Comparison of Extraction Methods for Determination of Polybrominated Biphenyl Residues in Animal Tissue

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Liver and renal adipose tissue from male Fischer rats previously given Firemaster FF-1 daily each work week for 180 days, by gavage in corn oil [1 and 10 (mg/kg)/day] and in rat chow [averaging 1 (mg/kg)/day], were analyzed by GLC for the major component 2,4,5,2′,4′,5′-hexabromobiphenyl (HxBB). The results from two extraction methods, hexane for both tissues and chloroform:methanol, 2:1 (C:M), for liver and methylene chloride (CH_2Cl_2) for adipose were compared. Total tissue bromine determined by neutron activation analysis (NAA) was included as an independent method to monitor the extraction methods. Hexane extracted tissues gave lower lipid and HxBB recoveries than those from C:M–CH_2Cl_2 extraction. Lipid values were dose related being lower at the low doses than the high dose. The pattern was similar for HxBB but coefficient of variance values (CV) were about three times higher indicating poorer reproducibility for the hexane extraction method. The HxBB recoveries from C:M–CH_2Cl_2 extracts were reproducible and quantitative when compared with total bromine for all dose levels. Similarly liver to adipose ratios of the HxBB expressed on lipid weight basis gave comparable means and CV's for the various treatment groups. Hexane extraction of HxBB however showed wide CV when compared with total bromine.

As a result of the ubiquitous occurrence of halogenated organic compounds in the environment as pollutants, including the lipophilic polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs), numerous extraction and cleanup methods have been devised for monitoring these compounds in tissue and body fluids in animals and humans. Validation of the methods is often based upon two criteria, viz., corroborative evidence of reproducibility between laboratories and sample spiking recovery data. Concentration of the compound is expressed on the basis of the whole sample weight and/or the lipid weight basis.

Firemaster FF-1, a polybrominated biphenyl mixture, was inadvertently mixed into the feed of livestock (*1*) in the state of Michigan which caused extensive damage and environmental contamination. Hass et al. (*2*) have found Firemaster BP-6, which is the same as Firemaster FF-1 but without the 2% calcium trisilicate additive, to be composed of 4% penta-, 63% hexa-, and 33% heptabromobiphenyl. The 2,4,5,2′,4′5′-hexabromobiphenyl (HxBB) isomer was estimated to be present at 56% of the total. Penta- and hexabromonaphthalenes were also present but in the parts per million range.

Various methods (*3–4*), usually based on hexane or petroleum ether extraction have been reported for analysis of PBBs in environmental samples. This study was designed to provide definitive analytical residue data on tissues of animals from the 6-month toxicological study of FF-1 in rats (*5*). The rats were dosed at different levels and upon sacrifice gross pathology and histological abnormalities were evaluated. In the same study, liver and perirenal adipose tissue of selected animals were analyzed by gas chromatography with electron capture (GC/EC) detection for the presence of 2,4,5,2′,4′,5′-hexabromobiphenyl. For validation comparisons the tissues were extracted by two methods. One was a modified petroleum ether extraction (*6*) which had been collectively studied for a number of years and had been found to yield interlaboratory relative standard deviation values of 15% or better for chlorinated pesticidal compounds. The second method followed the chloroform:methanol procedure described by Albro and Corbett (*7*) which gave quantitative lipid recoveries.

Fries et al. (*8*) showed that a steady state was reached in 20 days for hexa- and heptabromobiphenyls in milk fat of cows fed daily doses of 10 mg of PBB for a total of 60 days. Assuming that a steady state was also attained in the rat study, neutron activation analysis (NAA) was chosen as an independent method to monitor the efficiency of the extraction methods as compared to each other and to total bromine levels determined by neutron activation analysis.

EXPERIMENTAL SECTION

Apparatus. Gas liquid chromatograph (GLC) used was a Tracor Model MT 220 with linearized pulsed ^{63}Ni electron capture (EC) detector. The GLC column dimensions were 1.8 m × 4 mm i.d. for the hexane extract analysis and 1.8 m × 2 mm i.d. for the chloroform:methanol–methylene chloride extract analysis. Both columns were U-shaped glass tubes. They were packed with 3% OV-101 on 100/120 mesh GasChrom Q, conditioned 72 h at 250 °C with a nitrogen flow rate of 50 mL/min (*9*). The operating temperatures for the hexane extracted samples were column temperature 240 °C, inlet temperature 275 °C, and detector temperature 300 °C. For the chloroform:methanol extracted samples, they were column 260 °C, inlet 275 °C, and detector 350 °C. The nitrogen gas flow in both cases was 100 mL/min.

