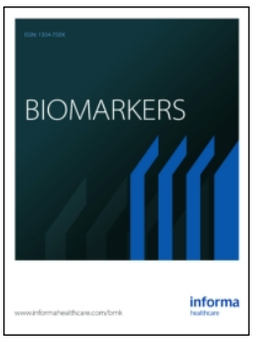

Biomarkers

ISSN: 1354-750X (Print) 1366-5804 (Online) Journal homepage: http://www.tandfonline.com/loi/ibmk20

# Quantification of cotinine in dried blood spots as a biomarker of exposure to tobacco smoke

Daniel Ladror, Bruce Pitt & William Funk

**To cite this article:** Daniel Ladror, Bruce Pitt & William Funk (2017): Quantification of cotinine in dried blood spots as a biomarker of exposure to tobacco smoke, Biomarkers, DOI: 10.1080/1354750X.2017.1375558

**To link to this article:** http://dx.doi.org/10.1080/1354750X.2017.1375558

Accepted author version posted online: 01 Sep 2017.
Published online: 18 Sep 2017.

Submit your article to this journal

Article views: 2

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=ibmk20

**Download by:** [Northwestern University] **Date:** 18 September 2017, At: 09:29

BIOMARKERS, 2017
https://doi.org/10.1080/1354750X.2017.1375558

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Check for updates

# Quantification of cotinine in dried blood spots as a biomarker of exposure to tobacco smoke

Daniel Ladror[a], Bruce Pitt[b] and William Funk[a#]

[a]Department of Preventive Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA; [b]Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA

**ABSTRACT**

**Objective:** We present an ultra-sensitive, minimally-invasive method for quantifying cotinine in dried blood spot (DBS) samples as a biomarker of exposure to tobacco smoke that can be collected using a simple heel or finger prick to obtain blood samples.

**Methods:** Cotinine levels were measured in matched plasma and reconstituted DBS samples from smokers and nonsmokers to evaluate assay parameters. In addition, we applied this new method to finger-prick DBS samples that were collected from infants, children and young adults ages 1–21 to estimate exposure to tobacco smoke. Partitioning of cotinine across red blood cells and haematocrit effects were investigated.

**Results:** Cotinine levels measured in matched plasma and reconstituted DBS samples from smokers and nonsmokers were found to be highly correlated ($R^2$=0.94), with 100% sensitivity and 94% specificity to differentiate reported smokers from nonsmokers. With this method, the LOQ is <0.25 ng/mL using a single 3.2 mm punch of a DBS, and haematocrit effects are negligible.

**Conclusions:** This sensitive, high-throughput and minimally-invasive method for quantifying cotinine in DBS samples provides a simple and cost effective means for estimating exposure to tobacco smoke in population based studies, and has particular advantages in studies involving infants and children.

**ARTICLE HISTORY**
Received 9 May 2017
Revised 18 August 2017
Accepted 30 August 2017

**KEYWORDS**
Cotinine; tobacco smoke; biomarkers; dried blood spots; tandem mass spectrometry

Downloaded by [Northwestern University] at 09:29 18 September 2017

## Introduction

Tobacco use is a leading preventable cause of death worldwide and is responsible for more than 5 million deaths per year (Lopez *et al.* 2006). In addition to exposures from direct smoking, exposure to environmental tobacco smoke (ETS) is a risk factor for cardiovascular disease, lung cancer and stroke (Taylor *et al.* 1992, Al-Zoughool *et al.* 2013). Children exposed to ETS are also at greater risk of ear infections, asthma, respiratory symptoms and infections, and sudden infant death. According to the US Department of Health and Human Services, prenatal exposures to tobacco smoke *in utero* can affect brain development, and exposures to tobacco smoke during foetal development is a risk factor for preterm birth and stillbirth. Consequently, sensitive and specific field-friendly methods for assessing tobacco smoke exposures in epidemiological studies are of critical importance.

