Human Aryl Hydrocarbon Receptor Ligand Activity of 31 Non-substituted Polycyclic Aromatic Hydrocarbons as Soil Contaminants

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Toxic equivalency factors (TEFs) of 31 non-substituted polycyclic aromatic hydrocarbons (PAHs) for the human aryl hydrocarbon receptor (AhR) ligand activity were measured using the yeast recombinant reporter gene assay established by Miller *et al*., in order to estimate the toxic equivalents of individual PAHs in environmental samples. Ten PAHs showed sigmoid-shape concentration-response curves. An effective concentration to achieve 50% activity obtained with 1 μmol/l benzo[*a*]pyrene (BaP) (EC_{50BaP}) was calculated by curve fitting, and TEFs were calculated from the ratio of EC_{50BaP} for BaP to that for the chemical studied. The highest TEF was found for naphthacene (NPC) (TEF = 35) followed by benzo[*b*]fluorene (BbFl) (19), benzo[*k*]fluoranthene (BkF) (11), benz[*a*]anthracene (BaA) (7.0), benzo[*j*]fluoranthene (BjF) (4.0), benzo[*a*]fluorene (BaFl) (1.9), benzo[*b*]fluoranthene (BbF) (1.4), chrysene (CHR) (1.1), and BaP (1.0). Concentration dependent activities were also observed for indeno[1,2,3-*cd*]pyrene (IP) and fluoranthene (FLT). The TEFs of IP and FLT were about 0.2 and 0.02, respectively. The other 19 PAHs, naphthalene (NPT), acenaphtylene (ACL), acenaphthene (ACT), fluorene (FLU), anthracene (ANT), phenanthrene (PHN), pyrene (PYR), triphenylene (TRI), benzo[*e*]pyrene (BeP), perylene (PER), benzo[*ghi*]perylene (BgP), dibenz[*a*, *h*]anthracene (DahA), picene (PIC), coronene (COR), dibenzo[*a*, *e*]pyrene (DaeP), dibenzo[*a*, *h*]pyrene (DahP), dibenzo[*a*, *i*]pyrene (DaiP), dibenzo[*a*, *l*]pyrene (DalP) and naphtho[2,3-*a*]pyrene (NaP), showed little or no activity in the 0.1–1,000 nmol/l range. The toxic equivalents (TEQs) of surface soil were calculated by multiplication of the TEFs with the concentrations in soil samples collected from Kyoto, Japan. The TEQ of BaA showed the highest value of the 16 US Environmental Protection Agency Priority Pollutant PAHs.

Key words —— aryl hydrocarbon receptor, polycyclic aromatic hydrocarbon, soil, toxic equivalency factor, human, yeast

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are components of fossil fuel and are also produced by incomplete combustion of organic matter such as fossil fuel.[1, 2] PAHs are widespread environmental pollutants[3–5] and some are suspected to be human carcinogens.[6] Some PAHs have been reported to bind to the aryl hydrocarbon receptor (AhR)[7] and induce cytochrome P4501A1 (CYP1A1).[8] Moreover, the agonist activated AhR/AhR nuclear translocator (ARNT) heterodimer has been reported to associate with the estrogen receptor, and this association leads to activation of the transcription and estrogenic effects.[9] Therefore, some PAHs are suspected to disrupt the human endocrine system.

Many studies on the AhR relating activity of PAHs have been reported.[7, 8, 10, 11] However, most of these studies were on 16 PAHs, which have been listed as priority pollutants by the US Environmental Protection Agency. There have been few reports on other minor PAHs, such as naphthacene and benzo[*b*]fluorene (BbFl).

In this study, in order to estimate the toxic equivalent of individual PAHs in environmental samples, we measured the toxic equivalency factors

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(TEFs) of 31 non-substituted PAHs for human AhR ligand activity using the yeast recombinant reporter gene assay established by Miller *et al.*[12–14] Moreover, we calculated the toxic equivalents (TEQs) of surface soil by multiplication of the TEFs and concentrations in soil samples collected in Kyoto, Japan.

