DEVELOPMENT OF AN INSTRUMENTAL FRACTIONATION AND QUANTITATION SCHEME FOR SELECTED POLYNUCLEAR AROMATIC HYDROCARBONS PRESENT IN EXTRACTS FROM WOOD-PRESERVING WASTE

K. Washburn, H. Huebner, S. Safe, T. Phillips, and K.C. Donnelly[*]*, Texas A&M University, College Station, TX, 77843-4458, Phone: 409-845-7956, FAX: 409-847-8981*

ABSTRACT The polynuclear aromatic hydrocarbons (PNAs) are components of wood-preserving waste (WPW) which is known to contain multiple classes of organic and inorganic compounds. Identification and quantitation of individual PNAs and classes of PNAs is a preliminary step in assessing both the health risk associated with WPW as well as remediation efficacy. Ag^+ from $AgNO_3$ interacts with the conjugated π electrons of PNAs, allowing for a chromatographic separation based on differing degrees of conjugation. In this study, a high-volume, low-pressure (HVLP) chromatographic system comprised of a 0.5 meter, high-bore $AgNO_3$/silica-packed column (in-house) and a Waters 600 HPLC system was used in tandem with a tunable (200-800 nm) photodiode-array detector (Waters 996) to offer a comprehensive instrumental package capable of separating PNAs by degree of aromaticity and identifying and quantifying individual components. The system was used to separate and quantify 115 individual PNAs from a complex WPW-extract. Sub-fractions collected were rich in 2-ring, 3-ring, 4-ring, 5-ring, and >5-ring PNA components. Fractionated sample material may facilitate testing in a range of biological systems to determine potential interaction of PNAs by class and establish the contribution of each to the toxicity of the whole. Additionally, the data may allow for a more comprehensive risk assessment based on constituent PNAs. While the first separation was very tedious, later separations can be tailored to collect specific subfractions of interest.

This research was funded by NIEHS grant P42-ES04917.

KEYWORDS: polycyclic aromatic hydrocarbons, HPLC, separation, detection

INTRODUCTION

The exposure of human populations to toxic organic compounds occurs mainly in the form of complex chemical mixtures rather than individual chemicals [1]. As a result, many researchers today are faced with the challenge of accurately characterizing environmental samples in terms of both chemical content and, ultimately, toxicological effects. Chemical analysis alone is an extremely difficult undertaking considering all of the possible constituents of a given complex mixture. Assessing the risk associated with complex mixtures is likewise difficult due to the possible interactions of those constituents and the toxicological effects they may promote. Therefore, methods are needed that allow the fractionation of complex mixtures into distinct chemical classes.

Chemical fractionation studies utilizing *Salmonella* mutagenicity assays have shown that a few compounds in a complex mixture are responsible for the majority of the mutagenic activity induced by the whole mixture [2, 3]. More recently, bioassay-

[*]To whom all correspondence should be addressed (email: kdonnelly@vetmed.tamu.edu).

directed fractionation research [4] has demonstrated that much of the mutagenic activity induced by environmental samples can be attributed to particular classes of chemicals within each mixture. Such findings support the utility of chemical separation methods to divide mixtures of chemicals into less complex subfractions for further study. These separations allow for a more accurate characterization of mixture components and toxicological interactions.

In order to facilitate the identification of individual chemicals and provide a better analytical scenario for quantifying those chemicals of interest, various analytical methods have been developed to separate crude mixtures into less complex fractions. Previous studies on chemical separation procedures have included fractional distillation of coal liquids [5, 6], size exclusion chromatography [7, 8], solvent extraction [9, 10], adsorption chromatography with alumina and/or silica [11, 12], normal phase high performance liquid chromatography (HPLC) [13, 14], and reverse phase HPLC [15]. Analytical methods using tandem separation methods are often required for a more complete chemical characterization and subsequent biological testing of highly complex mixtures. Multidimensional coupled-column HPLC techniques to produce distinct fractions have been utilized in the analysis of PNAs from complex coal liquids [16, 17]. Other studies have demonstrated the efficacy of a multi-step methodology for separating solvent-refined coal material using fractional distillation followed by adsorption chromatography on neutral alumina with subsequent reverse phase HPLC [18]. More recently, nonaqueous ion-exchange separation techniques have been utilized for bioassay-directed fractionation of wood smoke particle extracts [19]. Nonaqueous anion-exchange solid phase extraction methods for the subsequent chemical and biological analysis of ambient air particulate extracts have also been used [20]. Current separation techniques offer much needed approaches to the separation, identification, and quantitation of individual components in complex mixtures. However, current approaches are based on costly and labor-intensive techniques which do not yield quantities of subfractions necessary for biological testing.

