Quantification of Polycyclic Aromatic Hydrocarbons in Avian Dried Blood Spots by Ultra-performance Liquid Chromatography with Simple Liquid Extraction and Phospholipid Solid-phase Extraction Preparation

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ABSTRACT
Here, a simple, reliable method for the quantification of the 16 EPA priority polycyclic aromatic hydrocarbons in dried blood spots is outlined using liquid extraction and phospholipid solid-phase sample cleanup coupled with analysis by ultra-performance liquid chromatography with ultraviolet–visible detection. Whole blood spotted on Whatman FTA cards was efficiently quantified by extraction into acidified methanol and passed through a phospholipid solid-phase extraction well plate before injection into a liquid chromatography under reverse-phase conditions. The analyte recoveries in quality control samples ranged from 63.4 to 104.1%, with relative standard deviations from 0.48 to 2.04%. These figures of merit are comparable with measurements in whole blood or serum using similar techniques. The method detection limits were from 45.0 ng·g⁻¹ for benzo[g,h,i]perylene to 118.7 ng·g⁻¹ for chrysene, with matrix spike recoveries from 64.3 to 99.4%, demonstrating acceptable sensitivity and low matrix interference. With a simple liquid extraction approach and short 16-min liquid chromatography, the dried blood spots were effectively and rapidly analyzed.

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Introduction
Polycyclic aromatic hydrocarbons (PAHs) are organic compounds consisting of multiple fused unsubstituted aromatic rings. Although naturally occurring in oil and coal deposits, these compounds are frequently released into the environment through incomplete combustion processes of wood, fossil fuels, and the production of coal tar, and therefore, are ubiquitous in the general environment. Consequently, the emission of these compounds into the atmosphere has increased drastically over the past centuries due to the rapid increase in industries dependent on processes involving the burning of fossil fuels and coal (Boström et al. 2002; Srogi 2007). In addition, oil spills also significantly contribute to the distribution of PAHs in the environment. This has been increasingly
relevant since the Deepwater Horizon oil spill in the Gulf of Mexico, and as a result, PAHs were, and are still, monitored extensively in this area due to concerns of their impact on the biota of the Gulf (Seegar et al. 2015). PAHs have a generally low solubility in water but are absorbed by substances with high lipid and protein content, including biological tissue and fluids. Analyses of local sediment, seawater, and marine life for crude oil constituents showed that PAHs yielded one of the highest concentrations of these compounds. Specifically, seafood samples, which include commonly consumed species such as blue crab and oysters, showed concentrations of C1-benz[a]anthracene and chrysene well over the EPA limit of $1.80 \times 10^{-5}$ µg·g$^{-1}$ for human consumption (U.S. Environmental Protection Agency 2011; Sammarco et al. 2013).

It has been demonstrated that these compounds are carcinogenic and mutagenic (Dipple 1985), with benzo[a]pyrene being particularly notable for its carcinogenic and mutagenic character. The application of coal tar, which is a notable source of PAHs, to the inside of rabbits’ ears was shown to produce malignant tumors (Yamagiwa and Koichi 1918). More recently, several PAHs that are known to be highly dispersed in the environment were shown to have tumor-producing effects on rats and that certain PAHs, specifically benz[g,h,i] perylene, showed a strong, positive correlation between mutagenicity and concentrations in air samples from which they were found (Deutsch-Wenzel et al. 1983; Kuo et al. 1997).

