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Development of an Immunoassay for the Determination of Polyaromatic Hydrocarbons in Plasma Samples from Oiled Seabirds

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External exposure assessment of oiled seabirds is undertaken by assessment of the percentage oil coverage of the plumage. Nondestructive monitoring of the toxic fraction of petroleum oils and diesels (polyaromatic hydrocarbons, PAHs) which enters the general circulation (internal exposure burden) of oiled seabirds is rarely undertaken. This is because the traditionally used chromatographic methods for plasma PAH analysis require larger sample volumes than those that can be safely collected from smaller species, such as guillemots (*Uria aalge*). Furthermore, these methods are not a cost-effective or practical approach for analysis of large numbers of birds in a short time period as part of an oil spill response in wildlife rehabilitation centers. This study describes the modification and validation of a commercially available PAH immunoassay (cRaPID PAH) to enable high-throughput, cost-effective, simple, and rapid determination of total PAH concentrations in 50 μL volumes of plasma. The limit of detection of the assay was 0.1 ng/mL as benzo-α-pyrene (BaP) equivalents with a working range of 0.1−20 ng/mL. As further validation of the immunoassay, PAHs were determined by GC−MS. GC−MS data were significantly positively correlated with corresponding immunoassay data for the same birds ($r^2 = 0.976$, $p < 0.001$). The plasma PAH concentrations of 40 oiled guillemots stranded on U.K. shores were determined using the assay to demonstrate its usefulness for biomonitoring studies. The mean $\sum$PAH concentration observed was 1.05 $\pm$ 0.67 ppm (range 0.02−2.40 ppm as BaP equivalents). The modifications to the cRaPID PAH kit in this study enable nondestructive, high-throughput, semiquantitative determination of PAH concentrations in plasma samples suitable for exposure assessment of oiled seabirds during oil spill response and rehabilitation.

Introduction

Releases of petroleum to the marine environment are mainly from incidental discharges from day to day transport and refining activities, industrial and municipal discharges, disposal of waste oil and diesel (e.g., oil tanker contaminated ballast, contaminated oil-rig brines), atmospheric deposition, and river and urban runoff. Tanker accidents and other nonroutine releases of oil and diesels are also other important sources of marine oil pollution. Consequently, petroleum components such as polyaromatic hydrocarbons (PAHs), aliphatic hydrocarbons, and heavy metals are now ubiquitous marine pollutants. Seabirds, fish, and aquatic mammals especially become significantly oiled within minutes of contact with a crude oil or diesel spill. Marine wildlife are also indirectly exposed to the persistent and bioaccumulative components of petroleum, particularly PAHs, via the marine food chain from the consumption of contaminated prey. Mass mortalities of seabirds are a common feature of past and recent oil spill events, such as the Exxon Valdez in 1989 (Prince William Sound, Alaska), Sea Empress in 1996 (Milford Haven, U.K.), and Prestige spill in 2002 (Galician Coast, Spain).

The treatment and rehabilitation of oiled seabirds requires determination of the degree of oil exposure of each individual. Chemical analysis by gas chromatography (GC) with mass spectrophotometric (MS) detection or high-performance liquid chromatography (HPLC) with UV or fluorescence detection are most commonly used to determine PAH concentrations in biological samples (*1*). The drawback of these methods, although the most accurate, are that they are expensive and time-consuming, require large sample volumes, and must be undertaken by an analytical chemist in a dedicated analytical laboratory. To date, the level of oil exposure in oiled seabirds is most commonly assessed by estimating the percentage oil coverage of a bird's plumage and assigning a relative oil score. Clearly this approach does not accurately reflect internal exposure to the toxic components of oil, such as PAHs, because the time between oiling and exposure assessment can vary widely. The amount of time the bird has had to preen and remove oil from its plumage greatly influences the appearance of external oil coverage by the time the exposure assessment is conducted. A more accurate approach to assessing oil exposure is required for treatment and rehabilitation of oiled seabirds. PAH exposure biomarkers such as induction of CYP4501A and dependent mixed function oxidases in liver microsomes have been used to a limited extent in the past (*2*−*5*). These however are unsuitable for bird rehabilitation since, in the first place, they require destructive liver sampling.

Ideally, oil exposure in seabirds should be assessed using a nondestructive approach employing a high-throughput, simple, low-cost methodology for assessing large numbers of birds in a short time period. Plasma samples are ideal samples for exposure assessment because they represent that which is circulating to target organs and causing toxic effects, thereby providing a good indication of toxic exposure. Blood sampling is nondestructive and can be repeated to monitor decontamination over time. A number of PAH immunoassays have been developed to determine PAH concentrations (*6*, *7*) and are available as commercial test kits (e.g., RaPID, EnviroGard). However, PAH immunoassays have so far only been used to screen PAH concentrations in abiotic environmental samples (e.g., water and sediment) rather than biolgical samples (*8*, *9*) until recently, when the RaPID PAH kit was successfully adapted for determination of total PAH in crab urine and hemolymph (*10*). The advantage of immunoassay methods is that they can be adapted for high-throughput use (80-sample batch + standards and controls) using 96-well microtiter plates for cost-effective, rapid (compared with GC/HPLC analysis) analysis, using very small sample volumes as low as 50 μL. The existing commercially available PAH test kits have limitations which need to be overcome to increase their suitability for use in rehabilitation. The aim of this work was to modify an existing commercially available PAH test kit to produce a nondestructive, high-

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throughput, cost-effective, semiquantitative (swan) approach for PAH determination in microvolume plasma samples. The method was then used to determine PAH concentrations in plasma samples from stranded oiled guillemots, to demonstrate its suitability for use in oiled wildlife rehabilitation.

