Supporting information

A comparison of batch mode and dynamic physiologically based bioaccessibility tests for PAHs in soil samples

Mark R Cave*, Joanna Wragg, Ian Harrison, Christopher H Vane

British Geological Survey, Keyworth, Nottingham, NG12 5GG, UK

Tom Van de Wiele, Eva De Groeve

LabMet, University of Ghent, Coupure Links 653, 9000 Ghent, Belgium

C.Paul Nathanail, Matthew Ashmore

University of Nottingham, School of Geography, Sir Clive Granger Building, The University of Nottingham, University Park, Nottingham, NG7 2RD, UK

Russell Thomas, Jamie Robinson

Parsons Brinckerhoff Queen Victoria House, Redland Hill, Bristol, BS6 6US, UK

Paddy Daly

National Grid Property, Warwick Technology Park, Warwick, CV34 6DA, UK

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Soil Analysis

Total Organic Carbon (TOC)

The TOC content of the soils was determined using an Elementar VarioMax C/N analyser. Samples were dried to constant weight at 40°C and treated with excess acid (HCl, 50% v/v) until all effervescing stopped and oven dried at 105-110°C (at least 1.5 hours). The TOC was then determined by high temperature combustion with subsequent analysis of evolved CO_2.

Total PAH Content

The prepared soils were analysed to determine the concentrations of the six PAHs using an extraction process routinely employed for sediments [1-5]. Thus, for each soil, a known weight (*ca.* 0.2 g) was placed in a 125 ml amber glass bottle (with a polytetrafluoroethylene (PTFE)-lined screw cap) and 100 ml of 1:1 acetonitrile/tetrahydrofuran (THF) was added. The bottle was capped, heated to 50°C and sonicated in an ultrasonic bath (300W) for 45 minutes with periodic inversions to continually re-suspend the soil. After cooling, a sample of the supernatant was taken and injected into an HPLC system using an in-line syringe filter (0.2 µm nylon Millex 13 mm) to remove particulates. The quality of the PAH data was verified by analysis of a well-characterised proficiency testing material (Laboratory of the Government Chemist (LGC), Contaminated Land Proficiency Testing Scheme (CONTEST) Soil 58.3c) which had comparable PAH concentrations to those in the soils. All solvents used for HPLC and SPE, including water, were of HPLC grade.

The HPLC conditions were as follows: Mobile phase (isocratic): 90% acetonitrile and 10% water; Flowrate: 1ml min^{-1}; Column configuration: Hypersil filter 0.2µm, Phenomenex Security guard C18, Hypersil PAH Guard (10 mm x 4 mm id), Hypersil PAH Analytical (100 mm x 4.6 mm id); Sample Loop: 5 µl Rheodyne; Detection: Waters 474 Scanning Fluorescence Detector – Excitation 296 nm / Emission 408 nm changing at 23.5 minutes to Excitation 302 nm / Emission 506 nm (for IP detection). The PAH and TOC data for the soils are presented in Table S1.

Table S1 Soil concentrations of the 6 PAHs of interest and their summed totals (mg kg^{-1}) and the total organic carbon content (%)

Soil	BaA	BbF	BkF	BaP	DBA	IP	Total	TOC
1	2.25	2.18	1.45	2.21	0.41	1.43	9.93	1.27
2	11.8	15.8	8.75	15.0	2.79	13.3	67.4	8.84
3	22.1	30.3	17.2	25.0	4.39	22.3	121.3	8.24
4	55.9	66.7	38.0	68.0	11.1	46.8	286.6	6.94
5	28.7	29.3	17.4	30.1	4.63	19.1	129.3	3.29
6	18.0	23.3	14.3	23.7	3.80	17.7	100.8	11.64
7	56.7	70.3	42.7	56.7	9.01	43.1	278.5	7.76
8	30.6	50.9	23.5	54.1	7.76	33.7	200.5	12.91
9	26.8	52.0	25.4	56.4	8.34	34.1	203.0	3.85
10	61.1	44.1	29.4	49.3	6.60	29.9	220.4	4.91
11	55.5	52.0	33.7	64.3	8.22	36.0	249.7	5.04

Operating conditions for the Dynamic bioaccessibility gastro-intestinal simulator system (SHIME system):

Since toddlers are the most sensitive target groups, the physiological conditions of the LabMET system were adjusted to simulate the conditions of a 6 to 24 months child. For fasted conditions the composition of the gastric solution was selected from Oomen et al. [6], except that the medium was not acidified. For the fed conditions nutrition (50.8 g L^{-1}), sunflower oil (3.2 ml L^{-1}) and milli Q water (150 mL L^{-1}) were added to the fasted gastric medium. In both the batch and the dynamic methods, the composition of the nutrition (Hipp organic creamy porridge®) was the same and representative for the diet of a small child. The composition of the pancreatic and bile fluid was applied as described by Van de Wiele et al.[7]. No increase in enzyme and bile concentration was made for fed conditions [8]. The temperature inside the vessels was controlled at 37 °C.