Reagents. All solvents were either pesticide grade or distilled in glass (Burdick and Jackson, Muskegon, MI). Florisil 60/100 mesh, P.R. grade was stored at 130 °C until used (Flordin Co., Pittsburgh, PA). Sodium sulfate, anhydrous granular, was stored at 130 °C until used (Mallinckrodt No. 8024 or equivalent). The reference standard 2,4,5,2′,4′,5′-hexabromobiphenyl, 99.5% pure, was previously (*2*) purified from Firemaster BP-6 in our laboratory.

The hexane extract was cleaned up in a Chromaflex column (0.5–0.7 cm in diameter) plugged with a wad of clean glass wool (rinsed in hexane) supporting 3.2 g of Florisil and topped with a 2-cm layer of sodium sulfate. The chloroform:methanol extract cleanup column was packed with acidic alumina (aluminum oxide active, acidic activity I) and before use was activated at 130 °C overnight. The column was 0.5–0.7 cm in diameter and contained 3 g of alumina. Both solvent extracts were concentrated for GLC analysis in evaporative concentrator tubes of 10 or 25 mL sizes using a 4-mL capacity modified micro Snyder column (Kontes, Vineland, NJ).

Animal Feeding. Firemaster FF-1 (Firemaster BP-6 + 2% calcium trisilicate), Lot 1312 FT Batch 03 from Michigan Chemical Corp., St. Louis, MI, was given to Fischer 344N (CDF) male rats by gavage in 0.2 mL of corn oil, five times per week for a total of 125 doses (time period 180 days) at levels of 0.0 (given vehicle only), 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg of body weight calculated on the basis of the concentration of the brominated compound, i.e., adjusted for the silicate present. Each dose level was administered to 51 rats, housed as three animals per cage.

Another set of 51 rats were fed FF1 in the diet at a concentration equivalent to 1 mg of FF-1/kg of body weight (based on the average weight of the three caged animals). Rat chow was prepared as follows: 1 mg of FF-1/g of rat chow premix was prepared in a commercial food mixer for 1 h. The feed mix was

prepared in the same manner by mixing the premix with ground upspiked chow to obtain the desired concentration. The amount consumed was recorded once each week.

These were the same animals used for the toxicological study (*5*) and only a limited number could be made available for chemical study. At the end of the experiment randomly selected animals were sacrificed and liver and perirenal fat were removed for chemical analysis (GC/EC and neutron activation analysis). Tissues were stored frozen at −20 °C in screw cap scintillation vials. Tissues analyzed were taken from animals from control, 1 mg of FF-1/kg and 10 mg of FF-1/kg of body weight from the gavage fed groups and 1 mg of FF-1/kg of body weight from the diet fed group.

Tissue Preparation. *Spiking Procedure*: The pure hexabromobiphenyl isomer was dissolved in dimethyl sulfoxide and toluene for spiking liver and adipose using techniques previously described (*10*) for these sample types. Spike volume for each solvent was 100 μL/0.5 g of tissue. Control values (tissue and control) were subtracted from the spiked tissue values in reporting the results.

Hexane Extraction, Liver and Adipose: About 1 g of frozen tissue was transferred to a 250-mL steel beaker to which was added about 5 g acid washed and ignited sand (Mallinckrodt No. 7062 or equivalent) and about 5 g of anhydrous sodium sulfate. It was ground with a stainless steel stirring rod (8 in. × $^3/_8$ in.) and portions of sodium sulfate were added until a dry mass was produced. Twenty-five milliliters of hexane was added and warmed on a water bath at about 45 °C with stirring to dissolve the lipid and the solution was then filtered through Whatman No. 1 paper into a 100-mL volumetric flask by decantation. The extraction was repeated twice with 20-mL hexane portions. The beaker and paper were rinsed with hexane until the flask was nearly full. It was brought to the 100-mL mark and mixed well by inversion. A 20-mL aliquot was transferred to a tared 70 mm aluminum dish (A. H. Thomas Co., Philadelphia, PA) and evaporated just to dryness on a water bath and the remaining solid was stored under vacuum in a desiccator for 2 h and then weighed to determine the lipid content of the tissue.