In this paper, the authors describe an integrated multi-step chromatographic method developed specifically for PNA analysis. This method was used to separate and quantify 115 individual PNAs from a complex wood-preserving waste (WPW) which is known to contain multiple classes of polycyclic aromatic compounds. Fractions were chemically much less complex than the starting crude material and were more definable in terms of chemical composition and quantity. The described method may be used for generating relatively large quantities of fractionated sample material which will facilitate testing in a range of biological systems.

PROCEDURES

All solvents used, including water, were HPLC grade (Fisher Scientific, Pittsburgh, PA) and used without further purification or analysis. The 115 toluene-solvated PNA standards were purchased from AccuStandards (New Haven, CT). Other standard chemicals used, including $AgNO_3$, silica, HNO_3, and NaOH, were purchased from Sigma Chemical Co. (St. Louis, MO).

WPW extraction and solvation

A 1.5 g sample of homogenized WPW was sequentially extracted with 100 ml of 0.1 N HNO_3 and 0.1 N NaOH to remove residual polar components. Water was used to

remove residual polar components as well as residual base. This neutral WPW fraction was allowed to settle, and excess water was decanted. The sample was then dried in a dessicator over $CaCl_2$ for 2 h. The resulting 1.3 g of dry neutral WPW residue was solvated in petroleum ether to a total volume of 5 ml.

Sorbent preparation

One 100 g preparation of silica/$AgNO_3$ (10:1) sorbent was prepared as described previously [21] and stored in amber glass under N_2 at 4ºC.

Chromatographic elution

A 0.5 m (1.5 cm id) glass column (Bio-Rad Laboratories, Melville, NY) was filled with 28.5 g of silica/$AgNO_3$ sorbent and uniformly packed by elution with petroleum ether. The resulting low-pressure chromatographic column was fitted with HPLC-compatible inlet and outlet fittings, jacketed with aluminum foil, and clamped into a vertical position. The 5 ml of solvated WPW was placed on column and the inlet was connected to a Waters 600 Controller (Bedford, MA); the outlet was connected to a Waters fraction collector. Petroleum ether was introduced at 2 ml/min and eluant was collected in 3 ml aliquots. After the addition of 150 ml of petroleum ether, 10% water was added to the solvent system to deactivate Ag^+-PNA complexes and allow for the elution of more aromatic PNAs. Each collected fraction was air-dried and solvated with toluene. Any residual water was removed, and an aliquot was collected for analysis.

Analysis

HPLC analysis was performed with a Waters 600 controller in series with a Waters 996 photodiode array detector. The column used was a 15 cm x 3.9 mm PNA (modified silica) column (Restek, Belefonte, PA). Automated sample injection was performed with a Millipore (Bedford, MA) WISP 710B autoinjection system with a 20 µl injection volume. The solvent system was 50% acetonitrile/50% water (2 ml/min) linearly ramped to 100% acetonitrile at 25 minutes with a 10 minute hold. Photodiode array scans were collected from 200-600 nm with 2 nm resolution. The target PNAs present in each aliquot were identified and quantified. A running total and temporal elution profile for each analyte was recorded. Eluant from the second and third replicates was collected as 2-ring, 3-ring, 4-ring, 5-ring, and >5-ring subfractions for future analysis and biological testing.