Experimental Section

Chemicals. The RaPID carcinogenic PAH test kit (A0020/A00201) was obtained from Strategic Diagnostics Inc. (Newark, DE). Certified high-purity parent PAH standards were obtained from Sigma-Aldrich Inc. (St. Louis, MO): acenaphthene, phenanthrene, acenaphthalene, pyrene, fluorene, chrysene, naphthalene, anthracene, benz[*a*]-anthracene, fluoranthene, benzo[*a*]pyrene (BaP), and internal standard (IS) phenanthrene-d_{10}. Certified high-purity metabolite PAH standards were obtained from ChemService Ltd. (2-hydroxyfluorene, 1-hydroxypyrene, chrysene-5-carboxylic acid, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene, 4-hydroxyphenanthrene, phenanthrene-9,10-diol, and 6-oxo-7-oxa-benzo[*a*]pyrene). Solvents were high-purity glass distilled grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). All other chemicals were purchased from Sigma-Aldrich Inc. unless otherwise specified. All glassware was acid-washed and then hexane-washed prior to use.

Sampling and Plasma Preparation. A total of 40 adult common guillemots (*Uria aalge*) were sampled for this study by veterinary staff at the Royal Society for the Prevention of Cruelty to Animals (RSPCA) Wildlife Rehabilitation Centres (Mally Dams Wood, West Hatch Hospital, and Norfolk Wildlife Hospital) during winter 2002−2003. All of the birds sampled were oiled (crude oil or diesel) to some degree. Oil coverage scores derived from external oil coverage (0−5/25/50/75/100%) and oil type (light/heavy) assigned by RSPCA were available for 36 of the sampled guillemots. These were converted into oil scores as follows: 0 (unoiled), 1 (0−5% coverage with light oil), 2 (25% coverage with light oil), 3 (50% coverage with light oil), 4 (75% coverage with light oil), 5 (100% coverage with light oil), 6 (0−5% coverage with heavy oil), 7 (25% coverage with heavy oil), 8 (50% coverage with heavy oil), 9 (75% coverage with heavy oil), and 10 (100% coverage with heavy oil). Determination of the exact age and the sex of adult guillemots studied was not possible since this can only be achieved by pathological examination with this species (examination of gonads and measurement of bursa of fabricus and supraorbital ridge size (*11*)). According to the RSPCA veterinary staff, it is possible to collect from healthy unoiled guillemots 0.5−1 mL of whole blood. However, only a maximum of 0.5 mL was collected from oiled guillemots in this study, due to their emaciated, highly stressed, and hypothermic condition. It was therefore necessary to collect surrogate blood samples from swans ($n = 10$), where 1−5 mL sample volumes were possible/ethical, to enable parallel GC−MS analysis of duplicate samples for immunoassay validations. Samples were collected from live oiled birds 1−4 days after admission to the RSPCA centers. Collections were made in EDTA-treated (to prevent blood clotting) 0.5 mL pediatric (guillemot samples) or 5 mL standard (swan samples) blood tubes from the foot vein. Whole blood was centrifuged at 2500 rpm at 4 °C for 5 min to separate the plasma, which was transferred to cryopreservation tubes and stored at −20 °C until analysis.

Modified cRaPID. In preparation for cRaPID analysis, PAHs were extracted from 50 μL of swan plasma by the addition of 100 μL of methanol and 100 μL of PBS (10 mM phosphate-buffered saline containing 150 mM NaCl and 0.1% w/v sodium azide, pH 7.4) followed by centrifugation at 4000 rpm at 4 °C for 10 min. This facilitated the precipitation and sedimentation of sample proteins, producing a clear protein-free extract (supernatant) for analysis. This is a standard approach of plasma pretreatment for drug analysis by ELISA (*12*). All extracts were diluted 1:100 in the first instance since initial analyses showed plasma PAH concentrations tended to exceed the linear range of the assay. The ability of the pretreatment to remove plasma matrix effects was investigated by running replicate BaP standard curves in four subsamples of the same swan plasma sample on the same plate.