MATERIALS AND METHODS

Chemicals —— Naphthalene (NPT), acenaphthene (ACT), anthracene (ANT), naphthacene (NPC), fluoranthene (FLT) and pyrene (PYR) were purchased from Nacalai Tesque (Kyoto, Japan). Chrysene (CHR), triphenylene (TRI), benzo[*e*]pyrene (BeP) and benzo[*ghi*]perylene (BgP) were purchased from Sigma-Aldrich Co. (Milwaukee, WI, U.S.A.). Fluorene (FLU), phenanthrene (PHN), benzo[*j*]-fluoranthene (BjF), benzo[*b*] fluoranthene (BbF), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), perylene (PER), benzo[*a*]pyrene (BaP) and indeno[1,2,3-*cd*]pyrene (IP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acenaphthylene (ACL), benz[*a*]anthracene (BaA), dibenzo [*a*, *c*]anthracene (DacA), dibenzo-[*a*, *h*] anthracene (DahA), naphtho[2,3-*a*]pyrene (NaP) and coronene (COR) were purchased from Tokyo Kasei (Tokyo, Japan). Benzo[*a*]fluorene (BaFl), BbFl and picene (PIC) were purchased from AccStandard (New Heaven, CT, U.S.A.). Dibenzo[*a*, *e*]pyrene (DaeP), dibenzo[*a*, *h*] pyrene (DahP), dibenzo[*a*, *i*]pyrene (DaiP) and dibenzo[*a*, *l*]pyrene (DalP) were purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). All other chemicals were special grade and commercially available.

AhR Ligand Assay —— The yeast recombinant assay established by Miller *et al.*[12–14] was used in this study. The yeast strain YCM3 was grown overnight at 30°C in a shaking (120 min^{-1}) incubator in a synthetic glucose medium. The sample solution (vehicle, EtOH; 10 μl), overnight culture (50 μl) and synthetic galactose medium (950 μl) were mixed in a test tube and incubated at 30°C for 18 hr in the shaking incubator. The cell densities were determined by reading the absorbance at 600 nm. Culture (10 μl), Z-buffer containing 60 mmol/l Na_2HPO_4, 40 mmol/l NaH_2PO_4, 1 mmol/l $MgCl_2$, 10 mmol/l KCl, 2 mmol/l dithiothreitol, 0.2% salkosil (70 μl) and 4 mg/ml *o*-nitrophenol-*β*-D-galactopyranoside solution (20 μl) were mixed and incubated at room temperature for 1 hr. After incubation, the absorbance was measured at 420 nm. The transcriptional activity (%) was calculated by Eq. (1):

$$\text{Activity (\%)} = \frac{A_{SP} - A_{NC}}{A_{PC} - A_{NC}} \times 100 \quad (1)$$

where A_{SP}, corresponds to the absorbance intensity of the sample, and A_{NC} and A_{PC} correspond to the negative control and positive control, respectively. The positive control was 1 μmol/l BaP in EtOH, and the negative control was the liquid vehicle (EtOH).

Curve Fitting —— To describe the concentration-response curves by a simple mathematical equation, the sigmoid curve expression given by Eq. (2) was employed:

$$y = \frac{a}{1 + \left(\frac{x}{c}\right)^b} \quad (2)$$

where y is the activity (% of BaP), x is the concentration (μmol/l), a is the maximum intensity (% of E2), b is the slope factor and c is the medial effective concentration (EC_{50}, μmol/l). EC_{50} is the concentration eliciting 50% of the maximal response. The correlation coefficient (r) between the experimental data and the formulated curve were also calculated.