Another 20-mL aliquot was concentrated to a small volume (approximately 1 mL) with a modified micro Snyder column. The glassware was rinsed with hexane and the volume was adjusted to 0.5 mL in a nitrogen stream. The entire 0.5-mL sample was loaded onto a Florisil chromatographic column. The sample container was twice rinsed onto the column with 0.3 mL of petroleum ether. When the solvent reached the top of the absorbent material, an additional 29 mL of petroleum ether was added. The eluate was collected and concentrated to 1 mL volume for GLC analysis.

Chloroform:Methanol Extraction: Liver. One-half gram of tissue was blended with 20 mL of chloroform:methanol (2:1, v/v) and filtered with suction through glass fiber (Reeve Angel 934AH). The cake was returned to the blender and reextracted with 20 mL of the solvent mixture. The filtrates were combined. Nine and one-half milliliters of 1.2% aqueous KCl solution was added to remove the methanol without producing an emulsion. The aqueous phase was discarded and the solvent evaporated just to dryness at 40 °C with a rotary evaporator.

Methylene Chloride Extraction: Adipose. One-half gram of tissue was ground with 4 g of anhydrous sodium sulfate in a glass mortar or its equivalent to remove excess water. The resulting powder was extracted with methylene chloride in a Soxhlet extractor allowing at least eight cycles or preferably overnight and then the extract was evaporated just to dryness at 40 °C in a rotary evaporator. Methylene chloride was substituted for chloroform because it is less toxic and has been shown in our hands to quantitatively extract lipid.

The lipid residue from the liver and adipose was leached into 15 mL of carbon tetrachloride. A 1-mL aliquot of the CCl_4 extract was transferred to a tared flask and evaporated just to dryness at 40 °C on a rotary evaporator to determine the lipid content of adipose tissue. Any condensed moisture was wiped from the outside of the flask, and the flask was stored under vacuum in a desiccator for 2 h before weighing. For liver tissue the entire 15 mL of the CCl_4 was transferred to a tared flask and treated as for the adipose tissue. After weighting 15 mL of CCl_4 was added again to the liver sample for further cleanup prior to analysis.

As a preliminary cleanup, the CCl_4 lipid extracts were partitioned against an equal volume of concentrated sulfuric acid overnight and the layers separated by centrifuging at 2000 rpm for 30 min. As much of the CCl_4 layer was removed as possible and the volume recovered was recorded. The CCl_4 solution was passed through anhydrous sodium sulfate:potassium carbonate (1:1, w/w) in a glass plugged funnel to remove any residual sulfuric acid, rinsing with 2 mL of fresh CCl_4. The filtrate was concentrated just to dryness on a rotary evaporator at 40 °C and the residue leached into 1.5 mL of hexane:methylene chloride (97:3, v/v). The solution containing the residue was loaded onto the dry packed acid alumina column. The flask was rinsed with 1 mL of the hexane:methylene chloride mixture and the rinsings were added to the column. The column was then eluted with 28 mL of the same solvent. The eluate was blown to nearly dryness with nitrogen at room temperature. The samples were taken up in 2,2,4-trimethylpentane for analysis with GLC.

Neutron Activation Analysis. Bromine ^{82}Br of 35.87 h half-life and major γ-rays of 554.3 and 776.6 keV were used for analysis. Samples were encapsulated in both high-purity polyethylene and quartz and the unextracted liver and adipose tissues were irradiated for 2 h in the the wet water cooled, rotating exposure ports of the nuclear reactor at a flux of 1.5×10^{13} n/(cm^2 s). After a 5-day delay all samples were counted for 400 s count on an ORTEC 24% GE (LI) detector coupled to a completely computerized Nuclear Data 6620 gamma spectroscopy system. No significant interferences were encountered at the bromine levels of interest. About 100 mg amounts of the purified hexabromobiphenyl reference isomer and Firemaster FF-1 were also analyzed for bromine content by NAA. The NAA reference standard was Baker certified reagent ammonium bromide.