Preparation of blood samples for the analysis of PAHs can be both difficult and time consuming, with potential complications in sample collection and analysis. Collecting whole blood samples and blood fractions, including red blood cells, can be difficult for people inexperienced with handling blood and can easily be mishandled without proper training and equipment, ultimately compromising the analysis of any analytes of interest. If working in remote areas or in other countries, transporting and shipping samples to the laboratory can be difficult, because shipping biological substances necessitates strict packaging and handling, as well as potential chemical and physical treatment to allow samples to pass through customs, delaying sample analysis. Dried blood spots, however, provide an alternative method for sampling blood that overcomes the difficulties of using liquid whole blood (Vining et al. 2014; Zheng et al. 2015). Using dried blood spots as a sample matrix reduces the chance of contamination, as well as collection vessel breakage, eliminating the use of analyte preservatives or treatments due to customs and import requirements that may interfere with the analysis. Sample transportation does not require refrigeration or freezing, is economical, and requires minimal manipulation at the collection site. As an example, studies examining cholinesterase-inhibiting insecticides in liquid whole blood samples require strict sample collection steps to preserve the integrity of the analyte. Samples of this nature require centrifugation of blood and immediate isolation at low temperature to prevent reactivation of enzymatic activity. However, the required equipment is not always present at the collection site, and risk of thawing samples during transit is a concerning possibility. Replacing liquid whole blood samples with dried blood spots eliminates the need for such a regiment while still inhibiting enzymatic activity (Trudeau et al. 2007). In addition, a sample aliquot obtained from dried blood spots is expected to yield equivalent levels of accuracy and precision compared with a pipette volume of a liquid blood sample (Verplaetse and Henion 2016).

Preparing whole blood for analysis of PAHs has consistently been problematic, because these compounds are readily absorbed into biological fluids and tissue. Recently developed methods have utilized numerous modes of extraction, such as solid-phase extraction (SPE),
size exclusion chromatography, and gel permeation chromatography (Poon, Lam, and Lam 1999; Gilgenast et al. 2011). Several instrumental techniques have been employed to analyze PAHs, most of which are readily accessible in laboratories, such as ultra-performance liquid chromatography (UPLC) coupled with fluorescence detection, gas chromatography, and UPLC (Gilgenast et al. 2011). Although methods assessing PAH concentrations in whole blood and serum are detailed extensively in the literature, few methods have been developed on the quantification of PAHs in dried blood spots.

Previous investigations conducted by our research group reported the analysis of PAHs using whole blood and plasma as a sample matrix requiring the use of QuEChERS dispersive extraction prior to a phospholipid solid phase cleanup (Provatas et al. 2015). The methodology described in this manuscript however, is a refined development of our previous efforts to utilize the advantages of the dry blood sampling technique described above for environmental analysis.

**Experimental**

**Materials and reagents**

Methanol (≥99.9%), acetonitrile (≥99.9%), UPLC-grade water, formic acid (≥95%), bromobenzene (99%), 3-(trifluoromethyl)anisole (99%), chrysene-d$_{12}$ (98%), naphthalene-d$_{8}$ (99%), and perylene-d$_{12}$ (99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 16 PAH solution mixture was purchased from AccuStandard (New Haven, CT, USA). Chicken red blood cells were acquired from Lampire Biological Laboratories (Pipersville, PA, USA).

Red blood cells were spotted on Whatman 903 Proteinsaver cards, which were purchased from GE Healthcare (Chicago, IL, USA). The clear 8 mL disposable vials used for sample extraction were purchased from Fisher Scientific (Waltham, MA, USA). The Ostro 96-well phospholipid removal plate (25 mg sorbent, 2 mL plate well volume) used for sample cleanup was obtained from Waters (Milford, MA, USA).

**Instrumentation**

A Precisa XT 220A (Princeton, NJ, USA) analytical balance was used for the determination of sample and analyte masses. A bransonic 5510 R-DTH ultrasonic cleaner (Danbury, CT, USA) was used to sonicate the samples whereas a thermo scientific 945093 multitube vortexer (Waltham, MA, USA) was utilized for sample mixing. A Waters (Milford, MA, USA) plate vacuum manifold was utilized for the SPE application.