RESULTS

Sequential extraction of WPW with 0.1 N acid and 0.1 N base allowed for the removal of an average of 17.5% of the total mass of WPW as polar components. An elution profile for 115 target PNAs was established after the removal of the toluene baseline. The first 50 aliquots of petroleum ether-eluted components contained the 2-ring (represented by naphthalene, methylated naphthalenes, and biphenyls), 3-ring components (represented by anthracene, acenaphthalene, etc.) and 4-ring components (represented by chrysene). The 5-ring (represented by benzo(a)pyrene, benzo(e)pyrene, pentacene, etc.) and >5-ring (represented by dibenzanthracene congeners) did not elute until water was introduced to deactivate the sorbent-PNA complex. Total yield of target PNAs and substituted PNAs was 1.7% (m/m) with naphthalene being the most prevalent target PNA and picene being the least prevalent. Variance in elution for individual PNAs was very component- and concentration-dependent (Table 1).

DISCUSSION

The lewis acidity of Ag^+ which allows for separation of PNAs makes the sorbent susceptible to attack by all nucleophiles, even very weak ones. Extraction of crude WPW with acid and base, followed by aqueous washing, allowed for a less complex matrix and removal of polar components which could theoretically reduce chromatographic resolution by interacting with cationic Ag sites. PNAs are considered to be "benzenoid" compounds which are in fact built of benzene monomeric units. In the absence of strong nucleophiles, monovalent Ag from $AgNO_3$ interacts with the conjugated π system of benzenoid compounds binding in an additive fashion; thus, the phenanthrene (3-ring)-sorbent

TABLE 1. (CONTINUED ON NEXT PAGES) WPW FRACTIONATION.

Sample PNA	ppm (1)	ppm (2)	ppm (3)	Ave. (ppm)	s.d.	% Total PNA
Acenaphthene	312	289	290	297	13	1.73
Acenaphthylene	235	256	298	263	32	1.53
Anthanthrene	114	167	251	177	69	1.03
Anthracene	453	411	388	417	33	2.44
Azulene	112	134	98	115	18	0.67
Benz(a)anthracene	145	134	160	146	13	0.85
2,3-Benzanthracene	76	45	36	52	21	0.31
Benz(a)anthracene-7,12-dione	110	97	89	99	11	0.58
Benzo(b)chrysene	210	223	201	211	11	1.23
Benzo(b)fluoranthene	190	231	228	216	23	1.26
Benzo(j)fluoranthene	145	124	210	160	45	0.93
Benzo(k)fluoranthene	132	143	154	143	11	0.83
1,2-Benzofluorene	243	232	189	221	29	1.29
2,3-Benzofluorene	233	226	174	211	32	1.23
Benzo(g,h,i)perylene	103	120	153	125	25	0.73
Benzo(a)pyrene	264	235	270	256	19	1.50
Benzo(e)pyrene	243	246	233	241	7	1.40
2,3-benzofuran	322	267	289	293	28	1.71
5,6-Benzoquinoline	24	34	63	40	20	0.24
2,2'+A147-Binaphthyl	23	21	23	22	1	0.13
Biphenyl	34	56	32	41	13	0.24
Carbazole	150	126	132	136	12	0.79
Chrysene	226	235	231	231	5	1.35
Coronene	13	8	6	9	4	0.05
Dibenz(a,h)acridine	12	13	24	16	7	0.10
Dibenz(a,j)acridine	21	8	13	14	7	0.08
1,2:3,4-Dibenzanthracene	25	21	18	21	4	0.12
Dibenz(a,h)anthracene	31	24	41	32	9	0.19
7H-Dibenzo(c,g)carbazole	31	28	23	27	4	0.16
Dibenzo-p-dioxin	45	49	74	56	16	0.33
Dibenzofuran	231	215	190	212	21	1.24
Dibenzo(a,e)pyrene	267	259	271	266	6	1.55
Dibenzo(a,h)pyrene	258	259	241	253	10	1.47
Dibenzo(a,i)pyrene	264	237	278	260	21	1.52
Dibenzo(a,l)pyrene	132	167	139	146	19	0.85
Dibenzothiophene	12	26	17	18	7	0.11

TABLE 1. (CONTINUED FROM PREVIOUS AND ONTO NEXT PAGE) WPW FRACTIONATION.