The cRaPID assay procedure was followed according to the manufacturer's recommendations modified for use with 96-well microplates for high-throughput analysis by scaling down assay reagent volumes by a factor of 4 to enable use of 50 μL microsample volumes as follows: A 50 μL portion of sample/BaP standards/controls and 65 μL of enzyme conjugate were added to each well and aspirated to mix. A 100 μL portion of polyclonal PAH antibody-coupled paramagnetic particles (in PBS) was then added to all wells, and the plate was incubated at room temperature on a slowly moving plate shaker for 20 min. The plate was then placed on a Dynatech (U.K.) magnetic plate designed specifically for use with 96-well microtiter plates (in place of the magnetic tube rack recommended by the manufacturer) to facilitate sedimentation of the paramagnetic anti-PAH antibody complex. The plate was allowed to stand for 5 min and then tilted by 45° to allow the solution to be removed gently from all wells (avoiding contact with sedimented particles adhered to well bases), and then 200 μL of washing solution (deionized water) was added to all wells. The washing solution was removed from the wells and the wash step repeated with a further 200 μL of washing solution. A 100 μL portion of color solution was then added to the wells (H_2O_2 and 3,3′,5,5′-tetramethylbenzidine) and the plate incubated at room temperature on a plate shaker for 20 min. Reaction was stopped by addition of 100 μL of stop solution (2 M H_2SO_4) to all wells and the absorbance read within 2 min at 450 nm using a Bio-Rad ELISA plate reader. The standards provided in the kit by the manufacturer were 0.1, 1, and 5 ppb BaP in 50:50 (v/v) methanol/25 mM Tris−HCl, pH 7.0). To increase the working range of the kit and reduce the need to dilute the samples, standards of higher concentrations were prepared, diluting neat BaP with 50:50 (v/v) methanol/PBS. ΣPAH concentrations in the samples were calculated using the equation of the linear curve of log(BaP concentration) versus [observed absorbance of a standard/absorbance of a zero standard (negative control)] × 100 (% B/B_0). Values were corrected for dilution accordingly. The specificity of the cRaPID anti-PAH antibody (raised against BaP immunogen) was established by comparing the BaP concentration required for 50% inhibition of color formation (i.e., 50% B/B_0 or I_{50}) with respective values for 10 parent PAHs and 10 metabolites in the same diluent (50:50 v/v methanol/PBS washing buffer), as recommended by Marco et al. (*13*). Cross-reactivity (CR) values were calculated as follows: 50% B/B_0 PAH_x/50% B/B_0 BaP.

GC−MS Analysis. GC−MS analysis was used as a means of method validation for the modified cRaPID assay using surrogate swan plasma samples and to investigate matrix effects, which may interfere with the immunoassay analysis. To test the reliability of the modified cRaPID assay, 10 swan plasma samples were analyzed in parallel by this assay and GC−MS. Plasma samples were first subjected to protein precipitation pretreatment as previously described for modified cRaPID assay, with the addition of an internal standard (IS), as follows. To 1 mL of swan plasma were added 1 mL of methanol and 100 μL of IS (50 ppm phenanthrene-d_{10} in methanol) and the mixture was centrifuged for 10 min at 4 °C at 4000 rpm to pellet precipitated interfering proteins. Following pretreatment, 50 μL subsamples of each sample were analyzed by modified cRaPID assay for later comparison with GC−MS data for the same samples by the method

described. Further subsamples from four of these samples (with ∑PAH concentrations ≥20 ppb) were each diluted to cover the working range of ∑PAH concentrations (as BaP equivalents) of the modified cRaPID assay and analyzed in triplicate by the assay on the same plate. The resultant standard curves were compared with a standard curve derived from triplicate BaP standards of the same concentration range. Matrix effects are evident where there is a lack of parallelism between these standard curves and that of BaP standards (*13*).

Pretreated plasma extracts were cleaned up for GC−MS analysis by solid-phase extraction (SPE) as follows. Strata-X SPE tubes (100 mg of adsorbent, 1 mL capacity, Phenomenex) were conditioned with 2 × 1 mL of methanol and 2 × 1 mL of distilled water and dried for 10 min under high vacuum. The clear protein-free sample extract (0.9 mL) was transferred to the SPE tube and the tube washed with 2 × 0.5 mL of distilled water. The PAHs plus sample lipids were eluted with 2 × 0.5 mL of dichloromethane. Retained PAH metabolites were then eluted from the tube with 2 × 0.5 mL of acetonitrile. PAHs from the first fraction were cleaned up to remove coeluted lipid on Si SPE tubes (1 g of silica adsorbent, 5 mL capacity, Varian Inc.) which were conditioned with 2 × 2 mL of hexane. Lipid-free PAHs were eluted from the tube with 2 volumes (2 × 3 mL) of dichloromethane/hexane (30:70 v/v). With every batch of five plasma samples, one blank sample was included, whereby 1 mL of methanol spiked with 100 μL of internal standard was used in place of the sample and processed through the method as normal.

To ensure thermal stability of heat-sensitive PAH metabolites during GC−MS analysis and increase detection sensitivity, PAH and PAH metabolite fractions were derivatized with 20 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatizing agent plus 5 μL of pyridine (catalyst) and left overnight to ensure complete reaction before GC−MS analysis. Samples were analyzed with a GC−MS system (5890 Hewlett-Packard) fitted with a 30 m Zebron ZB-5 capillary column (5% phenylpolysiloxane, 0.25 μm, 0.25 mm i.d., Phenomenex) using helium as the carrier gas. Injector and detector temperatures were 280 °C. The column temperature program was as follows: initial temperature 60 °C, hold for 1 min, ramp at 50 °C/min to 130 °C, ramp at 4 °C/min to 280 °C, and hold for 1 min. The injection volume was 10 μL by splitless mode to achieve maximum sample loading to improve detection. Single-ion monitoring (SIM) was used for selective detection of molecular ions of major PAH and PAH metabolites. The limit of detection achieved by this method was 1 ppm. The recovery of PAHs and metabolites through the extraction and cleanup methods was found to be 70−80% as determined by the recovery of IS added to every sample. The identification of 11 parent PAHs and 8 metabolite PAHs in the samples was determined by matching molecular fragment patterns (Table 1) with certified reference standards. Concentrations of PAHs and metabolites were determined by comparing their response values with the respective target ions of the calibration standards.