Analysis was performed on a Waters acquity liquid chromatography system (Milford, MA, USA) coupled with a photodiode array and a tandem mass spectrometer. The photodiode array detector was set in continuous wavelength scan mode from 205 to 350 nm, with the specific absorption maxima used for the detection of individual PAHs presented in Table 1. Confirmatory analysis of the analytes was performed using the tandem mass spectrometer in multiple reaction monitoring mode, with an atmospheric pressure photoionization probe as the ionization source. A mixture of bromobenzene and 3-(trifluoromethyl) anisole (0.01% v v$^{-1}$) was used as a doping solution to enhance ionization (Robb, Covey, and Bruins 2000; Kauppila, Kostiainen, and Bruins 2004). Chromatographic conditions
were reverse phase, using an Acquity BEH C18 column (Waters, P/N 186002352) with a 16 min gradient elution of 80:20 water/methanol and 90:10 acetonitrile/methanol as mobile phases A and B as summarized in Table 2.

### Preparation of standards

The surrogate stock solution consisted of the two deuterated compounds, naphthalene-d8 and perylene-d12. Separate 1.0 mg·mL⁻¹ stock solutions of naphthalene-d8 and perylene-d12 were prepared by dissolving 10 mg of the compounds in 10 mL of acetonitrile. Then, 1.0 mL of each of stock solution was combined and diluted to 10 mL in acetonitrile to produce a 100,000 ng·mL⁻¹ solution. The spiking solution of concentration 10,000 ng·mL⁻¹ and calibration standards ranging from 5000 to 10 ng·mL⁻¹ were produced by further serial dilutions with acetonitrile. A 200,000 ng·mL⁻¹ spiking solution containing the PAHs was produced by diluting a commercial 2.0 mg·mL⁻¹ solution of PAH to 10 mL in acetonitrile. Calibration standards from 1000 to 10 ng·mL⁻¹ were produced by further serial dilution. The internal standard stock solution utilized the labeled compound chrysene-d12. The stock solution was prepared by diluting 4.0 mg·mL⁻¹ chrysene-d12 to 10 mL in acetonitrile to 400,000 ng·mL⁻¹. A 10,000 ng·mL⁻¹ spiking solution was prepared by subsequent serial dilution with acetonitrile.

### Sample preparation

#### Dry blood spot extraction

Avian whole blood, in replicates of four or five blood spots, were received by our laboratory with an unknown volume of blood spotted on the Whatman FTA cards, wrapped in aluminum foil, and stored at −18°C until extraction. For duplicate samples, one spot was chosen at random to be used as a matrix spike control, whereas the other spots were pooled and treated as a single sample. Method blank samples were prepared by spreading

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### Table 1. Retention time, wavelength of maximum absorbance (λ_max), and correlation coefficient over a 100-fold concentration range from 5 to 1000 ng·mL⁻¹.

| Compound          | Retention time (min) | λ_max (nm) | Correlation coefficient |
|-------------------|----------------------|------------|-------------------------|
| Naphthalene-d8    | 5.82                 | 219        | 0.9998                  |
| Naphthalene       | 6.02                 | 229        | 0.9998                  |
| Acenaphthylene    | 7.28                 | 227        | 0.9998                  |
| Fluorene          | 8.80                 | 227        | 0.9998                  |
| Acenaphthene      | 8.97                 | 264        | 0.9994                  |
| Phenanthrene      | 9.61                 | 252        | 0.9997                  |
| Anthracene        | 10.03                | 251        | 0.9998                  |
| Fluoranthene      | 11.02                | 236        | 0.9998                  |
| Pyrene            | 11.39                | 240        | 0.9997                  |
| Chrysene-d12      | 12.48                | 267        | Internal Standard       |
| Chrysene          | 12.77                | 268        | 0.9998                  |
| Benz[a]anthracene | 12.90                | 288        | 0.9997                  |
| Perylene-d12      | 13.44                | 250        | 0.9995                  |
| Benzo[b]fluoranthene | 13.56           | 256        | 0.9999                  |
| Benzo[k]fluoranthene | 13.62           | 256        | 0.9998                  |
| Benzo[d]pyrene    | 13.73                | 296        | 0.9998                  |
| Dibenz[a,h]anthracene | 14.03         | 297        | 0.9998                  |
| Indeno[1,2,3-cd]pyrene | 14.35        | 299        | 0.9993                  |
| Benzo[g,h,i]perylene | 14.37           | 299        | 0.9997                  |
| Column     | C18, 130 Å, 1.7 µm, 2.1 × 100 mm (P/N 186002352) |
|------------|--------------------------------------------------|
| Column temperature | Ambient |
| Solvent A   | 80% Water/20% Methanol |
| Solvent B   | 90% Acetonitrile/10% Methanol |
| Time (min)  | Initial 0.25 3.75 4.00 7.00 7.25 8.00 9.00 10.50 10.60 12.00 12.50 15.00 15.50 15.80 16.00 |
| Solvent A (%) | 66.0 66.0 66.0 61.0 56.0 48.0 48.0 44.0 40.5 37.0 35.0 14.0 14.0 0.0 0.0 66.0 |
| Flow rate (mL min⁻¹) | 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 0.400 |

