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QUANTIFICATION OF PETROLEUM-TYPE HYDROCARBONS IN AVIAN TISSUE

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SUMMARY

Methods were developed for the analysis of 16 hydrocarbons in avian tissue. Mechanical extraction with pentane was followed by clean-up on Florisil and Silicar. Residues were determined by gas–liquid chromatography and gas–liquid, chromatography–mass spectrometry. The method was applied to the analysis of liver, kidney, fat, and brain tissue of mallard ducks (*Anas platyrhynchos*) fed a mixture of hydrocarbons. Measurable concentrations of all compounds analyzed were present in all tissues except brain. Highest concentrations were in fat.

INTRODUCTION

Pollution of coastal areas by petroleum hydrocarbons has become a serious threat to waterfowl in recent years. Oil spills have caused the loss of large numbers of birds[1]. Less well-documented are the toxic effects of the chronic ingestion of petroleum hydrocarbons.

An important aspect of the elucidation of the toxic effects of petroleum is the development of methodology for the analysis of petroleum hydrocarbons in avian tissue. Methods for the analysis of petroleum in water[2] and in marine organisms[3] have been developed. Methods for the determination of petroleum residues in ducks also have been reported[4,5].

Initial attempts to adapt the Warner procedure[6] to the analysis of hydrocarbons in marine organisms to avian tissue resulted in the production of intractable emulsions. A procedure developed in our laboratory for the extraction of pesticides[7] from avian tissues was therefore adapted for use with hydrocarbon residues.

We have developed methods for the extraction and quantification of petroleum-type hydrocarbons in avian fat, brain, liver and kidney tissues. Unlike methods depending upon total area integration, our method involves individual quantification

of 16 specific compounds found in petroleum and in a mixture compounded to resemble petroleum. Constituents of this mixture are listed in Table I. Ethylbenzene was not analyzed because it coeluted from the gas chromatograph with the solvent. Three other compounds (2,3,3-trimethylindolenine, 2-methylbenzothiazole, and 2,6-dimethylquinoline) were not analyzed because of problems discussed below.

TABLE I

CONSTITUENTS OF MIXTURE FED TO DUCKS

Aromatics (Reconstructed Aromatic Mixture or RAM)
2,3,3-Trimethylindolenine (2,3,3-TMI)*
2-Methylbenzothiazole (2-MBT)*
2,6-Dimethylquinoline (2,6-DMQ)*
Ethylbenzene*
Tetralin
Dimethylnaphthalene (DNN)
Acenaphthylene
Acenaphthene
Dibenzothiophene (DBT)
Phenanthrene
Aliphatics
trans-Decalin
cis-Decalin
n-Tridecane (*n*-C_{13})
2,2,4,4,6,8,8-Heptamethylnonane (br-C_{15})
n-Pentadecane (*n*-C_{15})
n-Hexadecane (*n*-C_{16})
n-Heptadecane (*n*-C_{17})
Pristane
n-Octadecane (*n*-C_{18})
n-Nonadecane (*n*-C_{19})

* Not analyzed.

EXPERIMENTAL

Reagents

Pentane, dichloromethane, hexane, diethyl ether, and benzene (distilled in glass grade) were obtained from Burdick & Jackson Lab. (Muskegon, Mich., U.S.A.). Hydrochloric acid, sodium chloride, potassium hydroxide and sodium sulfate (anhydrous, granular) were ACS reagent grade. Silicar (CC-4) was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Florisil (60–100 mesh, Grade A, activated at 650°) was obtained from Floridin Co. (Berkeley, W. Va., U.S.A.). Pre-treatment of Florisil and Silicar were described previously[7]. Deuterated internal standards[8] were obtained from Merck Isotopes (St. Louis, Mo., U.S.A.).

Apparatus

Gas chromatographic analysis was performed with a Hewlett-Packard Model 5711 gas–liquid chromatograph equipped with a Model 18740 flame ionization detector, a glass capillary inlet system, and a Model 3852B laboratory data system. Gas–liquid chromatography–mass spectrometry (GLC–MS) was performed using a Finnigan Model 3200 GLC–MS instrument interfaced to a Finnigan Model 6100

data system. The GLC inlet was a Grob injector (Finnigan). The GLC instrument was interfaced to the mass spectrometer directly. Tissue was homogenized using a Polytron with a PT20ST probe (Brinkmann). Solvent volume was reduced on a Buchler rotary evaporator.