Determination of PAH Concentration in Guillemot Plasma Samples. The modified cRaPID assay was used to analyze 40 guillemot plasma samples for ∑PAH concentration accordingly. Where necessary, samples with high concentrations were diluted and reanalyzed and the resultant data corrected for dilution. It was not possible to analyze guillemot plasma samples by GC−MS due to the lack of an adequate sample volume.

Results and Discussion

Performance of Modified cRaPID PAH Assay. Calibration curves of triplicate BaP standards were obtained for PAH concentrations of 0.1−20 ppb (higher BaP concentrations were also tested, but saturation effects occurred) as shown in Figure 1. Following logit−log transformation and linear regression analysis, a linear range was observed over a PAH concentration (as BaP equivalents) range of 0.1−20 ppb ($r^2 = 0.99$, $p < 0.001$), with a limit of detection (assay sensitivity) of 0.1 ppb (Figure 1b). Within an assay the precision (the mean coefficient of variation (% CV) was 6.8 ± 1.8% (within an assay)), interassay reproducibility (% CV = 12.3 ± 2.7%), and BaP percent recovery (range 80−105%) were all within acceptable limits. The working range of the cRaPID assay used according to the manufacturer's recommendations for detecting PAHs in water samples is 0.2−10 ppb (as BaP equivalents). However, PAH concentrations in biological samples from polluted wildlife can exceed this range, requiring sample dilution. As a result of the modifications to the assay, plasma samples with ∑PAH concentrations up to 20 ppb could be accommodated, reducing the need for sample dilution and reanalysis, which is an advantage for reducing the analysis time of biological samples. The pretreatment method employed in this study to remove plasma matrix effects is an established approach to reduce interfering proteins present in plasma during ELISA drug analysis (*12*). Experiments to study matrix effects confirmed the pretreatment method was indeed effective. A high degree of parallelism was observed between a standard curve derived from swan plasma samples and a standard curve derived from BaP standards (slope 3.57 ± 0.17, $r^2 = 0.99 \pm 0.01$, $n = 4$; not shown), an accepted approach to assessing matrix effects in pollutant immunoassays (*13*).

According to calculated CR values, the cRaPID assay (raised against BaP immunogen) anti-BaP antibody recognized all 10 parent PAHs tested but none of the PAH metabolites. The 50% B/B_0 values (and derived CR values) observed for parent PAHs are presented in Table 2 and were highly similar to corresponding values reported by the cRaPID PAH kit manufacturer (Strategic Diagnostics Inc.). Due to the apparent inability of the assay anti-BaP antibodies to recognize PAH metabolites, it would be appropriate to develop/test alternative anti-PAH capture antibodies for determination of PAH concentrations in plasma where metabolites predominate (e.g., associated with plasma protein such as albumin), formed by hepatic biotransformation (*1*, *14*). The relative pattern of parent and metabolite PAHs in plasma at a given time is dependent on uptake, distribution, biotransformation, and excretion, which is

TABLE 1. Molecular Ions Used for Identification of Parent and Metabolite PAHs by GC−MS

PAH	molecular ions			
Parent PAHs				
naphthalene	128	102	101	129
acenaphthylene	152	151	150	76
acenaphthene	153	154	151	76
fluorene	166	165	163	82
phenanthrene	178	176	76	89
phenanthrene-d_{10} (IS)	188	100		
anthracene	178	176	76	89
fluoranthene	202	101	100	199
pyrene	202	101	100	198
chrysene	228	113	226	115
benz[*a*]anthracene	228	226	113	229
benzo[*a*]pyrene (BaP)	252	126	254	225
Metabolite PAHs				
2-hydroxyfluorene	254	100	239	68
4-hydroxyphenanthrene	266	100	235	67
phenanthrene-9,10-diol	251	100	266	99
3-hydroxyphenanthrene	266	100	251	67
2-hydroxyphenanthrene	266	100	251	85
chrysene-5-carboxylic acid	228	100	226	29
1-hydroxypyrene	290	100	73	34
6-oxo-7-oxa-BaP	270	100	106	25