To evaluate the AhR ligand activity, the effective concentrations to achieve 50% of the activity obtained with 1 μmol/l BaP (EC_{50BaP}) and BaP TEF were calculated. EC_{50BaP} (μmol/l) was calculated with Eq. (3):

$$EC_{50B} = c \times \sqrt[b]{\frac{50}{a - 50}} \quad (3)$$

TEF is the ratio of EC_{50BaP} for BaP (0.019) to that for the chemical studied.

Sampling and Pretreatment of Soil —— Three surface soil samples were collected in Kyoto, Japan. Sample no. 1 was collected at an athletic field of Kyoto Pharmaceutical University on July 31, 2001, sample no. 2 was at roadside of residential area on April 10, 2002, and sample no. 3 was in a campus of Kyoto Pharmaceutical University on March 1, 2002. The soils were first dried at room temperature for one day, and then screened through a 60 mesh sieved for 10 min. Organic matter was extracted from the sieved soil sample with acetone using a Soxhlet extractor over one day. Weight of the obtained extracts were as follows: no. 1, 270 mg from 5500 g soil; no. 2, 3970 mg from 3200 g soil; no. 3, 820 mg from 350 g soil. The extracts were evaporated to dryness and the residue was dissolved in dimethylsulfoxide. Aliquots of the solutions were applied to the AhR ligand assay and PAH analysis.

Determination of PAHs —— An HPLC system, consisted of two Shimadzu (Kyoto, Japan) LC-10AS pumps, a Rheodyne (Cotati, CA, U.S.A.) 7125 sample injector, a Shimadzu CTO-10A$_{VP}$ column oven, a Shimadzu SPD-10A UV detector and a Jasco (Tokyo, Japan) FP-1520S fluorescence detector, was equipped with an analytical column (Wakosil-PAHs; 4.6 mm i.d. × 250 mm, Wako). Acetonitrile/methanol/water (10 : 72 : 18) was employed as the initial mobile phase for 0–5 min, and then the acetonitrile concentration was increased for 5–15 min. The final mobile phase was acetonitrile/methanol/water (75 : 20 : 5), which was used for 15–50 min. The flow rate of the mobile phase was 1 ml/min. The fluorescence detection wavelengths were 220 (excitation) and 325 (emission) nm for NPT, 220 and 315 nm for ACT and FLU, 244 and 400 nm for ANT, 244 and 360 nm for PHN, 286 and 433 nm for FLT, 331 and 392 nm for PYR, 284 and 385 nm for BaA, 264 and 362 nm for CHR, 300 and 428 nm for BbF, 384 and 406 nm for BkF, BaP and BgP, 292 and 440 nm for DahA and 294 and 482 nm for IP. ACL was detected with a UV detector at a wavelength of 254 nm. The temperature in the column oven was maintained at 30°C.

RESULTS AND DISCUSSION

The structures of the 31 PAHs tested in this study are shown in Fig. 1. The structures included a 2-ring, five 3-rings, eight 4-rings, nine 5-rings, seven 6-rings and a 7-ring PAHs. Twenty-one of the selected PAHs are alternate while the other ten are non-alternate.

Concentration-response curves of the 31 PAHs are shown in Fig. 2. Nineteen of the PAHs, PIC, DahA, DahP, DaiP, NaP, DaeP, DalP, PYR, BgP,