Sample PNA	ppm (1)	ppm (2)	ppm (3)	Ave. (ppm)	s.d.	% Total PNA
1,2:8,9-dibenzpentacene	107	112	132	117	13	0.68
9,10-Dihydroanthracene	216	186	129	177	44	1.03
12,12A-Dihydro-3,9-Dimethylbenz[a]anthracene	31	20	10	20	11	0.12
Diindeno(1,2,3-cd-1',2',3'-1m)perylene	320	329	298	316	16	1.84
2,3-Dimethylanthracene	112	130	174	139	32	0.81
9,10-Dimethylanthracene	116	136	189	147	38	0.86
3,9-Dimethylbenz[a]anthracene	20	27	20	22	4	0.13
6,8-Dimethylbenz[a]anthracene	17	12	21	17	5	0.10
7,12-Dimethylbenz(a)anthracene	14	43	12	23	17	0.13
1,12-Dimethylbenzo[c]phenanthrene	162	143	118	141	22	0.82
5,8-Dimethylbenzo[c]phenanthrene	124	129	131	128	4	0.75
7,10-Dimethylbenzo[a]pyrene	218	216	229	221	7	1.29
1,2-Dimethylnaphthalene	218	209	222	216	7	1.26
1,3-Dimethylnaphthalene	261	223	180	221	41	1.29
1,4-Dimethylnaphthalene	243	274	215	244	30	1.42
1,5-Dimethylnaphthalene	240	319	200	253	61	1.48
1,6-Dimethylnaphthalene	310	314	287	304	15	1.77
1,8-Dimethylnaphthalene	276	225	274	258	29	1.51
2,6-Dimethylnaphthalene	221	239	248	236	14	1.38
3,6-Dimethylnaphthalene	265	269	265	266	2	1.55
Dodecahydrotriphenylene	19	4	23	15	10	0.09
Fluoranthene	340	289	296	308	28	1.80
Fluorene	189	176	190	185	8	1.08
Indan	125	114	132	124	9	0.72
Indene	32	45	41	39	7	0.23
Indeno(1,2,3-cd)pyrene	35	44	27	35	9	0.21
Indole	11	14	21	15	5	0.09
Isoquinoline	0	0	0	0	0	0.00
4-Methylchrysene	363	351	278	331	46	1.93
1-Methylanthracene	386	391	413	397	14	2.31
2-Methylanthracene	381	350	337	356	23	2.08
9-Methylanthracene	397	429	396	407	19	2.38
1-Methylbenz[a]anthracene	137	143	142	141	3	0.82
2-Methylbenz[a]anthracene	132	129	133	131	2	0.77
3-Methylbenz[a]anthracene	127	142	123	131	10	0.76
4-Methylbenz[a]anthracene	130	132	128	130	2	0.76
5-Methylbenz[a]anthracene	145	137	128	137	9	0.80
6-Methylbenz[a]anthracene	126	128	120	125	4	0.73
7-Methylbenz[a]anthracene	132	127	132	130	3	0.76
9-Methylbenz[a]anthracene	142	133	120	132	11	0.77
10-Methylbenz[a]anthracene	118	137	117	124	11	0.72
1-Methylbenzo[c]phenanthrene	43	38	17	33	14	0.19

interaction is 3/2 that of naphthalene (2-ring) sorbent. The bonding is a result of two events: first, the extended conjugation acts as an electron donor and transfers electrons to the unfilled metal orbitals; second, the resulting filled metal orbital interacts with the empty π-antiorbitals of the PNA. The linearity of this interaction can be observed by the order of elution. It is only when the interaction becomes so strong as to overcome elution by a non-polar solvent that deactivation of the sorbent becomes necessary. Addition of water released the Ag-PNA complex and allowed elution of the same. In fact, it may become necessary to use chloride anion to remove silver for the resulting organometallic complex.

TABLE 1. (CONTINUED FROM PREVIOUS PAGES) WPW FRACTIONATION.