RESULTS AND DISCUSSION

With respect to validation by spiking, Albro (*11*) points out that the endogenous compound may partition differently from that of the spiked (exogenous) sample, especially if the effect of the spiking solvent has not been considered. Choice of the spiking solvent has been primarily based on the solubility of the compound of interest and has ignored the effect of the solvent upon the distribution in the tissue. For example, choice of hexane for adipose tissue (lipophilic) is better than for tissue with high water content (hydrophilic) such as liver. In the latter case the solvent will not readily penetrate the tissue and may give recoveries equivalent to spiking of the extraction solvent(s) only.

In contrast, Mes and Campbell (*12*) examined the extractability of DDT, DDE, polychlorinated biphenyls, and phthalate esters from three human adipose tissues and found that the differences between apparent residue levels using nine different solvent systems were relatively small. Pesticide residue levels however did not correlate well when expressed on the lipid weight basis. Folch et al. (*13*) and Bligh and Dyer (*14*) developed chloroform–methanol systems that were essentially quantitative for lipid extraction. Albro and Corbett (*7*) used a similar system successfully for the quantitative recovery of PCB's, dibenzofurans, and dibenzodioxin and for the determination of lipid content in animal tissues and fluids.

Figure 1 shows a chromatogram of FF-1 in which eight peaks are distinguishable. Typical chromatograms of residues extracted from tissue of dosed animals match each of these peaks except that peaks A and C are missing. Peak D represents the 2,4,5,2′,4′,5′-hexabromo isomer. In addition another peak (U) common to both tissues is not evident in the original FF-1 mixture. The identity of this new peak is not known. These observations may have biological significance, but this cannot be determined until the materials are identified and their biological properties are investigated.

Table I compares NAA and GC/EC values for rat liver and perirenal adipose tissues which were spiked at 0.2 μg and 2.0 μg of 2,4,5,2′,4′,5′- hexabromobiphenyl/g of tissue. Percent lipid recovered by each method is presented in Table II. Table III shows the corresponding 2,4,5,2′,4′,5′-hexabromo-

Table I. Comparison of NAA and GC/EC of Rat Liver and Perirenal Adipose Tissues Spiked with 2,4,5,2′,4′,5′-Hexabromobiphenyl

Neutron Activation Analysis

type of sample	spike HxBB level, μg/g	amt of Br found, μg/g	equiv[a] HxBB found, μg/g	no. of replicates	% found	calcd % in sample	% found	% recovered
rat liver[b]	0.20	0.178	0.23	1	115			
rat adipose[b]	2.0	1.57	2.1	1	105			
HxBB[c]				3		76.1	72.0 ± 1.5	95
FF-1[d]				2			83.0 ± 0.3	
NH_4Br std[e]				3		81.6	81.6 ± 0.4	100

GC/EC $CHCl_3$:MeOH-CH_2Cl_2 Extraction Method

type of sample	spike HxBB level, μg/g	HxBB level found, μg/g	no. of replicates	% found
rat liver[b]	0.2	0.14 ± 0.02	3	70
rat adipose[b]	2.0	1.7 ± 0.2	3	80

[a] (Br found × 100)/[76.3(% Br in hexabromobiphenyl)] = equiv of HxBB found. [b] Rat tissues spiked with reference standard (2,4,5,2′,4′,5′-hexabromobiphenyl, 99.5% purity). [c] Reference standard 2,4,5,2′,4′,5′-hexabromobiphenyl, 99.5% purity. [d] Compound administered to animals. [e] Desiccated at 70 °C for 6 h.

Table II. Feeding of Firemaster FF-1, Comparison of Two Extraction Methods for Rat Liver and Renal Adipose Tissue—Percent Lipid Content

Liver

	control		1 mg/kg gavage		1 mg/kg diet		10 mg/kg gavage	
rat no.	HEX extn[a]	C:M extn[b]	HEX extn	C:M extn	HEX extn	C:M extn	HEX extn	C:M extn
1		6.3	2.2	12	1.9	10	4.0	14
2		4.6	2.0	14	1.7	10	3.5	15
3		6.4	2.3	11	1.9	10	4.3	14
4								15
5								15
mean		5.8	2.2	12	1.8	10	3.9	15
std dev		1.0	0.1	1.5	0.1	0	0.4	0.5
CV		17%	4.5%	12%	5.6%	0	10%	3%