Because of the chemical characteristics of BaP (hydrophobic; logKOW = 6), specific adaptations in the design of the system were necessary [9]. For this reason all tubings in contact with the BaP containing media (sampling tubes), inert PTFE-tubing was used.

Incubations according to the SHIME system: The PAH contaminated soils were incubated in gastro-intestinal suspensions simulating the stomach and small intestine (and large intestine)

for fasted and fed conditions respectively. Soil samples were processed in triplicate through the reactor. Also negative and positive (spiked) controls were performed.

Soil and stomach solutions were brought into the vessel (L/S = 100 for fasted and 115 for fed). Acid (1 M HCl) was gradually added to the suspension over 30 minutes till the pH was 2.5 for fasted conditions and 3.5 for fed conditions. The fasted pH was slightly higher than the pH used in the SHIME model for adults [10]. The pH for fed conditions was slightly higher to average the postprandial peak.

After 1 hour imposing the stomach conditions, the intestinal digestion was started: a fixed volume pancreatic and bile fluid was gradually added over one hour which caused a pH rise to 7 - 8. After addition of the pancreatic fluid the suspension was incubated for a further 2 hours.

To simulate in one of the replicates the conditions of the large intestine i.e. the colon descendens, SHIME suspension was added to small intestinal fluid in a ratio 30/45. This suspension was anaerobically incubated overnight.

Subsequently, gastric and intestinal incubation samples were taken and centrifuged at 3000 g for 5 minutes to remove the non-bioaccessible particles. The supernatants were stored at -20 °C before analysis by the extraction method described in the Analysis of PAHs in the bioaccessibility extracts section.

Extraction and analysis of PAHs from the *in-vitro* Bioaccessibility Test

The extraction vessels used were specially designed for the Fed ORganic Estimation human Simulation Test (FOREhST) methodology: these were modified from heavy duty round bottomed centrifuge tubes capable of withstanding 3000 g, rubber coated and adapted to accept a 20 mm diameter PTFE-faced silicone rubber septa (3.25mm total thickness supplied by Wheaton, UK) with a 20 mm unlined centre tear off aluminium seal (supplied by Grace-Division, UK).

Prior to use extraction vessels were cleaned using the following protocol: Tap-water rinsed to remove residual sediment/solid deposits; aqueous methanolic surfactant solution (10% $^{v}/_{v}$ Teepol-L in 50:50 Methanol/Pure Water) washed, completely filled with the surfactant

solution and placed in an ultrasonic bath at 50°C for 15 minutes; left overnight containing the Teepol solution in order to remove and greasy deposits; rinsed several times with sufficient reverse osmosis (RO) water to remove all of the Teepol (surfactant) solution; inverted and drained for at least 15 minutes to remove excess RO water; rinsed 3 times, each with a 10.0 ml aliquot of HPLC grade Acetone to remove any organic contaminants from the glass surface; for removal of acetone, inverted to drain and dry for a minimum of 0.5 days.

The extractor used for the purposes of both end-over-end agitation at 30 rpm and incubation at 37°C has been previously described by Kelley et al [11].

A pH electrode specific for biological applications, including proteins and food samples, was chosen for use with the FOREhST methodology because of the inclusion of food in the fed state simulation and the increased enzymatic status of the system. A Thermo-Russell high-performance glass bodied K-series thin stemmed electrode (KDCMAW11/A), 120 mm in length and 4.50 mm in diameter was paired with a Russell RL060P meter for this specific application. This electrode was chosen because of the polymer gel double junction reference system, which results in a faster response, better reproducibility and stability in both sample and buffer solutions in complex mixtures found in food applications. In addition, the dimensions allowed for direct pH measurement of the solution in the extraction vessels employed.