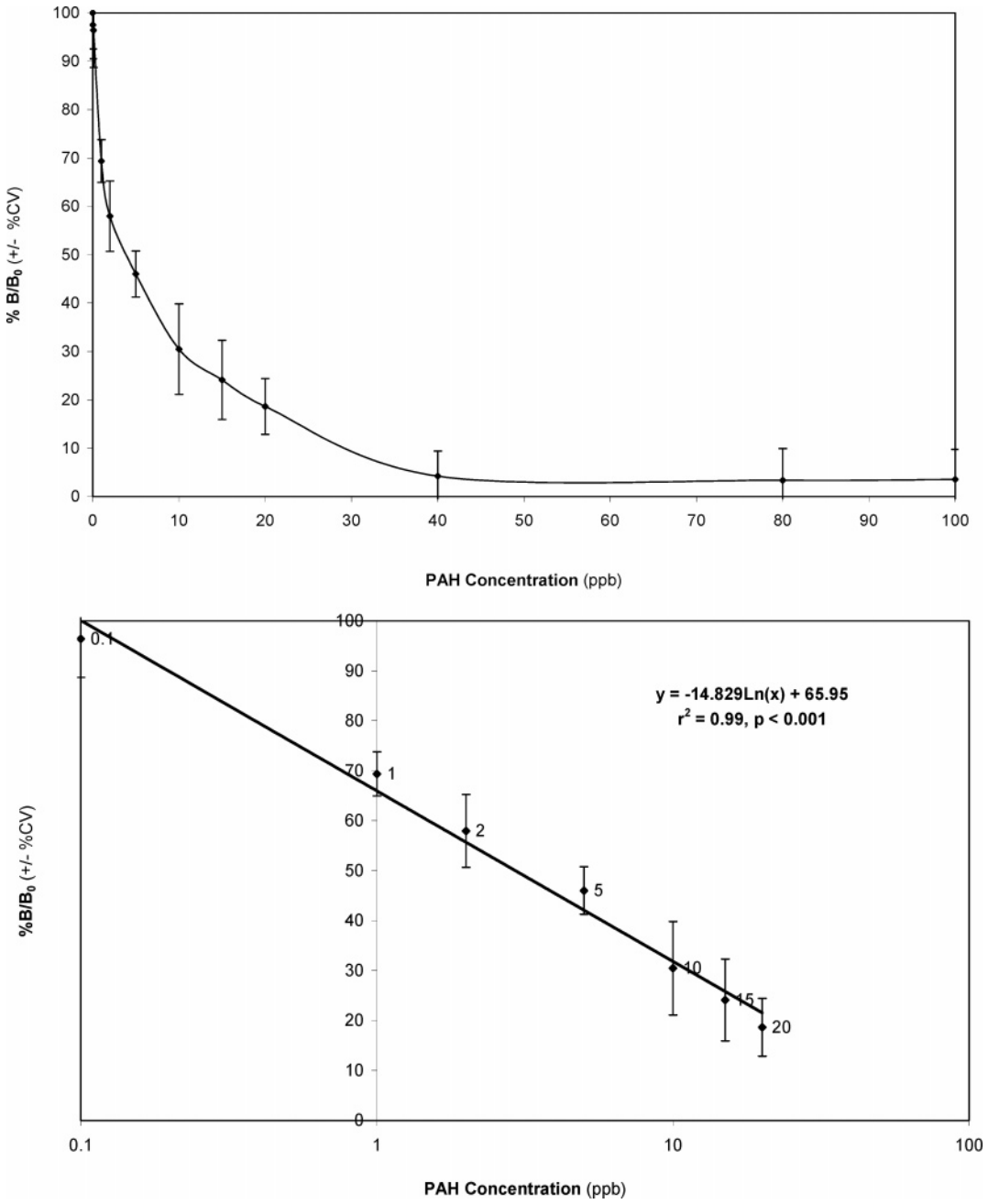

FIGURE 1. Standard curve for modified cRaPID assay (mean of triplicate data ± standard deviation): (a, top) % B/B_0 versus PAH (BaP equivalents) concentration; (b, bottom) log−logit transformation and linear regression analysis.

dependent, among other things (e.g., nutrition, intraspecific differences), on the amount of time passed since oiling/exposure, which can vary widely between individuals. Thus, it is not possible to accurately extrapolate total PAH (parent + metabolite) concentration estimates in plasma from data derived using the modified cRaPID assay.

GC−MS PAH concentrations (parent + metabolite) and immunoassay-determined ∑PAH concentrations in swan plasma samples were significantly positively correlated, as shown by linear regression analysis ($r^2 = 0.83$, $p < 0.001$; Figure 2). GC−MS concentrations were higher than respective values obtained by modified cRaPID assay. This was because the modified cRaPID recognized only parent PAHs, while PAH data from GC−MS were the sum concentration of 11 parent plus 8 metabolite PAHs. Similar discrepancies between PAH data from cRaPID (used according to the manufacturer's recommendations) and GC−MS for the same contaminated soil samples were reported by Chuang et al. (*15*). Clearly, the presence of PAH metabolites in plasma from oiled seabirds is confirmed here. Chromatographic analysis is therefore more appropriate for PAH metabolite analysis, until PAH metabolite-specific immunoassays can be developed. The correlation between GC−MS and immunoassay data was considerably improved when GC−MS parent PAH data and immunoassay ∑PAH data were compared ($r^2 = 0.976$, $p < 0.0001$; Figure 2). This demonstrates that the modified cRaPID assay can reliably be used for screening plasma PAH burdens in exposure biomonitoring and oiled wildlife rehabilitation.

To date, commercially available PAH immunoassays have only been used in oiled bird rehabilitation to assess oil exposure using feather samples (*16*). This is the first application of a commercial PAH immunoassay (RaPID and EnviroGard) for the analysis of plasma samples from oiled wildlife. One of the characteristics of commercial PAH assay kits that makes them less desirable for use in rehabilitation is that they are not high-throughput; for example, only 7

TABLE 2. Specificity of the cRaPID Anti-PAH Antibody toward Parent PAHs

parent PAH	concn for 50% B/B_0[a] (ng/mL)	cross-reactivity (CR)[b]
anthracene	41.0 ± 2.1	14 ± 0.70
acenaphthene	18171.5 ± 1635.4	6204 ± 558.36
phenanthrene	131.8 ± 9.2	45 ± 3.15
acenaphthylene	10605.9 ± 848.5	3621 ± 289.68
pyrene	442.3 ± 44.2	151 ± 15.1
fluorene	612.2 ± 24.5	209 ± 8.36
chrysene	1.5 ± 0.1	0.51 ± 0.04
naphthalene	9050.6 ± 362.0	3090 ± 123.6
fluoranthene	111.3 ± 5.6	38 ± 1.90
benzo[*a*]pyrene	2.9	1

[a] [(Absorbance of a standard/absorbance of a zero standard) × 100] for 50% inhibition of color formed by the zero standard. [b] CR = mean of triplicate 50% B/B_0/50% B/B_0 BaP data ± standard deviation.