Sample PNA	ppm (1)	ppm (2)	ppm (3)	Ave. (ppm)	s.d.	% Total PNA
2-Methylbenzo[c]phenanthrene	36	38	39	38	2	0.22
3-Methylbenzo[c]phenanthrene	46	27	33	35	10	0.21
4-Methylbenzo[c]phenanthrene	21	19	23	21	2	0.12
5-Methylbenzo[c]phenanthrene	33	41	20	31	11	0.18
7-Methylbenzo[a]pyrene	178	190	127	165	33	0.96
8-Methylbenzo[a]pyrene	196	222	210	209	13	1.22
9-Methylbenzo[a]pyrene	309	284	281	291	15	1.70
10-Methylbenzo[a]pyrene	253	308	299	287	30	1.67
3-Methylcholanthrene	37	20	29	29	9	0.17
6-Methylchrysene	389	229	420	346	103	2.02
2-Methylfluoranthene	231	242	229	234	7	1.37
1-Methylnaphthalene	451	442	476	456	18	2.66
2-Methylnaphthalene	378	452	409	413	37	2.41
9-Methyl-9-phenylfluorene	43	27	22	31	11	0.18
1-Methylphenanthrene	342	320	319	327	13	1.91
2-Methylphenanthrene	226	240	251	239	13	1.39
3-Methylphenanthro[3,4-C]Phenanthrene	12	61	40	38	25	0.22
1-Methylpyrene	165	172	182	173	9	1.01
4,5-Methylenephenanthrene	35	28	31	31	4	0.18
Naphthalene	643	540	673	619	70	3.61
Pentacene	23	14	19	19	5	0.11
Perylene	33	29	90	51	34	0.30
2-Phenylnaphthalene	32	114	142	96	57	0.56
Indeno(1,2,3-cd)pyrene	21	15	62	33	26	0.19
2-Phenylnaphthalene	118	123	121	121	3	0.70
Picene	0	1	7	3	4	0.02
Pyrene	229	208	241	226	17	1.32
Pyrrole	2	14	16	11	8	0.06
2,3:6,7-Tetraethylbiphenylene	2	3	8	4	3	0.03
1,2:3,4-Tetrahydrofluoranthene	12	16	4	11	6	0.06
Benz(a)anthracene	216	157	163	179	32	1.04
Thianaphthalene	0	0	0	0	0	0.00
4,6,8-Trimethylazulene	10	0	21	10	11	0.06
8,9,11-Trimethylbenz[a]anthracene	31	21	9	20	11	0.12
Triphenylene	9	1	0	3	5	0.02
Truxene	1	1	0	1	1	0.00

CONCLUSIONS

Separation and characterization of PNAs by degree of aromaticity from complex mixtures such as WPW in quantities large enough for subsequent chemical analyses and/or biological testing is highly desirable. This is the preliminary step in testing the role of individual PNAs and PNA classes to the overall toxicity of the complex mixture. The resulting data may aid in clarifying the role of interactions in toxicological studies as well as environmental risk assessment.

REFERENCES

1. National Research Council (NRC), Complex Mixtures, Methods for In-vivo Toxicity Testing, National Academy Press, Washington, DC, 1988.

2. D. Schuetzle and J. Lewtas, Bioassay-directed chemical analysis in environmental research, Anal. Chem., 58 (1986) 1060-1075.

3. R. Williams, C. Sparacino, B. Petersen, J. Bumgarner, H.R. Jungers, and J. Lewtas, Comparative characterization of organic emissions from diesel particles, coke oven mains, roofing tar vapors and cigarette smoke condensate, Intern. J. Environ. Anal. Chem., 26 (1986) 27-49.

4. D.M. DeMarini, M.L. Shelton, and D.A. Bell, Mutation spectra in salmonella of complex mixtures: Comparison of urban air to benzo(a)pyrene, Environ. Mol. Mutagen., 24 (1994) 262-275.

5. B.W. Wilson, R.A. Pelroy, and D.D. Mahlum, Chemical Characterization and Genotoxic Potential Related to Boiling Point for Fractionally Distilled SRC-I Coal Liquids, NTIS: Springfield, VA, PNL-4277, 1982.

6. R.A. Pelroy and B.W. Wilson, Fractional Distillation as a Strategy for Reducing the Genotoxic Potential of SRC-II Coal Liquids, NTIS: Springfield, VA, PNL-3787, 1981.