Adipose

	control		1 mg/kg gavage		1 mg/kg diet		10 mg/kg gavage	
rat no.	HEX extn[a]	CH_2Cl_2 extn[c]	HEX extn	CH_2Cl_2 extn	HEX extn	CH_2Cl_2 extn	HEX extn	CH_2Cl_2 extn
1		84	26	76	38	70	70	88
2		94	47	80	49	95	49	79
3		98	27	73	53	90	46	75
mean		92	33	76	47	85	55	81
std dev		7	12	3.5	7.8	13	13	6.6
CV		8%	36%	5%	16%	15%	24%	8%

[a] HEX = hexane extraction. [b] C:M = chloroform:methanol extraction. [c] CH_2Cl_2 = methylene chloride extraction.

biphenyl (HxBB) determinations by GC/EC. Total bromine determined by neutron activation analysis in the intact tissue is given in Table IV.

The data in Table II show that the mean percent lipid extracted by hexane is consistently lower than for chloroform:methanol by a factor varying between 5 and 6 times for liver at the two low doses and about 3 times at the higher dose. Adipose lipid values were 1.5–2.0 times smaller than for the methylene chloride and were less dependent upon dose of FF-1 than the liver.

A similar pattern is evident in comparing the two extraction methods when measuring the hexabromobiphenyl content (Table III). Levels for liver are about 3 times lower at both low doses. In the 10 mg/kg group levels are about 1.5 times lower. For adipose, differences were about 2 times lower at the lower doses and relatively no difference was seen at the higher dose. In addition the coefficients of variation for the low doses are about 3 times higher for the hexane method than for the corresponding chloroform:methanol procedure, suggesting not only incomplete but less reproducible recoveries.

In Table IV two means were calculated for total bromine at the 10 mg/kg level. One mean (480 μg/g) includes all five values. The other mean (283 μg/g) excluded the two highest values. The ratios of total bromine to the HxBB for the excluded values at this dose level indicated some unidentified contamination of these two samples.

Three approaches to data analyses were examined to more adequately evaluate the data presented in Tables II–IV: (1) assuming the total bromine values to be "accurate" compare both solvent extraction methods with it; (2) check for linearity in dose levels; (3) compare correlation on lipid weight basis between liver and adipose.

Ratio of Total Bromine to 2,4,5,2′,4′,5′-Hexabromobiphenyl (HxBB). It was assumed that because Firemaster FF-1 had been fed daily that a steady state had been reached by the end of the experiment. Since total bromine by NAA

Table III. Feeding of Firemaster FF-1, Comparison of Two Extraction Methods for Rat Liver and Renal Adipose Tissue—2,4,5,2′,4′,5′-Hexachlorobiphenyl (HxBB) Content (μg/g)

Liver

	control		1 mg/kg gavage		1 mg/kg diet		10 mg/kg gavage	
rat no.	HEX extn,[a] μg/g	C:M extn,[b] μg/g	HEX extn, μg/g	C:M extn, μg/g	HEX extn, μg/g	C:M extn, μg/g	HEX extn, μg/g	C:M extn, μg/g
1	0.040	0.065	9.6	29	12	31	190	350
2	0.008	0.10	8.6	34	13	30	200	455
3	0.050	0.20	12.0	31	5.6	24	220	380
4	0.053		6.4				300	
5	0.070		8.5				280	
mean	0.044	0.13	9.0	31	10	28	238	395
std dev	0.022	0.08	2.0	2.5	4	4	49	54
CV	50%	62%	22%	8%	40%	14%	20%	14%

Adipose

	control		1 mg/kg gavage		1 mg/kg diet		10 mg/kg gavage	
rat no.	HEX extn, μg/g	CH_2Cl_2 extn,[c] μg/g	HEX extn, μg/g	CH_2Cl_2 extn, μg/g	HEX extn, μg/g	CH_2Cl_2 extn, μg/g	HEX extn, μg/g	CH_2Cl_2 extn, μg/g
1	0.78	0.85	110	210	53	240	2700	2206
2	0.52	0.60	210	200	130	295	2500	2650
3	0.49	0.50	100	250	150	255	2400	2650
4	0.95		120					
5			100					
mean	0.68	0.65	128	220	111	263	2533	2506
std dev	0.21	0.18	46	26	51	28	153	260
CV	31%	28%	36%	12%	46%	11%	6%	10%

[a] HEX = hexane extraction. [b] C:M = chloroform:methanol. [c] CH_2Cl_2 = chloroform:methanol.