(triplicate sample testing) to 20 (single sample testing) samples can be tested at once according to the manufacturer's recommendations in cRaPID (30 test kit size), requiring the use of a magnetic tube rack. Also, the minimum sample volume is 200 μL, limiting sample availability for other analyses (e.g., blood chemistry), and they are developed for use with abiotic (water and sediment) samples not plasma, whereby plasma proteins may interfere with the assay reagents. As a result of modifications to the cRaPID assay, a nondestructive, high-throughput, simple, and rapid analytical technique is available for semiquantitative determination of plasma ∑PAH concentrations in oiled seabirds, suitable for use in oil response and bird rehabilitation. Both immunoassays require plasma samples of very small volume (50 μL for minimum invasiveness/disturbance), enabling them to be used for assessment of internal PAH exposure of oiled seabirds, down to 0.2 ppb as BaP equivalents. From 24 (triplicate) to 88 (no replicates) samples in a 96-well microtiter plate can be analyzed at one time using a spectrophotometric ELISA plate reader to yield total PAH concentration values as BaP equivalents within a maximum of 24 h of sampling. The cost of these methods equates to ~£1.50 per sample. The benefits of immunoassays over traditional chromatographic analyses (e.g., GC−MS and HPLC) are that they require smaller sample volumes and are more user-friendly, quicker to execute, more cost-effective, and high-throughput. Moreover, PAH analysis using these immunoassays can be undertaken by personnel other than analytical chemists in a rehabilitation setting (following training). The modified cRaPID assay is easily adaptable for use in the field, and portable equipment is available, such as hand-held manual centrifuges for plasma preparation and pretreatment and hand-held battery-powered ELISA plate readers.

Although the modified cRaPID assay was not able to provide fully quantitative concentrations for individual PAH compounds, semiquantitative ∑PAH data are more than adequate for obtaining an understanding of circulating PAH burdens for supporting/providing information for appropriate rehabilitation treatment/action. Other forms of bioassay in addition to immunoassays can also be modified for measuring PAH concentrations in plasma samples. These include aryl hydrocarbon receptor (AhR) binding assays (*17*) and reporter gene induction assays (*18*). Despite modifications, for example, to the CALUX (chemically-activated luciferase expression) assay to quantify PAHs in serum samples (*19*), still none of these bioassays are suitable for use in oil spill response and/or bird rehabilitation. This is mainly because these assays require cultured cell lines and because of their greater cost compared with that of immunoassays. Fluorescence techniques have also been successfully used for high-throughput rapid screening and present another novel analytical approach for PAH exposure assessment in oiled bird rehabilitation. For example, Watson et al. (*20*) used fluorescence detection to quantify parent and metabolite PAHs in biological fluids of only 20−400 μL.

∑PAH Concentrations in Guillemot Plasma. The modified cRaPID was used to investigate ∑PAH concentrations in plasma samples collected from 40 oiled U.K. common guillemots. It is well established that PAHs are ubiquitous contaminants of the marine environment, not only from anthropogenic sources (pollution) but also from natural sources (e.g., forest fires). Therefore, it would not be expected that PAH levels would be completely absent, even in unoiled seabirds. ∑PAH concentrations observed in guillemots were in the range of 0.02−2.40 ppm, with a mean of 1.05 ± 0.67 ppm (Table 3). Naturally, PAH levels in each bird are dependent on the type and level of oil exposure and how soon after oiling the birds were sampled, which influences the amount of time preening and ingesting oil from plumage. Thus, the variation in PAH burdens reflected the different exposure scenarios of the birds. Due to the small sample