The simulated GI extraction fluids used were prepared as described by Wragg et al 2009 [12]. In comparison to the standard fasted state UBM, the FOREhST method is a fed state system and eating foods stimulates physiological changes within the GI tract, such as increased secretions of gastric acid, bile and pancreatic fluids, changes in pH and modifications in gastric and intestinal motility (Versantvoort et al, 2004). The composition of the GI fluids, such as the salival fluid, is influenced by the flow rate of the GI fluid, and for the salival fluid production is stimulated by chewing. At higher salival flow rates the composition of this fluid has an increased amylase content, but decreased mucin concentration. For other GI fluids the increase in enzyme concentration ranges by a factor of 2-5 depending on the activity of specific enzyme producing organs. Pancreatin and lipase concentrations increase as these juices are secreted in order to start the digestion of the major food groups (Brunner et al, 1974) and when fats begin to enter the duodenum, bile secretion is increased by the liver. All of the , additional modifications to the pH and the enzymatic concentrations in the individual

extraction fluids, in order to simulate the fed state, were based on the work of Versantvoort et al [13]. The pH of all gastro-intestinal fluids were increased compared to those used in the BARGE UBM: the 'Saliva' pH was increased from 6.5 ± 0.5 to 6.8 ± 0.5; the 'Gastric' fluid from 0.9-1.0 to 1.3 ± 0.5; the 'Duodenal' fluid from 7.4 ± 0.2 to 8.1 ± 0.2 and the 'Bile' fluid from 8.0 ± 0.2 to 8.2 ± 0.2. The pH adjustments were made by increasing the initial addition of 1.0M NaOH (2.80 ml) or reducing the amount of 37% HCl (6.5 ml) to the saliva or gastric fluids respectively or making minor adjustments to the pH of the prepared duodenal and bile solutions by the drop wise addition of 1.0M NaOH. The 'Saliva' fluid contained double the amount amylase compared to the UBM methodology, conversely the mucin concentration was reduced by half in the FOREhST (fed state) methodology. In comparison to the UBM 'Gastric' fluid, the concentration of both mucin and pepsin were increased by a factor of 3 and 2.5 respectively, requiring the addition of 9000 mg l^{-1} mucin and 2500 mg l^{-1} pepsin. Increases were also made to the concentrations of pancreatin and lipase (by a factor of 3) in the 'Duodenal' fluid and the bile concentration in the 'Bile' fluid (by a factor of 5).

All reagents used in the FOREhST extraction were of analytical grade or better. Each individual extraction fluid (Saliva, Gastric, Duodenal and Bile) was a composite of 500 ml each of an 'inorganic' and an 'organic' fluid, which were then combined with additional constituents (enzymes etc) in 1000 ml glass Duran® bottles with polypropylene lids with (PTFE) liners to form the final extraction solutions. All extraction fluids were prepared the day prior to soil extraction, to ensure that all of the additional constituents had dissolved, and stored at <8°C overnight. Prior to undertaking the FOREhST extraction, the pH of each extraction solution was adjusted with drop wise addition of 1.0M NaOH or 37% HCl to ensure that it was within the required tolerance (see above). Additionally, the pH of the 'Saliva & Gastric' fluids (1.6±0.2) and the 'Saliva & Gastric & Duodenal & Bile' (6.0±0.2) fluids at the ratio of 1:2 and 1:2:2:1 respectively were checked before carrying out the extraction. If the pH criteria of the mixed extraction fluids were not met, then the individual pH of one or more extraction fluids were adjusted (whilst remaining within the required tolerances) to meet the necessary requirements.

The test samples were extracted in batches of five unknown samples with one extraction blank. Each sample, including sample blanks, was extracted in triplicate, with the extraction carried out on separate days in order to estimate the repeatability of the extraction phase. As

there are no reference materials currently available for the bioaccessibility of PAHs, no reference materials were extracted in the current work.