Table IV. Feeding of Firemaster FF-1, Total Bromine (μg/g) in Rat Liver and Adipose Tissue[a]

	liver				adipose			
rat no.	control	1 mg/kg gavage	1 mg/kg diet	10 mg/kg gavage	control	1 mg/kg gavage	1 mg/kg diet	10 mg/kg gavage
1	4.28	23.3	28.3	281	5.16	180	282	2177
2	4.41	29.9	19.7	959	5.54	191	234	2026
3	5.03	18.8	36.3	598	3.63	220	254	2167
4	3.27	46.9		305	1.78	305		
5	2.97	19.7		264	3.20	124		
mean	3.99	27.7	28.1	481,[b] 283[c]	3.86	204	257	2123
std dev	0.85	11.6	8.3	300, 20	1.53	66	24	84
CV	21%	42%	30%	62%, 7%	40%	32%	9%	4%

[a] Values given are for total bromine after subtracting mean of control values. [b] Mean of five samples. [c] Mean of three samples, excluding the two highest values.

does not depend on extraction, the bromine method should reflect the "accurate" amount of bromine in the tissues. If either extraction method for GC is quantitative, the ratio of total bromine to the hexabromo isomer should approach some constant. Table V compares this ratio, and the values are calculated from the means shown in Tables III and IV. The chloroform:methanol–methylene chloride method shows a mean of 1.1 and 1.2 for liver and adipose respectively for the three dose treatments (when using the 283 μg/gm mean for total bromine at the 10 μg/g level). The hexane method, on the other hand, does not tend toward a constant between dose treatments for either tissue nor are the means for liver and adipose the same.

Dose Levels vs. Linearity. In Table VI means of high and low doses by gavage are compared for for both solvent methods and for total bromine. The table shows the ratio to be approximately 10:1 for total bromine in each tissue. Chloroform:methanol–methylene chloride ratios are slightly higher than 10:1 but agreement between liver and adipose is essentially the same. Hexane ratios however are askew with ratios of about 25:1 for liver and about 20:1 for adipose.

Correlation of Lipid Weight with 2,4,5,2′,4′,5′-Hexabromobiphenyl in Liver and Adipose. Since the bromobiphenyls show the same lipophilic tendencies as the chlorobiphenyls, an adequate extraction should extract quantitatively not only the bromobiphenyls but also the lipids. On the lipid basis the ratio of liver/adipose should approach some

Table V. Comparison of Total Bromine Mean/HxBB[a] Mean Ratios for Chloroform, Methanol-Methylene Chloride and Hexane Extraction Methods

	hexane extraction		$CHCl_3$-MeOH	CH_2Cl_2
dose	liver	adipose	liver	adipose
1 mg/kg gavage	4.0	2.1	1.2	1.2
1 mg/kg diet	3.6	3.0	1.3	1.3
10 mg/kg gavage	1.6,[b] 2.6[c]	1.1	0.9,[b] 1.6[c]	1.1
mean	3.1,[b] 3.4[c]	2.1	1.1,[b] 1.4[c]	1.2
std dev	1.3, 0.7	0.9	0.2, 0.2	0.1
CV	42%, 20%	43%	18%, 14%	8%

[a] HxBB = 2,4,5,2′,4′,5′-hexabromobiphenyl × 76.39% = equiv Br. [b] Based on 283 mean of total bromine. [c] Based on 481 mean of total bromine.

Table VI. Test for Linearity between High Dose and Low Dose

	Liver								
	total Br			$CHCl_3$-MeOH extn HxBB			hexane extn HxBB		
gavage	mean	std dev	CV	mean	std dev	CV	mean	std dev	CV
10 mg/kg	283[a]	20	7%	395	54	14%	238	49	20%
1 mg/kg	27.7	12	43%	31	2.5	8%	9.0	2.0	22%
10/1	10.2			12.7			26.4		
	Adipose								
	total Br			CH_2Cl_2 extn HxBB			hexane extn HxBB		
	mean	std dev	CV	mean	std dev	CV	mean	std dev	CV
10 mg/kg	2103	72	3%	2506	260	10%	2533	153	6%
1 mg/kg	204	66	32%	220	26	12%	128	46	36%
10/1	10.3			11.4			19.4		

[a] Based on three replicate mean.