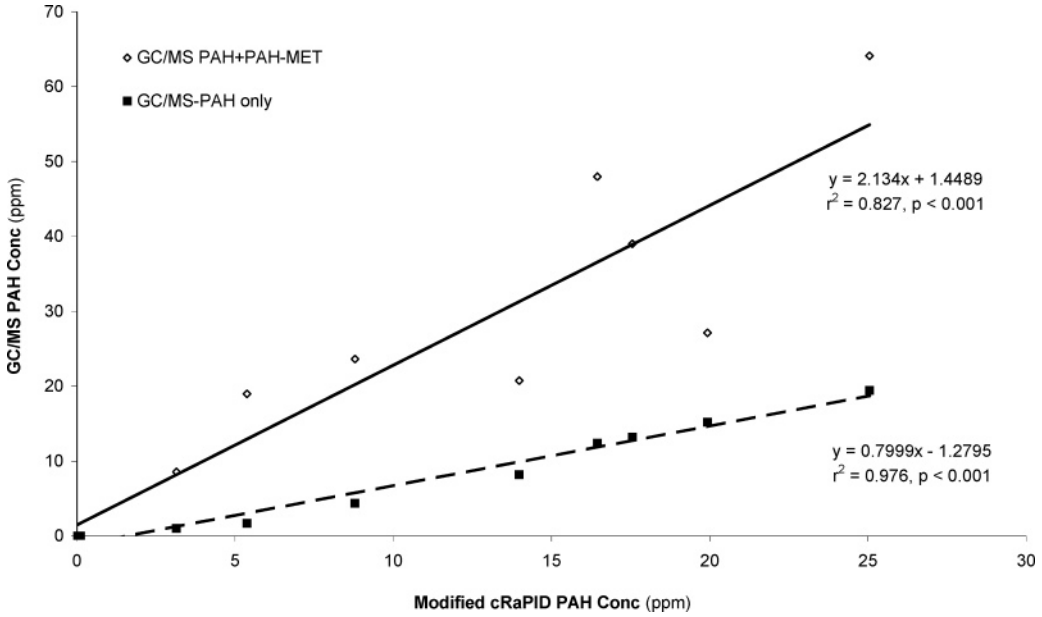

FIGURE 2. Linear regression of swan plasma PAH concentration data as determined by modified cRaPID and GC−MS methods.

TABLE 3. ∑PAH Concentrations in Guillemot Plasma Determined by ∑PAH ELISA

bird no.	∑PAH[a] (ppm)	bird no.	∑PAH[a] (ppm)	bird no.	∑PAH[a] (ppm)	bird no.	∑PAH[a] (ppm)
1	2.10	11	0.02	21	0.07	31	0.76
2	0.56	12	0.18	22	1.68	32	0.99
3	0.91	13	0.62	23	1.25	33	1.47
4	0.67	14	0.98	24	1.33	34	1.89
5	1.83	15	2.40	25	1.99	35	0.04
6	1.09	16	1.76	26	2.10	36	0.02
7	1.20	17	1.93	27	1.91	37	0.54
8	0.06	18	0.41	28	1.07	38	0.81
9	0.88	19	1.05	29	1.22	39	0.60
10	1.49	20	0.68	30	0.32	40	1.08
mean ± std dev (range)				1.05 ± 0.67 (0.02−2.40)			

[a] Total PAH concentration expressed as BaP equivalents.

volume availability, duplicate analysis of guillemot plasma samples was not possible, so methodological error associated with these data may account for some of the variation observed. It was not possible to investigate the influence of age and sex on PAH levels since this information was not available.

This is the first study of plasma PAH concentrations in seabirds. Plasma PAH concentrations in guillemots of this study were generally higher than levels reported in tissues (liver and muscle) and eggs of other seabirds chronically exposed to PAHs through the food chain, such as cormorant (*Phalacrocorax carbo*; range of ∑PAH in eggs 0.02−0.08 ppm), gulls (*Larus* spp.; range of ΣPAH in eggs n/d to 0.33 ppm), shag (*Phalacrocorax aristotelis*; range of ∑PAH in eggs 0.16−0.38 ppm), and chough (*Pyrrhocorax pyrrhocorax*; range of ∑PAH in eggs 0.01−0.10 ppm) from U.K. coastlines (*21*) and pelicans (*Pelecanus conspicullatus*; range of ∑PAH in muscle 0.05−0.09 ppm) and gulls (*Larus* spp.; range of ∑PAH in muscle 0.05−0.11 ppm) from the Brisbane River Estuary (Australia) (*22*). This is mainly due to differences in exposure history, since the guillemots of this study were directly exposed to crude oil over a short time period as a result of oil spill incidents, while the birds of the cited studies were exposed to PAHs bioaccumulated in the aquatic food chain. Guillemot plasma PAH concentrations were closer to PAH concentrations reported in lesser scaup carcasses (*Athya affinis*; range of ∑PAH 0.08−0.20 ppm) from the Indiana Harbour Canal, Lower Michigan, a heavily polluted water body due to intense localized industrial activity (*23*).

PAHs are relatively well-metabolized in birds by hepatic CYP4501A-dependent enzymes, which have been shown to be significantly induced in guillemots following pollutant exposure (*3*, *5*). However, oxidative intermediary metabolites, known as reactive oxygen species (e.g., arene oxides), are formed from CYP4501A metabolism, which are more toxic than parent PAHs (*24*). The toxicity of PAHs in birds is well established, including reproductive toxicity (*25*), adrenal hypertrophy, bursa atrophy, weight loss (*26*−*29*), thyroid hypertrophy (*26*), hemolytic anemia (*30*, *31*), changes in salt gland weight and ATPase activity (*2*, *27*), gut damage, immunotoxicity (*26*, *27*, *33*), and renal and hepatic damage (*28*). The biological monitoring of internal PAH burdens is clearly an advantage in the rehabilitation of oiled seabirds to ascertain health risks resulting from PAH toxicity. Due to its practical advantages, the modified cRaPID assay will encourage greater use of internal exposure monitoring for rehabilitation of oiled birds.