UPLC, ultra-performance liquid chromatography.
15 µL of analyte-free chicken red blood cells on clean FTA cards and allowing them to dry for 30 min. After drying, each spot was center punched from the FTA card using a disposable punch and transferred to an 8 mL glass sample vial. The laboratory control sample was similarly prepared by obtaining a punch from a blank area of the filter card and transferring it to an 8 mL glass sample vial. The blank spot was treated with 15 µL of chicken red blood cells and immediately fortified with PAH stock solution to ensure mixing of the solution with the blood. The laboratory control sample was allowed to dry for 30 min. Once all samples and controls were punched and transferred to individual vials, 1.920 mL of methanol were added to each. An additional 80 µL of formic acid was then added to assist in lysing dried blood cells and enhancing the extraction. Samples were sonicated for 15 min, followed by vortexing at 2500 rpm for 15 min. A 500 µL aliquot of each extract was removed for phospholipid SPE sample cleanup (Provatas et al. 2013).

**Sample purification**

An Ostr 96-well phospholipid removal plate was placed on the vacuum manifold with the pressure stabilized at approximately 10 psi. A 500 µL aliquot of each sample was transferred to individual wells and eluted. An additional 250 µL of methanol was used to wash each well, yielding a final volume of 750 µL of purified extract. 190 µL were transferred directly to an autosampler vial with a 300 µL glass insert and fortified with 10 µL of the chrysene-d$_{12}$ internal standard. A flow diagram of the phospholipid cleanup protocol is presented in Figure 1.

**Results and discussion**

The validity of the procedure was assessed by determining method detection limit (MDL), precision, and accuracy. The MDL involved the preparation and analysis of seven replicate samples in the same manner as the laboratory controls. The MDL was calculated according to the U.S. Code of Federal Regulations Title 40 part 136, which defines the MDL as:

\[
\text{MDL} = \frac{t_{(n-1,1-\alpha = 0.99)} \times S}{\sqrt{n}}
\]

where \(t_{(n-1,1-\alpha = 0.99)} = 3.143\) and is equal to the Student’s \(t\) value at 99% confidence for six degrees of freedom, and \(S\) is equal to the standard deviation of the seven replicate measurements. At a 100 ng·mL$^{-1}$ fortification concentration, the recoveries and method detection limits for the PAHs ranged from 68.1 ng·g$^{-1}$ for dibenz[a,h]anthracene to 133.1 ng·g$^{-1}$ for acenaphthene as shown in Table 3.

Similarly, precision and accuracy were determined at a fortification level of 1000 ng·mL$^{-1}$ with four replicates. The sample recoveries were utilized to document method accuracy and

![Figure 1. Abbreviated flow diagram of the phospholipid cleanup method.](image)
standard deviation as a measure of precision. The recoveries in Table 4 varied from 63.4% in indeno[1,2,3-c,d]pyrene to 104.1% in benzo[g,h,i]perylene.