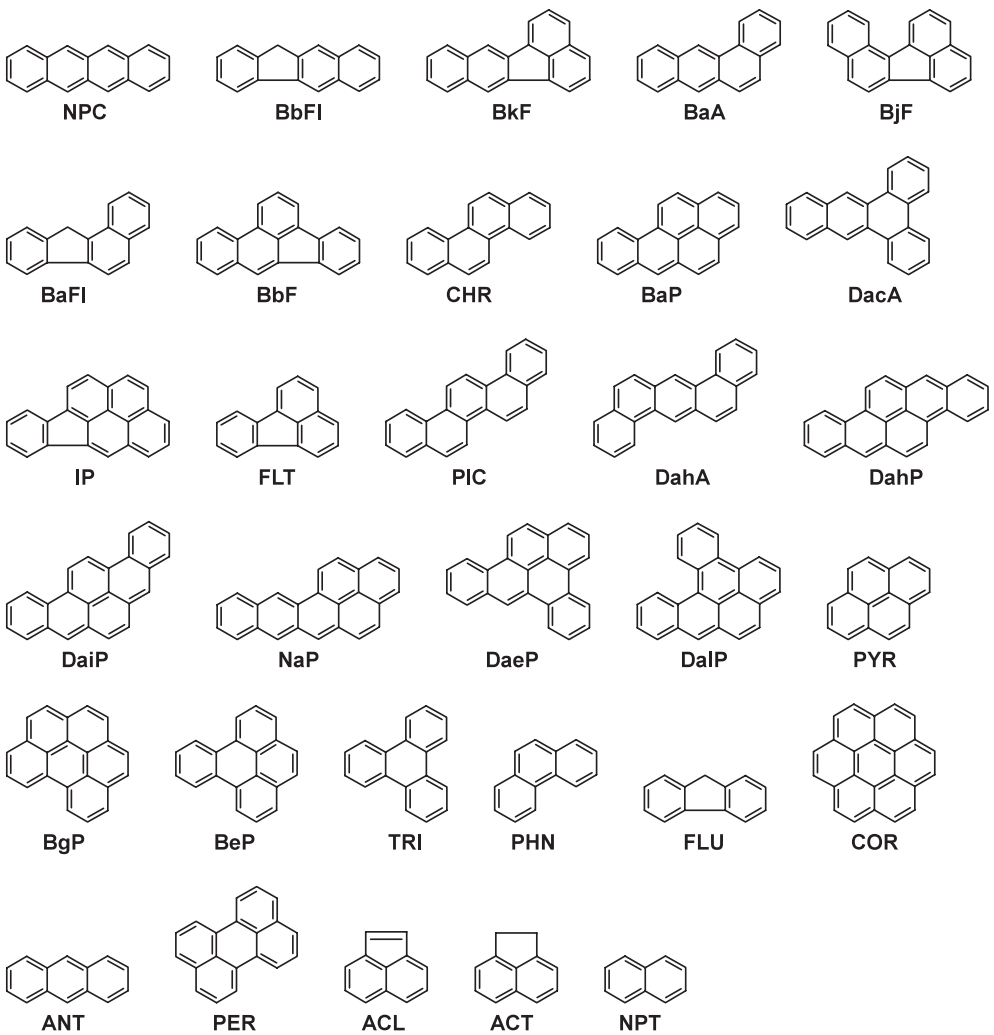

Fig. 1. Structures of PAHs Tested in This Study

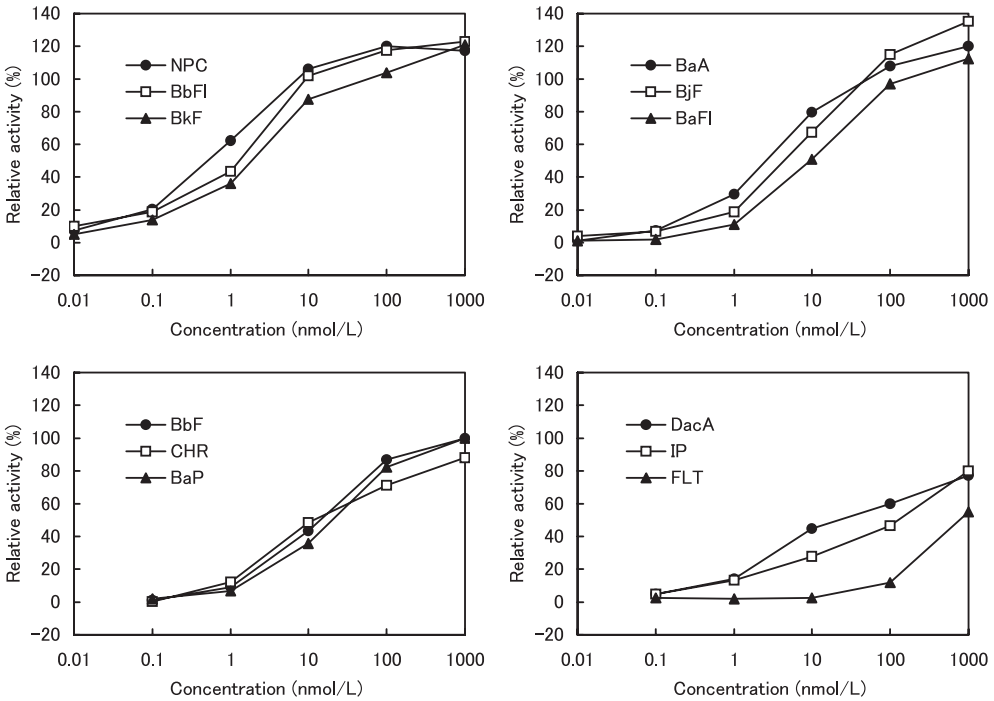

Fig. 2. Concentration-response Curves of Human Aryl Hydrocarbon Receptor Ligand Activity for PAHs
Relative activity (%) was an activity when an activity of 1000 nmol/l BaP was set as 100%.

Table 1. Parameter of Dose-response Curves and EC_{50BaP} for PAHs

PAHs	*a*	*b*	*c*	R^2	EC_{50BaP} *a)*
NPC	121	0.76	0.00087	0.9978	0.00055
BbFl	127	0.68	0.0019	0.9859	0.0010
BkF	121	0.65	0.0030	0.9929	0.0017
BaA	123	0.71	0.0047	0.9985	0.0028
BjF	140	0.71	0.011	0.9989	0.0048
BbF	102	0.89	0.014	0.9999	0.013
BaFl	116	0.84	0.014	0.9999	0.010
CHR	98	0.61	0.016	0.9896	0.017
BaP	104	0.85	0.021	0.9999	0.019
DacA	79	0.60	0.0089	0.9876	0.022
IP					≒ 0.1
FLT					≒ 1

a) μmol/l

BeP, TRI, PHN, FLU, COR, ANT, PER, ACL, ACT and NPT, showed little or no response over the whole concentration range. IP and FLT showed a dose dependent response, and their EC_{50BaP} values were about 1 and 0.1 μmol/l, respectively. The other 10 PAHs, NPC, BbFl, BkF, BaA, BjF, BaFl, BbF, CHR, BaP and DacA, all showed sigmoid shape concentration-response curves. The EC_{50BaP} values for the above ten PAHs studied were calculated by curve fitting. The parameters for the regression curves of the PAHs are listed in Table 1. Parameter "*a*" is the maximum response, ranging from 79 to 140%. Parameter "*b*" is slope factor, ranging from 0.60 to 0.89. Parameter "*c*" is the medial effective concentration (EC_{50}), ranging from 0.00087 to 0.021 μmol/l. The correlation coefficient between the experimental data and the formulated curves (r) ranged from 0.9859 to 0.9999. EC_{50BaP} was calculated with Eq. (3) using the above parameters, and the results listed in Table 1. The EC_{50BaP} values ranged from 0.022 μmol/l for DacA to 0.00055 μmol/l for NPC. TEF was calculated from the ratio of EC_{50BaP} for BaP with respect to that for the PAHs, and the results listed in Table 2. The highest TEF was found for NPC (TEF = 35) followed by BbFl (19), BkF (11), BaA (7.0), BjF (4.0), BaFl (1.9), BbF (1.4), CHR (1.1), BaP (1.0), DacA (0.87), IP (≒0.2) and FLT (≒0.02). The PAHs that showed the strongest AhR ligand activities are those comprising four or five rings, whose molecular weights ranged from 216 to 252.