For the FOREhST method 0.3g of each prepared soil and 0.813g of HIPP organic creamy porridge were accurately weighed into an extraction vessel (as described above). 2.45 ml of de-ionised water, 50 µl of sunflower oil and 4.50 ml of 'Saliva' fluid were added via pipette. The samples were then capped with PTFE-faced silicone rubber septa in an aluminium seal and rotated in a water bath at 37°C at 30 rpm for 5 minutes to ensure that the soil, food constituents and simulated fluid had thoroughly mixed. On removal from the extractor, 9.00 ml of 'Gastric' fluid was added, the extraction vessels re-capped and replaced in the extractor and incubated for a further 2 hours to represent simulation of the 'stomach' phase. After the 2 hour stomach phase extraction, the extraction vessels were removed from the extractor and the pH of each individual suspension measured. The mean stomach pH, measured across all extracted soils in triplicate was 4.15 ± 1.08 (1 sd) and 3.93 ± 1.14 including the measurement of the extraction blanks. By pipette, 9.00 ml of 'Duodenal' fluid and 4.50 ml of 'Bile' fluid were added to each extraction vessel and the pH of each suspension measured. The mean intestinal phase pH, measured across all soil samples in triplicate was 6.20 ± 0.59 and 6.16 ± 0.55 when the blanks measurements were included. After re-capping each vessel was further incubated for 2 hours, followed by rechecking of the 'intestinal' phase pH. The final average 'intestinal' phase pH was 6.24 ± 0.46 and 6.23 ± 0.43 on the inclusion of the blank measurements. The soil samples were then separated from the chime by centrifugation at 3000 g for 5 minutes.

Analysis of PAHs in the bioaccessibility extracts

Following FOREhST extraction, the PAHs leached from the soils were contained in a matrix that included food constituents (e.g. lipids, proteins, carbohydrates etc.), natural organic matter (e.g. humic acids, humin etc.) colloidal soil particles (e.g. clays). In order to release the PAHs from the various components of the FOREhST matrix saponification was employed, this process being generally regarded as a rigorous means of extracting PAHs into solution from otherwise intractable matrices e.g. Soils -[14-17] : Biota - [18]: Sewage sludges – [19]: Food substances – [20, 21]. For each soil, after the FOREhST extract had been centrifuged (5 minutes at 3000 g) a 1 ml sample of the supernatant was transferred into a Hach chemical oxygen demand (COD) vial by means of a glass pipette. The pipette was

rinsed into the tube with 1 ml methanol to ensure complete transfer of the PAHs. Then 3 ml of saturated potassium hydroxide in methanol (ca. 5.6 M KOH –analytical reagent grade) were added and the screw cap tightened firmly onto the vial before placing in an oven at 100°C for one hour. The resulting alkaline amber fluid was processed by SPE after cooling. It was found that once the methanolic KOH had been added the PAHs in the FOREhST extracts remained stable (for about a week), whereas, if left in the FOREhST matrix or in their final state after SPE, i.e. as an acetonitrile solution they were not stable even for relatively short periods; BaP appeared to be particularly susceptible to losses.

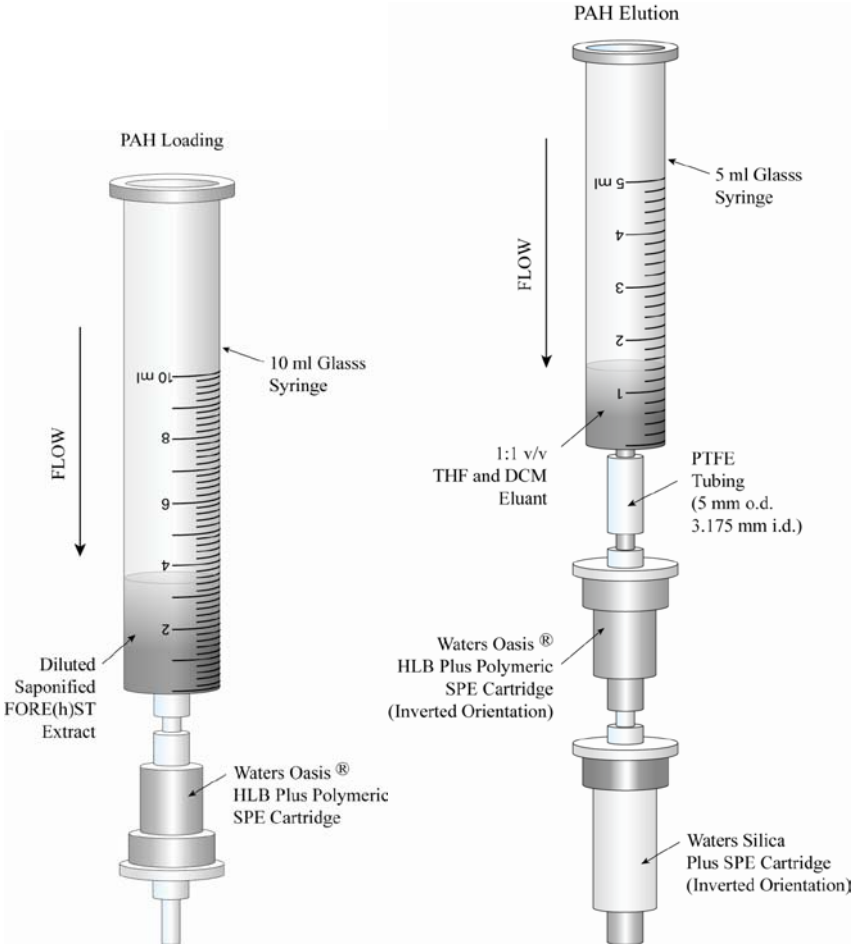

Figure S1 Experimental set up for i) the for separation and preconcentration of the PAH from the saponified extract; ii) for elution of the separated and preconcentrated PAHs