Tissues

Forty-eight mallard drakes (*Anas platyrhynchos*) were randomly assigned to one of four treatment groups, as described previously[9]. Liver, brain, kidney, and fat tissue from drakes in Group I, which had received breeder mash and served as the control, and Group IV, which had received a 1% mixture of 0.600% (6000 ppm) paraffin and 0.40% (4000 ppm) aromatic hydrocarbons in the breeder mash were analyzed for the presence of hydrocarbons. The individual compounds that comprised the paraffin and aromatic mixtures are listed in Table I.

Extraction and clean-up procedure

The sample tissue was cut into small pieces, put into a 250-ml centrifuge jar and weighed. Typically, the following quantities were used: fat, 2 g; liver, 15 g; kidney, 5–7 g; and brain, 3–5 g. It was necessary to pool two brains to obtain the required sample size. Pentane (80 ml) was added and the sample was extracted in the Polytron. The Polytron probe was rinsed with pentane (20 ml) and this rinse solution was added to the centrifuge tube. After centrifugation, the extract was decanted into a 300-ml flask. The residue was then extracted twice more as described above. The combined extract was reduced in volume to 150–200 ml on a rotary evaporator. The nitrogen heterocycles were extracted with HCl (three extractions of 5 ml, 3.0 *M* HCl). Quantitation of these compounds is not reported here because of difficulty encountered in obtaining reproducible recoveries.

Extracts from fatty tissues (fat and brain) were saponified following extraction of the bases. The pentane solution was placed in a 250-ml flask and the solvent was carefully removed on the flash evaporator. Methanol (135 ml) and 13 *M* KOH (15 ml) were added and the sample was refluxed 5–7 h. The saponification mixture was extracted with hexane, and the hexane solution was washed with water and brine. After being concentrated on the rotary evaporator to 5 ml the saponification product was subjected to Florisil clean-up.

Extracts of tissues with a lower fat content (liver and kidney) were subjected to Florisil clean-up directly, without saponification. The Florisil column was prepared by adding 21 g of Florisil to a glass column, topping with anhydrous sodium sulfate, and prewashing with pentane (100 ml). The extract then was placed on the column and eluted with 200 ml of 6% diethyl ether in pentane.

The volume of the eluate was reduced to 5 ml on a rotary evaporator and the aromatic and aliphatic fractions were separated on a column consisting of silicic acid (20 g, deactivated with 3% water) prewashed with pentane. Aliphatics were eluted with 100 ml pentane; aromatics with 100 ml benzene. Volumes were carefully reduced under a stream of air and the fractions were analyzed by GLC and/or GLC–MS.

GLC

The sample was introduced via splitless injection to a 50 m × 0.75 mm I.D

glass capillary column, OV-101 (Applied Science Labs., State College, Pa., U.S.A.). A flow-rate of 1.5 ml/min of helium was maintained through the column with nitrogen make-up gas added to produce a flow-rate of 51 ml/min through the detector. The temperature of the column was held at 70° for 2 min following injection and then programmed to 220° at 4°/min. Quantification was by comparison of the peak areas with those of an internal standard (perdeuterotetracosane). Peak integration and calculations were done using the software package of the Hewlett-Packard 3352 laboratory data system. One GLC run represented an injection of 100 mg of tissue. The lower limit of detection by GLC was approximately 1 ng for all compounds.

GLC–MS

The sample was introduced via splitless injection onto a 30 m × 0.75 mm I.D. high-performance glass capillary column, SP-2100 (Supelco, Bellefonte, Pa., U.S.A.). The flow-rate was 8.5 ml/min of helium. The column effluent was introduced directly into the mass spectrometer source. The column was held at room temperature for 2 min and then programmed from 160° to 200° at 2°/min. Ionization was at 70 eV. Operation of the mass spectrometer in the mass fragmentography mode was controlled by the data system. Quantification was by comparison of peak areas with those of an internal standard following establishment of relative response factors for the compounds to be quantified. For quantification of aromatic compounds perdeuteromethylnaphthalene was used as the internal standard. Perdeuterohexadecane was used as the internal standard for the aliphatic compounds. Mass peaks and relative retention times for aliphatic and aromatic compounds are presented in Tables II and III. Typically for a 10-ng; tissue sample, one GLC–MS run represented an injection of 20 mg of tissue. The lower limit of detection by GLC–MS was 0.05 ng for aromatic hydrocarbons and 0.1 ng for aliphatic hydrocarbons.