According to the American Cancer Society, tobacco smoke is comprised of thousands of chemicals, including more than 70 chemicals that are classified as human carcinogens. However, exposures to harmful chemicals that are present in tobacco smoke can come from other environmental sources as well. For example, tobacco smoke constituents including polycyclic aromatic hydrocarbons (PAHs) are present in automobile exhaust and can be produced by other combustion sources (Alves *et al.* 2015); heavy metals (e.g. lead, arsenic and cadmium) are commonly found in polluted water, dust and air (Dinis and Fiuza 2011); and formaldehyde is commonly released into the air from building materials containing formaldehyde-based resins (Xiong *et al.* 2016). As a results, biomarkers that are specific to tobacco smoke exposure (e.g. cotinine in dried blood spot (DBS) samples) can also provide an important tool for helping to differentiate tobacco smoke exposures from other environmental exposures in epidemiological research.

Self-reporting is a common means of obtaining smoking status. However, such indirect assessment of smoking is prone to reporter bias leading to exposure misclassification (Murray *et al.* 1993). Exposures to ETS can come from multiple sources and are often very difficult to measure using indirect exposure assessment methods. As an alternative, biomarkers can be measured in blood and other biospecimens to provide a more precise estimate of tobacco smoke exposure (Joseph *et al.* 2013, Searles Nielsen *et al.* 2014, Spector *et al.* 2014). While nicotine – a primary constituent in tobacco – has a biological half-life of 0.5–3 hours, its primary metabolite, cotinine, has a biological half-life of 15–20 hours and is the preferred biomarker for estimating tobacco smoke exposure in blood samples (Murphy *et al.* 2013b). However, the logistics associated

CONTACT William E. Funk w-funk@northwestern.edu Department of Preventive Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA
#William Funk was responsible for statistical design and analysis. w-funk@northwestern.edu

© 2017 Informa UK Limited, trading as Taylor & Francis Group

Downloaded by [Northwestern University] at 09:29 18 September 2017

with venipuncture for obtaining blood samples can be a challenge in community-based studies, and is often a significant barrier in studies involving infants and children. This has motivated the use of DBS sampling in public health research as a minimally-invasive alternative to venipuncture, where blood samples can be easily collected using a simple heel or finger stick. DBS samples are also low in cost, easily transported, require minimal sample processing prior to shipment, and can be shipped at room temperature through standard US mail. While these advantages have motivated the use of DBS sampling in several large-scale health surveys in the US (McDade *et al.* 2007), the use of DBS for measuring exposure biomarkers has been more limited and is just starting to gain traction within the scientific community.

While plasma and serum are the gold standard biospecimens for measuring cotinine in blood, cotinine levels have also been measured in whole blood samples (Lewis *et al.* 2003). However, additional research is needed to evaluate matrix effects in whole blood that can help to establish whole blood-specific cotinine guidelines. While DBS methods have been previously developed and applied to quantify cotinine in DBS samples in population-based studies (Murphy *et al.* 2013b, Yang *et al.* 2013), here we report an improved method for estimating exposure to tobacco smoke in DBS samples that has high analytical sensitivity, requires minimal blood volumes, includes simplified analyte extraction, and utilizes DBS-based calibration standards to account for matrix effects.

## Statement of clinical significance

- Provides a simple and minimally-invasive means for assessing exposure to tobacco smoke.
- Approach is applicable to population-based epidemiological research.

## Materials and methods

### Reagents

Native cotinine ((–)-cotinine, ≥98%), ammonium acetate (≥99.0%), d3-cotinine ((±)-cotinine-$d_3$ solution) and methanol (CHROMASOLV, ≥99.0%) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride (≥99.0%) was obtained from J.T.Baker (Charleston, SC). Bovine serum albumin (OmniPur, ≥98.0%) was obtained from Millipore (Temekula, CA). Acetonitrile (HPLC Grade, ≥99.9%) was purchased from Fisher Scientific (Bartlett, IL), and Whatman 903 Protein Saver cards were purchased from GE Healthcare (Pittsburgh, PA).

