Alachlor	20807 22832 23737	22459	8070 7992 7975	8012	34 ± 2.0
0.97 mg/g Atrazine	11666 13725 15192	13527	xxxx		0
6.6 mg/g Chlordane	48135 48403 52865	49801	41749 43152 41471	42124	86 ± 3.7
2.5 mg/g Catechol	23192 27422 39158	29924	xxxx		0

TABLE XI
THE CONCENTRATION OF CHLORDANE ISOMERS DETECTED IN TURTLE LIVERS

	Weight (g)	Concentration (mg/g) (for isomers with different t_r (min))					
		7.77	8.70	8.78	9.26	10.3	10.5
DET-165-1	16.878	0.17	1.7	0.39	n.d	n.d	7.1
DET-173-1	18.402	n.d	0.82	0.14	n.d	0.23	0.79
DET-173-2	36.624	n.d	n.d	20	n.d	5.7	n.d
DET-116-5	13.510	n.d	n.d	2.4	n.d	n.d	9.5
DET-116-7	9.935	n.d	n.d	23	n.d	n.d	n.d
DET-116-9	22.23	n.d	n.d	42	0.59	n.d	52
DET-116-?	41.795	n.d	n.d	n.d	n.d	2.9	n.d

indicated by the recovery values shown in Table X. The most important part of this analytical method is the clean-up.

Figure 17. Gas Chromatogram of A Blank Sample



STOP

RUN # 215 FEB/19/9[illegible] 22:39:22

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.68	3.4630E+07	[illegible]	0.967	99.991
1.88	433	[illegible]	0.017	0.001
4.09	263	[illegible]	0.017	[illegible]E-04
8.39	2559	[illegible]	0.030	0.007

TOTAL AREA= 3.4634E+07
MUL FACTOR= 1.0000E+00

Since the internal standard, catechol, was not recoverable after the clean-up procedure, a new internal standard should be selected. Further, since atrazine was not recoverable either, and the recovery of alachlor was very low (34%) through the overall procedure, the method with the more effective recoveries for both compounds should be tried. There are some reports about the clean - up of various oily extracts by gel permeation chromatography (GPC) (16, 17), which provides excellent removal of lipids and other large molecules. This technique may be tried to reduce the interference of fat in liver, which is difficult to achieve by Florisil alone.

Due to a shortage of normal turtles, the control analysis have not been carried out. When available, the results obtained from normal turtles should be compared with that of the deformed groups, so that we can know if the pesticides - alachlor, atrazine and chlordane are actually related to the deformity.

It is further suggested that the water samples of the lakes where the deformed turtles were trapped are tested. Therefore, the concentrations of alachlor, atrazine and chlordane in the water can be compared with that in the turtle tissues, so that we can determine if alachlor and atrazine existed in the environment in which the turtle lived, then metabolized by aquatic animals, or if they didn't exist in the lakes at all.

CHAPTER 4. REFERENCES

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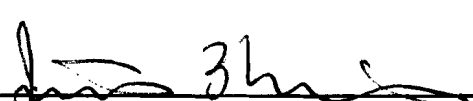


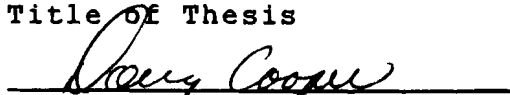
Signature of Author

Oct. 21, 1993

Date

Development of A Screening Test for Atrazine, Alachlor, and Chlordane in Turtles by Gas Chromatography.

Title of Thesis



Signature of Graduate Office Staff Member

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Date Received