To the 4 ml of cooled methanolic saponified FOREhST extract, water was added to give 10 ml of diluted extract. The diluted extract (ca. 40% aqueous methanol) was transferred to a 10 ml glass syringe barrel inserted into a conditioned polymeric sorbent SPE cartridge (Waters OASIS HLB Plus Sep-Pak®) and the Hach COD vial rinsed in with 1 ml 40% aqueous

methanol (Figure S1 i). The cartridge had been conditioned immediately prior to use by the consecutive passage of 5 ml dichloromethane (DCM), 5 ml methanol and 2 x 5ml water. By means of vacuum, the diluted extract was drawn through the cartridge at 1-2 ml min^{-1}. Once through, the cartridge was washed with 5 x 2 ml of pure water and then allowed to dry by drawing air through for 10 minutes under maximum vacuum. The dried cartridge was connected to a silica sorbent cartridge (Waters Plus Silica Sep-Pak®) such that the flow through the polymeric cartridge was reversed compared with sample loading, i.e. it was backflushed by the eluting solvent into the silica cartridge [16]. After initiating flow with a slight positive pressure the eluting solvent (1:1 DCM/THF) was allowed to flow through the coupled cartridges under gravity and the eluate was collected in a 15 ml amber vial (Figure S1 ii). Following the collection of 6 ml of eluate the contents of the vial were blown-down under a gentle stream of nitrogen to dryness. The residue was taken up in a known volume of acetonitrile. The volume employed, chosen to ensure on-scale chromatographic peaks, was based on the concentrations of PAHs in the soils previously determined by HPLC. Thus, suitable volumes of acetonitrile were found to be 1 ml for soil 1, 2 ml for soils 2, 3, 5, 6 and 5 ml for soils 4, 7, 8, 9, 10 and 11. The same isocratic method was utilised for the analysis of the acetonitrile solutions of the FOREhST extracts. The acetonitrile solutions were analysed using the same HPLC method as used for the soils. The method is equally applicable for use when subsequent analysis by GC/MS, rather than HPLC, is to be undertaken. The residue resulting from the nitrogen blow-down step merely needs to be dissolved in a GC/MS compatible solvent (e.g. hexane) instead of acetonitrile.

Comparison of the SHIME and FOREhST bioaccessibility results

Table S2 gives the BAF of the total PAH in the soil for each soil and each PAH as measured by the FOREhST and SHIME methods.

Table S2 Average values (n=3) for the bioavailable fraction of the totalPAHs as measured by the two methods.