### Biospecimens

*Smoker and nonsmoker plasma:* Non-fasting blood samples were obtained from 78 male and 80 female healthy smokers and nonsmokers in heparin as previously described (Cai *et al.* 2014). Plasma was separated from red blood cells by centrifugation, and red blood cells were washed with an equal volume of phosphate buffered saline (PBS) and combined with the plasma fractions to maximize plasma recovery. Plasma samples were stored at –80 °C for approximately 13 years before being thawed and aliquoted into 35 pooled samples of smokers and nonsmokers with 4–6 subjects in each pooled sample (required by institutional review board to ensure anonymity of participants), stratified by race, sex and smoking status.

*Smoker and nonsmoker reconstituted DBS:* Blood was collected via venipuncture from a male, nonsmoker volunteer in vacutainers containing EDTA. Plasma and buffy coat were removed and discarded, and the red blood cells were washed three times with equal volumes of PBS, discarding the PBS solution after each wash. Reconstituted whole blood samples were then prepared from each pooled plasma sample by combining 30 µL of plasma with equal volumes of washed nonsmoker red blood cells. Reconstituted whole blood samples were then pipetted onto Whatman 903 Protein Saver cards in 50 µL aliquots and dried overnight before storage at –80 °C with desiccant.

*Finger-prick DBS samples:* Finger-prick DBS samples were obtained from 50 male and female children, adolescents and young adults at the Northwestern Lurie Children's Hospital between the ages of 1 and 21, representing a subset DBS samples from a previous study conducted by the corresponding author under an approved human-subjects protocol (Funk *et al.* 2015). The broad range of participant ages was originally selected to represent the age range of interest in the National Children's Study. After obtaining parental consent (or direct consent if the participant was 18 years of age or older), approximately five drops of blood were collected on Whatman 903 Protein Saver cards. Blood was dried at room temperature for a minimum of four hours before storage at –80 °C with desiccant. All samples were collected and stored devoid of personal identifiers. A description of the biospecimens used in this study are provided in Figure 1.

### Cotinine DBS calibration standards

To minimize matrix effects and maximize comparability between calibration standards and DBS samples, DBS calibration materials were prepared according to methods described by McDade (2014). Blood was collected via venipuncture from a male, nonsmoking volunteer in vacutainers containing EDTA. Plasma and buffy coat were removed and discarded, and the red blood cells were washed three times with equal volumes of PBS, discarding the PBS solution after each wash. Solutions mimicking plasma were then prepared in normal saline (0.86 g NaCl/100 mL deionized $H_2O$) with 5% BSA, spiked with cotinine concentrations at 0, 0.5, 2.5, 5, 8, 10, 50, 100 or 300 ng/mL. Plasma-like calibration standards were then mixed with equal volumes of washed red blood cells, and applied to Whatman 903 Protein Saver cards in 50 µL aliquots, allowed to dry overnight, and stored in gas impermeable plastic bags at –80 °C with desiccant.

### Sample extraction and cotinine quantification

Extraction of metabolites from DBS is commonly performed by extracting the DBS in aqueous solutions, sometimes using