7. A.P. Toste, D.S. Sklarew, and R.A. Pelroy, Partition chromatography—high performance liquid chromatography facilitates the organic analysis and biotesting of synfuels, J. Chromatography, 249 (1982) 267-282.

8. T.K. Rao, B.E. Allen, D.W. Ramey, J.L. Epler, I.B. Rubin, R.M. Guerin, and B.R. Clark, Analytical and biological analyses of test materials from the synthetic fuel technologies, III. Use of Sephadex LH-20 gel chromatography technique for the bioassay of crude synthetic fuels, Mutation Research, 85 (1981) 29-39.

9. M.J. Mima, H. Schultz, and W.E. McKinstry, Method for the determination of benzene insolubles, asphaltenes and oils in coal derived liquids, In: C. Karr (Ed.), Analytical Methods for Coal and Gas Products, vol. 1, Academic Press, New York, 1978, p. 557.

10. R.J. Boduszynski, R.J. Hurtubise, and H.F. Silver, Separation of solvent-refined coal into compound-class fractions, Anal. Chem., 54 (1982) 375-381.

11. J.A. Leary, A.L. Lafluer, J.P. Longwell, W.A. Peters, E.L. Kruzel, and K. Biemann, Chemical characterization of domestic fuel oil and its combustion products, In: M. Cook and A.J. Dennis (Eds.), Polynuclear Aromatic Hydrocarbons: Formation, Metabolism, and Measurement, Batelle Press, Columbus, OH, 1983, pp. 799-808.

12. D.W. Later, M.L. Lee, K.D. Bartle, R.C. Kong, and D.L. Vassilaros, Chemical class separation and characterization of organic compounds in synthetic fuels, Anal. Chem., 53 (1981) 1612-1620.

13. B.A. Tomkins, W.H. Griest, J.E. Canton, and R.R. Reagan, Multicomponent isolation and analysis of polynuclear aromatics, In: M. Cook, A.J. Dennis, and G.L. Fisher (Eds.), Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry, Batelle Press, Columbus, OH, 1982, pp. 813-824.

14. A.P. Toste, D.S. Scalrew, and R.A. Pelroy, The role of partition chromatography/HPLC in the organic and mutagenic characterization of synfuels, In: C.W. Wright, W.C. Weimer, and D.W. Felix (Eds.), Advanced Techniques in Synthetic Fuels Analysis, NTIS, Springfield, VA, PNL-SA-11552, 1983, pp. 74-94.

15. W.A. Dark and W.H. McFadden, The role of HPLC and LC-MS in the separation and characterization of coal liquification products, J. Chromatogr. Sci., 16 (1978) 289-229.

16. K. Ogan and E. Katz, Analysis of complex samples by coupled-column chromatography, Anal. Chem., 54 (1982) 169-173.

17. W.J. Sonnefeld, W.H. Zoeller, W.E. May, and S.A. Wise, On-line multidimensional liquid chromatographic determination of polynuclear aromatic hydrocarbons in complex samples, Anal. Chem., 54 (1982) 723-727.

18. R.B. Lucke, D.W. Later, C.W. Wright, E.K. Chess, and W.C. Weimar, Integrated, multi-stage chromatographic method for the separation and identification of polycyclic aromatic hydrocarbons in complex coal liquids, Anal. Chem., 57 (1985) 633-639.

19. D.A. Bell, H. Karam, and R.M. Kamens, Nonaqueous ion-exchange separation technique for use in bioassay-directed fractionation of complex mixtures: Application to wood smoke particle extracts, Environ. Sci. Technol., 24 (1990) 1261-1264.

20. D.J. Thompson, L. Brooks, M.G. Nishioka, J. Lewtas, and R.B. Zweidinger, Bioassay and chemical analysis of ambient air particulate extracts fractionated by using nonaqueous anion-exchange solid phase extraction, Inter. J. Environ. Anal. Chem., 53 (1993) 321-335.

21. J.S. Warner, Determination of aliphatic and aromatic hydrocarbons in marine organisms, Anal. Chem., 48 (1976) 578-584.