Table 2 also lists the TEFs for the binding assays calculated from EC_{50} in Ref. 7, the TEFs for the reporter gene assay in Ref. 10, the TEFs for the ethoxyresorufin-*O*-deethylase (EROD) induction assay in rat hepatocytes calculated from EC_{50} in Ref. 8, and the TEFs in the rainbow trout liver cell line in Ref. 11. Twelve PAHs, FLT, PYR, BgP, BeP, TRI, PHN, FLU, ANT, PER, ACL, ACT and

Table 2. Comparison of TEFs for PAHs

PAHs	Binding	Reporter gene assay		EROD induction	
	Rat[a)]	Human[b)]	Rat[c)]	Rat[d)]	Trout[e)]
NPC	—	35	—	—	0.47
BbFl	—	19	—	—	0.29
BkF	—	11	67	9.6	3.4
BaA	—	7.0	0.39	0.10	0.14
BjF	—	4.0	2.2	—	—
BaFl	—	1.9	—	—	—
BbF	—	1.4	8.8	1.6	0.64
CHR	2.8	1.1	3.3	0.26	0.16
BaP	1.0	1.0	1.0	1.0	1.0
DacA	—	0.87	1.0	—	—
IP	—	0.2	44	2.7	0.93
FLT	—	0.02	0.01	0.00	n
PIC	8.0	n	0.12	—	—
DahA	—	n	11	5.4	1.2
DahP	—	n	2.8	—	—
DaiP	—	n	2.6	—	1.3
NaP	—	n	1.1	—	—
DaeP	—	n	0.49	—	—
DalP	—	n	0.02	—	—
PYR	0.00	n	0.01	n	n
BgP	0.00	n	0.01	0.00	—
BeP	—	n	0.00	—	0.1
TRI	—	n	—	—	0.03
PHN	0.00	n	—	n	n
FLU	—	n	n	n	n
COR	—	n	—	—	—
ANT	0.00	n	n	n	n
PER	0.00	n	—	—	n
ACL	—	n	—	n	n
ACT	—	n	—	0.00	n
NPT	—	n	—	n	n

a) TEF = BaP EC_{50}/PAH EC_{50}; based on cytosolic binding assay from Ref. 7. *b*) TEF = BaP EC_{50BaP}/PAH EC_{50BaP}; based on human aryl hydrocarbon receptor ligand assay in this study. *c*) TEF = BaP EC_{50}/PAH EC_{50}; based on reporter gene assay from Ref. 10. *d*) TEF = BaP EC_{50}/PAH EC_{50}; based on ethoxyresorufin-*O*-deethylase (EROD) induction assay in rat hepatocytes from Ref. 8. *e*) TEF = BaP EC_{50}/PAH EC_{50}; based on EROD induction assay in trout liver cells from Ref. 11. Dash (—), not tested. n, negative.

NPT, showed little or no activities in all five assays. BaA and CHR both showed strong activities in the two reporter gene assays, but only weak activities in the two EROD induction assays. BbF, BkF and BaP all showed strong activities in both the reporter gene assays and the EROD induction assays. In contrast to DacA, which showed strong activities in the two reporter gene assays, DahA showed very different results. DahA showed very weak activity in this study, but showed strong activities in the reporter gene assay using the rat liver cell line and the EROD induction assays using rat and rainbow trout liver cells. A similar result was shown in IP and DaiP.

In order to estimate the toxic equivalents of individual PAHs in the soil samples, we collected three soil samples from Kyoto, Japan, and determined 16 US Environmental Protection Agency priority pollutant PAHs in the soil samples.

Figure 3 shows the concentration–response curves of the soil samples. All samples showed sigmoid shape concentration–response curves. The maximum activities were 98–130% of the BaP activity, and EC_{50BaP} values were in the 0.5–1.3 mg/l range. The PAH concentrations determined by HPLC with fluorescence and UV detection are listed in Table 3. PAHs were detected in the range of 0.02–29 μmol/g of soil extract.