Table VII. Liver/Adipose Ratio on a Lipid Basis for 2,4,5,2′,4′,5′-Hexabromobiphenyl

	hexane extract			$CHCl_3$-MeOH extract		
rat no.	1 mg/kg gavage	1 mg/kg diet	10 mg/kg gavage	1 mg/kg gavage	1 mg/kg diet	10 mg/kg gavage
1	1.03	4.55	1.23	0.88	0.90	1.00
2	0.96	2.89	1.12	0.97	0.97	0.90
3	1.41	1.04	0.98	0.82	0.85	0.77
mean	1.13	2.82	1.11	0.89	0.91	0.89
std dev	0.24	1.75	0.12	0.08	0.06	0.11
CV	21%	62%	11%	9%	7%	12%

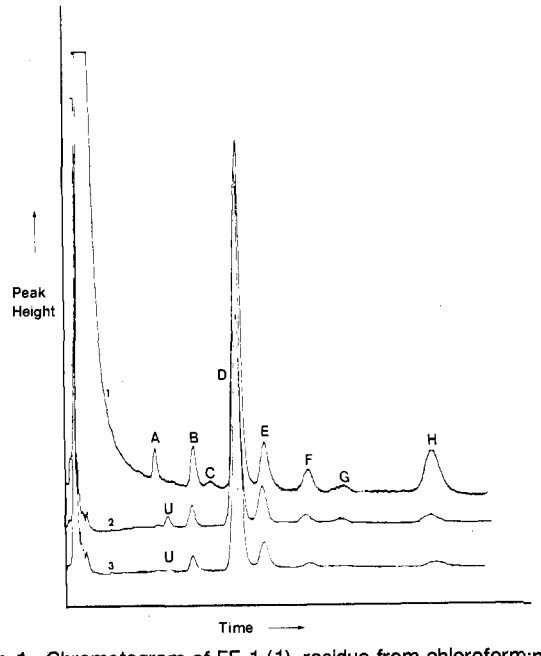

Figure 1. Chromatogram of FF-1 (1), residue from chloroform:methanol 2:1 extract of rat liver (2), and from methylene chloride extract of adipose (3). (This is a composite figure, redone and relabeled from original traces.)

constant. In Table VII ratios of liver/adipose are expressed on the lipid basis for each animal and the means for each dose calculated. Results for the hexane method do not correlate well as would be expected since lipid and bromobiphenyl extractions were not complete. The chloroform:methanol results however are close to each other regardless of dose treatment.

A small amount of the hexabromobiphenyl isomer was found to be present in control tissue of liver and adipose as characterized by GC chromatograms (Table III). These values however are at least 200–300 times smaller than the levels found for the animals dosed at the 1 mg/kg level. The control results therefore were not considered to be a significant interference which would affect the outcome of these validation experiments. For these same controls total bromine results (Table IV) are quite large and are of about equal concentration in both liver and adipose. This 1:1 ratio would not be expected for lipophilic compounds such as the PBBs for which the ratios are much greater than 1:1 (see Table II). Whether these bromine compounds are xenobiotic (exogenous) or natural (endogenous) in origin has yet to be determined.

The results obtained by NAA account for total bromine in the tissue samples. Since a steady-state concentration for HxBB has been assumed after 180 days, the total bromine (by NAA) should be related by a constant to HxBB content (by GC/EC) in these tissue samples. The deviation from a one to one relationship for total bromine and HxBB content (expressed as equivalent bromine) reflected in Table V is primarily a reflection of the fact that the total bromine number is a measure of all the brominated compounds present in the tissue whereas the HxBB is a measure of the major brominated biphenyl component of FF-1. The ratio is also affected to some extent by the yield of extraction in the HxBB determination from Table I.

In summary this work clearly demonstrates that the PBB determination method based on hexane extraction gives incomplete recoveries as well as erratic recoveries and results. While these problems appear to diminish at higher concentrations of PBB's in the tissues, they are most pronounced at lower concentrations which are usually found for residues in unknown environmental samples. The Michigan Department of Public Health Laboratories (*4*) routinely used a hexane extraction procedure for analysis of chlorinated pesticides in serum (*6*) but found recoveries for PBB in spiked samples to be invariably less than 50%.