TABLE II

MASS PEAKS AND RELATIVE RETENTION TIMES OF ALIPHATIC COMPOUNDS

Aliphatics	*Mass peaks*	*Relative retention time***
trans-Decalin	66, 138*	0.53
cis-Decalin	66, 138*	0.55
n-C_{13}	57, 113*	0.66
Br-C_{15}	57, 113*	0.70
n-C_{15}	57, 113*	0.87
n-C_{16}	57, 113*	1.04
n-C_{17}	57, 113*, 240	1.26
Pristane	57, 113*, 183	1.29
n-C_{18}	57, 113*, 254	1.54
n-C_{19}	57, 113*, 268	1.87

* Peaks used for quantitation against *m/e* 130 in the spectrum of the deuterated standard, or by comparison with external standard.

** Relative to $C_{16}{}^{2}H_{34}$ which has a retention time of 10.1 min.

RESULTS AND DISCUSSION

Recoveries

Recoveries were determined for all compounds analyzed in all tissues. Spiking

TABLE III

MASS PEAKS AND RELATIVE RETENTION TIMES OF AROMATIC COMPOUNDS

Aromatics	*Mass peaks*	*Relative retention time***
Tetralin	104*, 132	0.84
DMN	141*, 156	1.19
Acenaphthylene	152*	1.24
Acenaphthene	152, 154*	1.32
Dibenzothiophene	139, 152, 184*	2.19
Phenanthrene	152, 184*	2.31

* Indicates peaks used for quantitation against *m/e* 152 in the mass spectrum of the deuterated standard or by comparison with external standard.

** Retention times relative to perdeutero-1-methyl naphthalene which has a retention time of 7.07 min.

was done at lower levels than those actually encountered in the tissues. Although recoveries varied from 27–100%, most were between 40 and 80% (Tables IV, V, VI, VII). High losses (and therefore low recoveries) were observed for the more volatile compounds. High, non-reproducible interference with the determination of dimethylnaphthalene was observed in liver samples. Ethylbenzene was not analyzed because it coeluted with solvent.

Comparison of GLC and GLC–MS quantification

Although quantification by GLC was much more convenient than by GLC–MS, the latter method is much more specific. Because of high levels of interference in liver, GLC could not be used. Some interference also was encountered in the analysis of fat, but the residue levels were also very high and these GLC results are

TABLE IV

RESIDUES (PPM, WET WEIGHT) IN LIVERS OF MALLARD DRAKES FED PETROLEUM HYDROCARBONS FOR SEVEN MONTHS

	*Fed: 4000 ppm RAM**		*Controls**		*% Recovery*** GLC–MS*
	Average	*Range*	*Average*	*Range*	
Tetralin	0.59	0.07–1.4	0	0–0	55
DMN	0.97	0.12–2.5	0.02	0–0.07	64
Acenaphthylene	1.7	0.31–3.9	0.01	0–0.02	68
Acenaphthene	2.3	0.28–5.3	0	0–0.02	61
DBT	1.51	0.81–2.3	0.05	0–0.11	52
Phenanthrene	1.7	0.29–3.4	0.06	0–0.13	98
n-C_{13}	0.75	0.02–2.0	0	0–0.02	73
br-C_{16}	0.91	0.03–2.2	0	0–0	41
n-C_{15}	1.4	0.05–3.2	0.06	0–0.19	102
n-C_{16}	1.1	0.04–2.4	0.04	0–0.14	44
n-C_{17}	0.77	0.03–1.7	0.03	0–0.08	65
Pristane	3.7	0.38–9.7	0.03	0–0.08	85
n-C_{18}	0.65	0.04–1.5	0.01	0–0.03	82
n-C_{19}	0.76	0.04–1.7	0.03	0–0.05	90

* Based on four determinations.