TEQs were calculated by multiplication of the

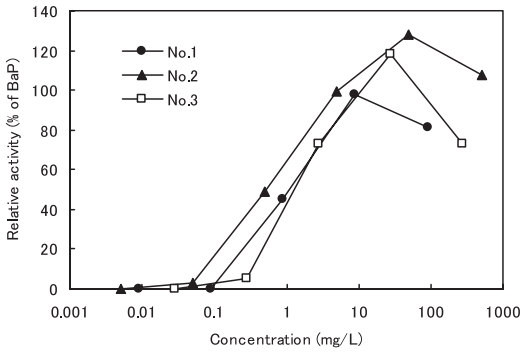

Fig. 3. Concentration-response Curves of Human Aryl Hydrocarbon Receptor Ligand Activity for the Soil Extract

Concentration, mg of extract/l of ethanol as vehicle. No. 1, at an athletic field of Kyoto Pharmaceutical University on July 31, 2001; No. 2, at roadside of residential area on April 10, 2002; No. 3, in a campus of Kyoto Pharmaceutical University on March 1, 2002.

Table 3. PAH Concentrations in Soil Extracts

PAHs	No.1	No.2	No.3
BkF	0.12	1.5	4.2
BaA	0.42	5.0	18
BbF	0.33	9.2	17
CHR	0.41	4.1	8.9
BaP	0.10	1.8	9.0
IP	0.22	4.8	11
FLT	1.3	29	29
DahA	0.04	0.30	1.1
PYR	1.0	8.7	18
BgP	0.33	2.8	6.4
PHN	1.6	19	8.9
FLU	0.08	8.3	N.D.
ANT	0.06	1.4	3.1
ACL	N.D.	N.D.	N.D.
ACT	0.04	N.D.	N.D.
NPT	0.02	10	N.D.

Unit, μmol/g of extract. N.D., not detected. No. 1, at an athletic field of Kyoto Pharmaceutical University on July 31, 2001; No. 2, at roadside of residential area on April 10, 2002; No. 3, in a campus of Kyoto Pharmaceutical University on March 1, 2002.

TEFs in this study listed in Table 2 and PAH concentrations in soil samples listed in Table 3, and TEQs were listed in Table 4. We used human's TEF because of estimation for human health. The TEQs were observed in the range of 0.03–130 μmol/g for the soil extracts. The TEQs of BaA were the highest of all samples studied, because of the higher TEF and higher concentration. CHR, BbF and BkF also showed high values. Although we did not determine values for the minor PAHs, we expect NPC, BbFl, BjF and BaFl are also likely to have high TEQs, because they have high TEFs in this study. Now, we are developing a new determination method for the minor PAHs using a comprehensive two-dimensional HPLC method, from which we will be able to determine the TEFs in surface soil and their TEQs.

Table 4. TEQs of PAHs in Soil Extracts

PAHs	No.1	No.2	No.3
BkF	1.3	17	46
BaA	2.9	35	130
BbF	0.46	13	24
CHR	0.45	4.5	9.8
BaP	0.10	1.8	9.0
IP	0.04	0.96	2.2
FLT	0.03	0.58	0.58
DahA	—	—	—
PYR	—	—	—
BgP	—	—	—
PHN	—	—	—
FLU	—	—	—
ANT	—	—	—
ACL	—	—	—
ACT	—	—	—
NPT	—	—	—
Sum	5.3	73	220

TEQ, PAH concentration (Table 3) × TEF in this study (Table 2). Unit, μmol/g of extract. No. 1, at an athletic field of Kyoto Pharmaceutical University on July 31, 2001; No. 2, at roadside of residential area on April 10, 2002; No. 3, in a campus of Kyoto Pharmaceutical University on March 1, 2002.

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