Quality control samples were used to establish assay precision and accuracy for each sample batch. These included a system blank, method blank, matrix spike, laboratory control sample, a duplicate of a collected sample, initial calibration verification, and a continuous calibration verification with example chromatograms shown in Figure 2. The results for PAHs in seven avian blood samples (n = 7) and associated quality control sample recoveries are shown in Table 4, with anthracene detected at 120 ng·g⁻¹.

An important aspect of quality control sample preparation is proper spiking of the blood spot to ensure acceptable surrogate and PAH spike recoveries. In early attempts at establishing this method, recoveries were as low as 40% due to diffusion of the spiking solutions across the surface of the card outside the radius of the sample spot. This effect was exacerbated by the immediate coagulation of fresh blood upon contact with the organic solvent in the spiking solutions during preparation of these samples. In a comparison of dried blood spots spiking techniques, Li and Lee (2014) observed similar behavior, with direct spiking of dried blood spots yielding accuracies with a −45% bias. The preferred method involves

| Compound                  | Recovery (%) | Standard deviation (ng·mL⁻¹) | Relative standard deviation (ng·g⁻¹) |
|---------------------------|--------------|------------------------------|-------------------------------------|
| Naphthalene               | 80.9         | 13.7                         | 1.48                                |
| Acenaphthylene            | 82.7         | 14.8                         | 0.77                                |
| Fluorene                  | 79.4         | 7.7                          | 0.93                                |
| Acenaphthene              | 78.0         | 9.3                          | 1.02                                |
| Phenanthrene              | 82.4         | 10.2                         | 1.29                                |
| Anthracene                | 78.6         | 12.9                         | 1.00                                |
| Fluoranthene              | 85.7         | 10.0                         | 1.32                                |
| Pyrene                    | 90.5         | 13.2                         | 0.48                                |
| Chrysene                  | 71.3         | 4.80                         | 1.54                                |
| Benzo[a]anthracene        | 71.5         | 15.4                         | 0.74                                |
| Benzo[b]fluoranthene      | 76.3         | 7.40                         | 0.73                                |
| Benzo[k]fluoranthene      | 66.4         | 7.30                         | 1.76                                |
| Benzo[a]pyrene            | 69.8         | 17.7                         | 1.42                                |
| Dibenzo[a,h]anthracene    | 85.9         | 14.2                         | 1.44                                |
| Indeno[1,2,3-c,d]pyrene    | 63.4         | 14.4                         | 2.40                                |
| Benzo[g,h,i]perylene      | 104.1        | 24.0                         | 1.48                                |
the spiking of blood before spotting on cards that produced recoveries with a +7.9% bias. However, this method is impractical, as requires the individual performing the initial field sampling to spike the blood with the quality control standards prior to spotting on the

Figure 2. Chromatograms of dried blood spot extracts. (a) Matrix fortified with 16 PAHs at 500 ng·mL⁻¹; (b) internal calibration verification of 16 PAHs at 500 ng·mL⁻¹; (c) Collected sample. Naphthalene-d₈, perylene-d₁₂, and chrysene-d₁₂ were fortified at 500 ng·mL⁻¹. The retention times of the 16 PAHs and analytical conditions are displayed in Tables 1 and 2. Note: PAHs, polycyclic aromatic hydrocarbons.
Table 5. Determination of PAHs in a batch of seven avian blood samples \( (n = 7) \) with quality control recoveries.