** Average of two determinations corrected for background.

TABLE V

RESIDUES (PPM, WET WEIGHT) IN KIDNEYS OF MALLARD DRAKES FED PETROLEUM HYDROCARBONS FOR SEVEN MONTHS

	*Fed: 4000 ppm RAM**				*Control*			*% Recoveries****	
	GLC		*GLC–MS*		*GLC***	*GLC–MS**		*GLC*	*GLC–MS*
	Average	*Range*	*Average*	*Range*	*Average*	*Average*	*Range*		
Tetralin	0.54	0.49–0.63	0.48	0.16–0.84	—	0	0–0	76	67
DMN	0.86	0.67–1.2	0.89	0.40–1.6	—	0	0–0.01	71	71
Acenaphthylene	1.6	1.5 –1.6	1.6	0.30–2.5	—	0	0–0	55	63
Acenaphthene	2.2	1.8 –2.4	2.3	1.1 –3.9	—	0	0–0	63	60
DBT	1.1	0.78–1.4	0.92	0.21–1.2	—	0	0–0	73	77
Phenanthrene	1.5	1.4 –1.8	1.1	0.43–1.3	—	0	0–0	75	91
trans-Decalin	0.03	0 –0.10	0.03	0 –0.08	—	0	0–0	‡	‡
cis-Decalin	0	0 –0	0.03	0 –0.07	—	0	0–0	‡	‡
n-C_{13}	0.78	0.53–0.91	0.67	0.30–0.90	—	0	0–0.04	46	43
br-C_{15}	1.4	0.57–2.3	1.4	0.33–2.2	—	0	0–0	43	38
n-C_{15}	1.5	1.3 –1.6	1.2	0.65–1.9	0.10	0.01	0–0.03	58	45
n-C_{16}	2.2	1.4 –3.6	1.0	0.69–1.6	—	0.01	0–0.02	62	51
n-C_{17}	1.6	1.4 –1.7	0.96	0.60–1.5	—	0.01	0–0.03	70	60
Pristane	7.4	4.1 –13	5.3	3.0 –12	—	0.01	0–0.02	70	56
n-C_{18}	1.7	1.3 –2.0	1.0	0.58–1.5	—	0.01	0–0.02	75	58
n-C_{19}	2.4	1.6 –3.0	1.3	0.88–1.8	—	0.01	0–0.03	72	63

* Based on three determinations.
** Based on two determinations.
*** Average of two determinations corrected for background.
‡ Interference.

TABLE VI

RESIDUES (PPM, WET WEIGHT) IN FAT OF MALLARD DRAKES FED PETROLEUM HYDROCARBONS FOR SEVEN MONTHS

	*Fed: 4000 ppm RAM**				*Control*			*% Recoveries**** ± *standard deviation*	
	GLC		*GLC–MS*		*GLC***	*GLC–MS**			
	Average	*Range*	*Average*	*Range*	*Average*	*Average*	*Range*	*GLC*	*GLC–MS*
Tetralin	6.0	3.3–10	2.0	2.8–3.8	0.25	—	0. –0.08	‡	53 ± 15
DMN	4.2	3.4–6	3.3	0.87–4.6	0.05	—	0. –0.17	76 ± 11	41 ± 23
Acenaphthylene	7.1	5.5–8.5	6.6	1.7–10	0.06	—	0 –0	55 ± 5	44 ± 13
Acenaphthene	9.4	7.0–11	9.6	2.4–14	—	0 –0	50 ± 5	54 ± 14	
DBT	2.1	1.5–3.1	2.1	1.3–3.7	0.45	—	0 –0	‡	65 ± 10
Phenanthrene	6.8	5.5–10	7.4	3.9–13	0.55	0.02	0.02–0.04	71 ± 19	76 ± 12
n-C_{13}	9.8	8.5–12	10	0.5–18	0.03	—	0 –0.05	67 ± 24	40 ± 18
br-C_{16}	13	9.5–20	14	1.5–23	0.01	—	0 –0.02	55 ± 22	54 ± 28
n-C_{15}	17	12 –20	24	6.3–51	0.05	0.06	0.01–0.11	75 ± 24	65 ± 16
n-C_{16}	14	10 –17	22	7.4–45	0.08	0.06	0.02–0.08	88 ± 25	76 ± 15
n-C_{17}	12	9.0–15	19	7.8–38	0.10	0.07	0.04–0.13	63 ± 23	79 ± 15
Pristane	42	26 –60	80	35 –130	0.11	0.06	0.05–0.15	72 ± 21	82 ± 6
n-C_{18}	11	8.5–13	17	8.2–35	0.26	0.07	0.05–0.17	82 ± 29	73 ± 11
n-C_{19}	15	10 –18	21	13 –42	0.05	0.10	0.05–0.17	70 ± 28	76 ± 18