The relationship between plasma PAH concentrations and external oil coverage scores was investigated by comparing the mean plasma ∑PAH concentrations of guillemots in each oil score category (Figure 3). Samples from unoiled guillemots (score 0) and scores of 4 and 5 (light oil) were not available for this study. With the limited data available, no statistically significant relationship was observed between mean plasma ∑PAH concentrations and oil scores. This would suggest no apparent relationship between internal and external oiling. The presence of any relationship may however be masked by the considerable variation (as indicated by standard errors) in mean plasma ∑PAH concentrations for each score (particularly score 1, light oil). Other confounding factors may also influence the data, such as preening time prior to blood sampling. The latter influences the proportion of plumage oil ingested and entering the circulation. For example, birds preening considerably prior to sampling are superficially free of oil and hence assigned low oil scores, but ingested oil elevates their plasma PAH concentration. Other influential factors include error associated with oil scores being assigned by different persons and also methodological error associated with plasma PAH determinations (due to lack of replicates). A more accurate investigation of the relationship between internal and external oiling is required, employing a higher number of birds with larger

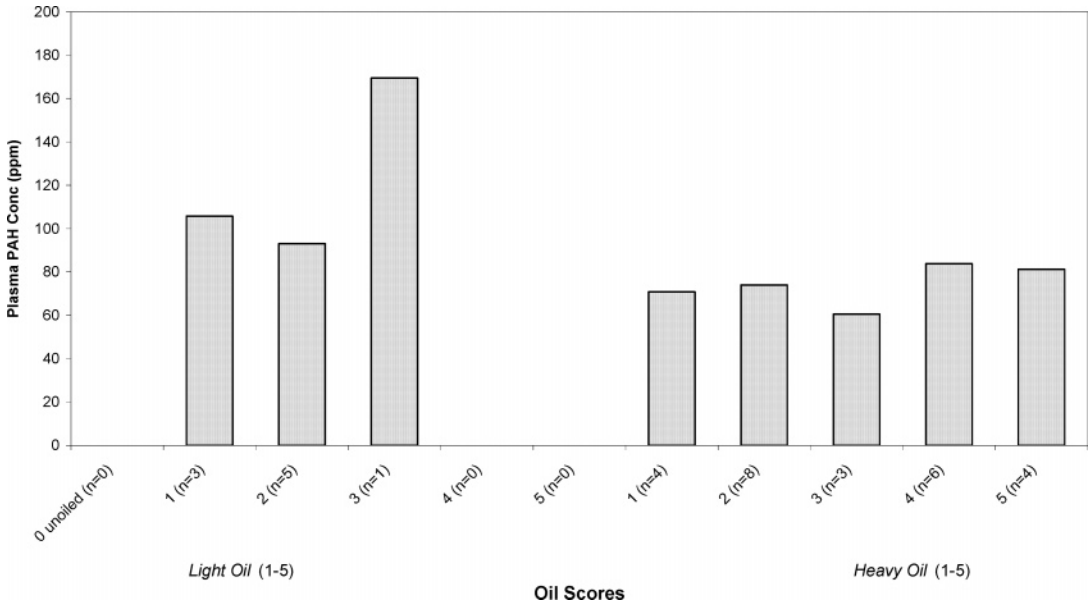

FIGURE 3. Comparison of internal plasma ∑PAH concentration versus external oil coverage scores of a sample of oiled U.K. guillemots.

plasma samples (to allow replicate PAH analysis) representing an even spread of oil scores (particularly unoiled guillemots).

Due to the physical effects of oil on seabirds (loss of insulation, entrapment, loss of buoyancy), external oil coverage scoring remains one of the most important methods of exposure assessment for triage of oiled guillemots admitted to rehabilitation centers. It is used in the first instance to decide the level of washing and as an indication of the severity of oiling. However, internal PAH exposure assessments are also important for several reasons. First, plasma PAH assessment of apparently unoiled birds (score 0) admitted to rehabilitation centers due to emaciation or generally poor condition can be used to determine whether this may be due to recent oiling. Following ingestion of oil, there may be extensive damage to the gastrointestinal mucosa, resulting in intestinal inflammation and reduced nutrient absorption (*34*, *35*). Birds are less able to gain sufficient body weight for release, despite the availability of food and feeding by a stomach tube. However, rehabilitators would be able to refer to plasma PAH assessments at least to exclude internal PAH contamination as the cause. Second, internal PAH exposure assessments enable rehabilitators to monitor efficacy of any detoxification treatments employed (e.g., charcoal administration) since plasma PAH levels can be monitored at regular intervals during treatment. Third, plasma PAH assessment can be used to ensure PAH burdens of birds ready for release are below toxic thresholds for long-term health effects such as reproductive toxicity and immunotoxicity, which influence postrelease survival and population recovery (*25*, *32*, *33*). Fourth, plasma PAH measurements are a vital prerequisite for confirming dose dependence of toxic responses in their validation as biomarkers of oil-induced health effects. Clearly, both external and internal exposure assessment methods are necessary for a complete understanding of the short- and long-term health impacts of oiling on seabirds, and are not interchangeable.

In conclusion, the modified cRaPID assay developed in this study provides a nondestructive, high-throughput, cost-effective, simple, and rapid analytical technique for semi-quantitative determination of ∑PAH concentrations in oiled seabirds, suitable for use in oil response and bird rehabilitation where traditional chromatographic techniques are not feasible or practical. With this assay, down to 50 μL volume plasma samples can be used to assess internal exposure of seabirds to toxic oil components, PAHs, down to 0.1 ppb (as BaP equivalents). The method can be used to ascertain total internal PAH exposure in oiled seabirds, to provide information for treatment decisions for oiled seabirds as part of oil spill response and bird rehabilitation action.

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