			FOREhST		SHIME	
Soil no	PAH name	**MW**	BAF (%)	RSD (%)	BAF (%)	RSD (%)
1	Benz(a)-anthracene	228	39.0	6.2	25.3	15.9
1	Benzo(b)-fluoranthene	252	32.2	8.5	19.9	9.2
1	Benzo(k)-fluoranthene	252	23.1	5.7	14.6	12.1
1	Benzo(a)-pyrene	252	33.1	5.9	18.2	21.8
1	Dibenz(ah)-anthracene	278	17.3	16.0	12.8	12.1
1	Indeno(123cd)-pyrene	276	35.6	15.5	18.6	8.9
2	Benz(a)-anthracene	228	39.2	4.0	53.0	3.6
2	Benzo(b)-fluoranthene	252	41.5	4.8	49.5	3.3
2	Benzo(k)-fluoranthene	252	30.2	1.9	34.7	1.9
2	Benzo(a)-pyrene	252	46.8	0.8	51.6	1.9
2	Dibenz(ah)-anthracene	278	26.3	2.1	30.6	0.2
2	Indeno(123cd)-pyrene	276	53.3	3.6	58.7	2.8
3	Benz(a)-anthracene	228	30.6	9.8	44.2	14.1
3	Benzo(b)-fluoranthene	252	33.9	11.3	41.5	11.8
3	Benzo(k)-fluoranthene	252	22.8	5.4	27.7	12.5
3	Benzo(a)-pyrene	252	34.5	7.0	39.9	11.1
3	Dibenz(ah)-anthracene	278	22.3	8.8	24.4	12.5
3	Indeno(123cd)-pyrene	276	41.2	5.4	45.5	11.5
4	Benz(a)-anthracene	228	41.2	4.9	35.1	13.6
4	Benzo(b)-fluoranthene	252	33.9	7.4	28.1	15.9
4	Benzo(k)-fluoranthene	252	26.2	5.5	20.9	17.8
4	Benzo(a)-pyrene	252	35.6	8.8	25.5	13.4
4	Dibenz(ah)-anthracene	278	21.4	2.4	20.4	12.5
4	Indeno(123cd)-pyrene	276	37.9	4.4	29.5	11.9
5	Benz(a)-anthracene	228	53.4	8.7	44.7	2.4
5	Benzo(b)-fluoranthene	252	43.0	4.3	33.6	4.9
5	Benzo(k)-fluoranthene	252	30.3	4.6	26.5	4.4
5	Benzo(a)-pyrene	252	36.2	5.2	32.3	3.9
5	Dibenz(ah)-anthracene	278	19.8	7.3	20.0	3.7
5	Indeno(123cd)-pyrene	276	42.6	3.4	36.0	6.6
6	Benz(a)-anthracene	228	55.6	6.5	43.0	15.9
6	Benzo(b)-fluoranthene	252	46.5	3.5	38.3	14.7
6	Benzo(k)-fluoranthene	252	34.3	5.4	27.8	15.9
6	Benzo(a)-pyrene	252	42.2	5.9	31.1	17.5
6	Dibenz(ah)-anthracene	278	28.3	9.1	23.6	13.8
6	Indeno(123cd)-pyrene	276	52.0	6.0	42.7	14.5
7	Benz(a)-anthracene	228	38.8	2.3	37.1	5.4
7	Benzo(b)-fluoranthene	252	34.7	2.2	33.5	4.4
7	Benzo(k)-fluoranthene	252	26.7	2.1	25.4	4.7
7	Benzo(a)-pyrene	252	20.7	3.5	17.8	7.1
7	Dibenz(ah)-anthracene	278	16.5	7.5	17.3	6.4
7	Indeno(123cd)-pyrene	276	33.4	1.3	30.3	7.9
8	Benz(a)-anthracene	228	32.8	9.8	35.5	11.6
8	Benzo(b)-fluoranthene	252	30.0	18.7	28.7	16.8
8	Benzo(k)-fluoranthene	252	19.1	9.5	20.1	12.7
8	Benzo(a)-pyrene	252	25.6	8.2	24.8	13.0
8	Dibenz(ah)-anthracene	278	12.1	12.5	14.5	11.4
8	Indeno(123cd)-pyrene	276	29.6	7.1	27.8	11.5
9	Benz(a)-anthracene	228	32.2	28.5	27.5	4.7
9	Benzo(b)-fluoranthene	252	30.9	28.2	22.3	5.3
9	Benzo(k)-fluoranthene	252	21.2	28.6	16.9	6.0
9	Benzo(a)-pyrene	252	25.6	31.3	19.1	3.0
9	Dibenz(ah)-anthracene	278	13.1	32.7	13.1	14.6
9	Indeno(123cd)-pyrene	276	31.3	31.3	24.0	6.3
10	Benz(a)-anthracene	228	60.9	13.3	53.7	26.3
10	Benzo(b)-fluoranthene	252	54.5	16.8	45.5	24.1
10	Benzo(k)-fluoranthene	252	37.4	10.7	32.4	25.3
10	Benzo(a)-pyrene	252	46.1	12.2	38.8	23.9
10	Dibenz(ah)-anthracene	278	29.2	10.2	25.5	19.4
10	Indeno(123cd)-pyrene	276	55.2	9.7	48.9	23.9
11	Benz(a)-anthracene	228	60.4	10.3	33.4	7.0
11	Benzo(b)-fluoranthene	252	53.1	13.8	29.4	5.4
11	Benzo(k)-fluoranthene	252	38.3	14.5	21.3	7.8
11	Benzo(a)-pyrene	252	50.4	15.9	27.0	6.8
11	Dibenz(ah)-anthracene	278	29.9	20.9	17.5	7.0
11	Indeno(123cd)-pyrene	276	59.3	16.8	32.9	5.5

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