* Based on five determinations.
** Based on two determinations corrected for background.
*** Average of eight determinations corrected for background.
‡ Interference.

TABLE VII

RESIDUES (PPM, WET WEIGHT) IN BRAINS OF MALLARD DRAKES FED PETROLEUM HYDROCARBONS FOR SEVEN MONTHS*

	*Fed: 4000 ppm RAM***				*Control***				*% Recoveries****	
	GLC		*GLC/MS*		*GLC*		*GLC/MS*		*GLC*	*GLC/MS*
	Average	*Range*	*Average*	*Range*	*Average*	*Range*	*Average*	*Range*		
Tetralin			0.05	0.03–0.08	0.02	0–0.05	0	0–0	37	45
DMN	0.20	0.17–0.27	0.10	0.07–0.16	0.10	0–0.32	0.01	0–0.04	32	54
Acenaphthylene	0.21	0.09–0.29	0.15	0.10–0.23	0	0–0	0	0–0	27	50
Acenaphthene	0.33	0.14–0.45	0.25	0.18–0.38	0	0–0.01	0	0–0	37	57
DBT	0.28	0.06–0.38	0.17	0.86–0.26	0	0–0	0.01	0–0	33	35
Phenan-	0.32	0.18–0.46	0.20	0.14–0.34	0.04	0–0.01	0.01	0–0.1	96	72
trans-Decalin	0.01	0–0.02	0.01	0–0.02	‡	‡	‡	‡	‡	‡
cis-Decalin	0.07	0–0.025	—	0–0.01	‡	‡	‡	‡	‡	‡
n-C_{13}	0.16	0.06–0.30	0.08	0.03–0.12	0.07	0–0.15	0	0–0	37	56
br-C_{16}	0.45	0.18–0.71	0.26	0.11–0.42	0	0–0	0	0–0	27	41
n-C_{15}	0.23	0.11–0.40	0.13	0.04–0.27	0.94	0.06–2.3	0.17	0.1–0.29	71	55
n-C_{16}	0.02	0–0.04	0.02	0.01–0.04	0.11	0.02–0.23	0.05	0.02–0.10	42	50
n-C_{17}	0.24	0.13–0.40	0.14	0.04–0.27	1.1	0.10–2.7	0.18	0.12–0.31	90	73
Pristane	0.20	0.03–0.46	0.06	0.04–0.07	0.04	0–0.11	0.03	0.01–0.05	46	61
n-C_{18}	0.05	0–0.14	0.03	0.02–0.04	0	0–0.02	0.02	0–0.03	54	55
n-C_{19}	0.08	0–0.25	0.04	0.02–0.06	0.01	0–0.04	0.02	0–0.06	65	78

* Two brains were pooled for each determination.
** Based on four determinations.
*** Average of two determinations corrected for background.
‡ Interference.

therefore included. The use of one standard for the quantification of several compounds of different classes has been reported previously[10]. In this study relative response factors were highly reproducible (relative standard deviation, 3–7%). Use of internal standards in which the concentration of the standard approximately matched the concentration in the sample produced meaningful results.

It is evident that residues do accumulate in tissue (Tables IV, V, VI, VII), although large variations were observed among individuals in the same treatment group. As expected, highest residues of lipophilic compounds were found in fat. Lowest residues were found in the brain. Aromatic compounds were accumulated in concentrations of 0.05–0.38 ppm. Reisdue levels in kidney and liver were intermediate. Pristane, a branched aliphatic, accumulated in the highest concentration of any compound. Branched aliphatics also have been shown to selectively accumulate in marine organisms[11].

Methodology developed in this project will be applied to the analysis of petroleum hydrocarbons in avian tissue and to the assessment of the impact of petroleum pollution on avian species.

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