Neutron activation analysis was used in the study as a measure of the "accuracy" of the PBB residue data from GC analysis. It is not recomended as an alternative procedure for PBB analysis even though there may be situations in which

its use as a screening method would be justified. As can be seen from the data in Table IV, this method suffers from some background interference from bromine containing materials in control tissues. The nature of this material is also of interest, but the identification of this material is beyond the scope of this work. However, for this reason the NAA method alone would not be useful for determining residue levels of PBBs in environmental samples, but at higher levels as used in this validation work it is a useful ancillary method. These findings further emphasize the need to design and develop tissue residue methodology with attention to answering the basic question of how accurate are the results in addition to how reproducible and reliable is the method. Interlaboratory studies may show good reproducibility but this does not necessarily mean the results are "accurate".

ACKNOWLEDGMENT

The experimental portion of this project concerned with polybrominated biphenyl analysis was performed by Analytical Bio Chemistry Laboratories, Inc., Columbia, MO, and that concerned with total bromine analysis by the Department of Nuclear Engineering, North Carolina State University, Raleigh, NC.

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RECEIVED for review December 10, 1981. Accepted June 11, 1982.

Characterization of Middle Petroleum Fractions by Nuclear Magnetic Resonance Spectrometry

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A method for characterization of petroleum fractions (220–500 °C) is presented. The structural characteristic and the paraffinic and aromatic hydrocarbon content are determined on the basis of the ^{1}H NMR spectral data and boiling range data. The method is tested on middle fractions (220–390 °C), as well as on a number of Yugoslav and imported samples with wider boiling range and higher boiling points (up to 500 °C). The aromatic carbon content was compared with the results obtained by the IR method. The aromatic and paraffinic hydrocarbon content was compared with the results obtained by ASTM D-1319-70 and D-2549-68. The standard deviation and the difference of the average for the results obtained by the NMR method were computed, as opposed to the results of the forementioned referent methods.

In the analysis of complex hydrocarbon compounds in petroleum with respect to the types of functional groups, the technique of NMR spectrometry has a very distinctive position, due to its specific characteristics. Numerous methods have been developed (*1–10*), based on the ^{1}H and ^{13}C NMR spectrometry data. Any analysis is significantly improved by application of ^{13}C NMR spectrometry, provided that all the conditions for obtaining quantitative results are satisfied (*11, 12*). However, the NMR technique is rarely applied to hydrocarbon-type analysis of petroleum. A successful method of gasoline analysis is discussed (*13*). Von Deutsch (*14*) has established relationships by which the aromatic and paraffinic hydrocarbons content, as well as the carbon content in functional groups, could be determined for petroleum fractions above 220 °C, using the ^{1}H NMR spectral data of the analyzed sample and of the aromatic fraction of examined petroleum. The obtained results were not compared in his paper with the results of any alternative method for verification purposes.

In this paper, the relations established by Von Deutsch are used as the starting point, and a method for determination of aromatic and paraffinic (*n*-, iso-, cyclo-) hydrocarbons content, as well as the carbon content in functional groups, is developed. This is based, on one hand, on the ^{1}H NMR spectral data and, on the other hand, on boiling-range value of the analyzed fraction.

The equations introduce also ring junction carbons, which are not derivable directly from the ^{1}H NMR spectrum. This follows from the assumed structure of aromatic hydrocarbons in petroleum, taking into account the hydrogen content in condensed aromatic rings. The assumption is made that the average structure of aromatic hydrocarbons in various petroleum types does not significantly differ within a given boiling-point range. Therefore, the established dependence between a boiling-point range and the corresponding α-alkyl to β,γ-alkyl ratio is used to determine the portion of aromatic alkyls in the section of spectrum, which is overlapped by corresponding paraffinic signals of the analyzed fraction.

The method is tested on middle fractions (220–390 °C) of Yugoslav petroleum with narrow boiling point range, as well as on a number of Yugoslav and imported samples with wider boiling range and higher boiling points (up to 500 °C). The aromatic and paraffinic content is compared with the results obtained by standard liquid chromatography methods (ASTM

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