| Compound                  | Naphthalene | Acenaphthylene | Fluorene | Acenaphthene | Phenanthrene | Anthracene | Fluoranthene | Pyrene | Chrysene | Benz[\(a\)] anthracene | Benz[\(b\)] fluoranthene | Benz[\(k\)] fluoranthene | Benz[\(a\)] pyrene | Benzo[\(g,h,i\)] anthracene | Indeno[1,2,3-c,d] pyrene | Benzo[\(k\)] perylene |
|---------------------------|-------------|----------------|----------|--------------|--------------|------------|--------------|--------|----------|------------------------|------------------------|------------------------|---------------|----------------------------|------------------------|----------------------|
| System blank              | ND          | ND             | ND       | ND           | ND           | ND         | ND           | ND      | ND       | ND                     | ND                     | ND                     | ND            | ND                         | ND                     | ND                   |
| Method blank              | ND          | ND             | ND       | ND           | ND           | ND         | ND           | ND      | ND       | ND                     | ND                     | ND                     | ND            | ND                         | ND                     | ND                   |
| Matrix spike (%)          | 99.4        | 91.8           | 86.7     | 85.9         | 91.2         | 89.2       | 96.0         | 72.5    | 79.2     | 86.4                   | 84.1                   | 72.0                   | 64.3          | 74.7                       | 94.2                   | ND                   |
| Laboratory control standard (%) | 111.0       | 106.2          | 102.2    | 102.2        | 106.1        | 102.5      | 109.5        | 112.3   | 87.6     | 92.3                   | 93.3                   | 98.1                   | 95.0          | 77.1                       | 89.3                   | 109.6               |
| Internal calibration standard (%) | 98.6        | 96.6           | 92.1     | 92.2         | 95.9         | 93.7       | 100.0        | 102.5   | 97.2     | 101.7                  | 102.2                  | 104.2                  | 103.3         | 98.7                       | 97.9                   | ND                   |
| Continuing calibration verification (%) | 95.9        | 97.3           | 95.0     | 91.2         | 96.6         | 97.1       | 101.3        | 106.5   | 90.1     | 101.4                  | 101.3                  | 99.8                   | 107.6         | 100.4                      | 96.8                   | ND                   |
| Actual sample (ng·g\(^{-1}\)) | ND          | ND             | ND       | ND           | ND           | 120.0      | ND           | ND      | ND       | ND                     | ND                     | ND                     | ND            | ND                         | ND                     | ND                   |

ND, not detected; PAHs, polycyclic aromatic hydrocarbons.
cards. Although this may not be an issue for a laboratory entirely performing a study starting from the initial blood sampling through quantitative analysis, this is highly impractical for obtaining field-based samples. Consequently, to obtain acceptable PAH spike recoveries without prespiking blood, the dried blood spots were punched from the card and transferred to a glass vial before the addition of the spiking solution to the dried blood spots. This allowed the spots to retain all of the spiking solution as the only area for diffusion to occur was on the dried blood spot itself. This method produced acceptable recoveries (Table 5) similar to results obtained in whole blood or plasma by other methods developed in our laboratory (Yeudakimau et al. 2013; Provatas et al. 2015).

As a result of this approach, it is possible that the surrogate and PAH spike recoveries are artificially high due to the incomplete mixing with the sample matrix. The likelihood of this, however, is low, as the aggressive extraction conditions in the presence of methanol and formic acid were observed to have liberated nearly all blood from the dried spot, yielding deep brown-red extracts with visible particulates and cards free of any dried blood mass. Variations in recovery are most likely due to losses from the phospholipid SPE step and matrix interferences. Due to the proprietary nature of the sorbent used in the plate, predicting how the analyte will behave is difficult. In addition, the plate is only offered in a 25 mg packing, which may explain the slightly greater recoveries observed for the method detection limit in comparison with the precision and accuracy measurements. Nevertheless, future investigations may benefit from comparing the merits of directly spiking blood before spotting the cards to observe differences in recovery.

**Conclusion**

Liquid extraction with phospholipid cleanup coupled with high-throughput UPLC analysis allowed for the rapid quantification of PAHs in avian dried blood spots at the low ng·mL$^{-1}$ range. The use of an FTA card for the sampling of blood offers multiple unique advantages over traditional samples for analysis. As the dried blood spot is a solid sample, customs requirements are nominal, and the validated analytical methodology may simplify sample preparation and analysis. In addition, as little as 15 µL of blood spotted on a card is sufficient for analysis, which is difficult because of viscosity and volume constraints of whole blood.

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