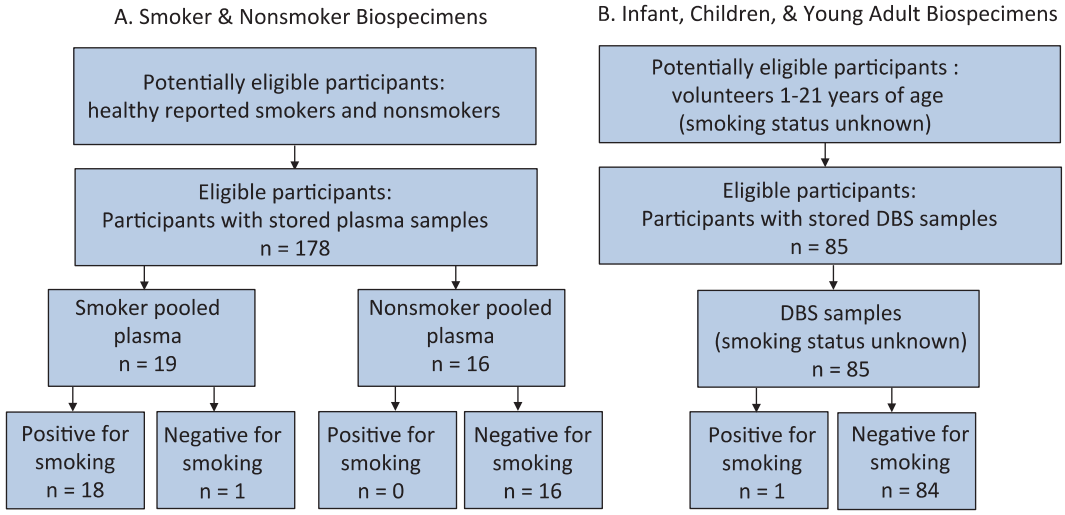


Figure 1. Flow diagram of biospecimens collected from study participants.

Downloaded by [Northwestern University] at 09:29 18 September 2017

sonication to improve recovery (Yang *et al.* 2013), followed by solid phase extraction (Murphy *et al.* 2013b, Tretzel *et al.* 2016) or liquid/liquid extraction (Yang *et al.* 2013) to clean up the sample and reduce sample complexity. However, these procedures are time and labour intensive and thus are prohibitive to high-throughput experiments. To address these limitations, we applied an extraction method using methanol, which has the advantage of extracting cotinine from DBS samples while excluding larger molecules (e.g. proteins), thus simplifying the sample matrix using a single extraction step.

*Cotinine extraction from dried blood spots:* 3.2 mm DBS punches (Figure 2) were extracted in 100 µL of methanol with 25 ng/mL of d3-cotinine added as an internal standard. Extraction was performed using a Barozyme HT48 (Pressure Biosciences, Easton, MA) cycled between atmospheric pressure and 20 kpsi once per minute for a total of 5 min. Each cycle included 50 seconds at high pressure followed by 10 seconds at atmospheric pressure.

*Cotinine extraction from whole blood and plasma:* Cotinine was extracted from whole blood and plasma samples by denaturing 5 µL of plasma or whole blood with 100 µL of methanol to precipitate proteins. Samples were incubated for five minutes at room temperature and centrifuged for 10 minutes at 3280×*g*. The supernatant was then removed and retained for subsequent analysis.

*Cotinine quantification:* Methanol extracts were dried under vacuum and resuspended in 20 µL of 5 mM ammonium acetate (pH 5.4). To remove particulates, resuspended samples were filtered through a Millex-LG 0.20 µm hydrophilic PTFE syringe filter (Millipore, Temecula, CA) and centrifuged for 10 minutes at 3280×*g* before transferred to autosampler vials. Samples were analysed by LC-MS using a 150 mm C18 large capacity LC chip (Agilent Technologies, Santa Clara, CA). Quantification of cotinine was performed using an Agilent 6490 QQQ mass spectrometer integrated with an Agilent 1260 Infinity HPLC-Chip Cube Interface. Chromatographic separation was conducted using 5 mM ammonium acetate buffer at pH 5.4 (A) and 100% acetonitrile (B). One microlitre of each sample was loaded onto the chip's enrichment column using the capillary pump at 4 µL/min, 1% B and eluted on to the analytical column using the nano pump at 0.6 µL/min. Samples were separated over a 15 minute gradient using the following conditions: 0 min 1% B, 5 min 1% B, 12 min 98% B, 12.9 min 98% B, 13 min 1% B, 15 min 1% B. Electrospray ionization was performed using 11 L/min gas flow at 275 °C. MRM transitions monitored were 177.2 *m/z* -> 80.1 *m/z*, 177.2 *m/z* -> 92.0 *m/z*, and 177.2 *m/z* -> 97.9 *m/z* for cotinine, and 180.2 *m/z* -> 80.1 *m/z*, 180.2 *m/z* -> 92.0 *m/z*, and 180.2 *m/z* -> 100.9 *m/z* for d3-cotinine, with collision energy 24, 30 and 23, respectively. All transitions were monitored with unit resolution for both MS1 and MS2, 100 ms dwell time, 380 fragmentor voltage, 5 V cell accelerator voltage, and positive polarity.

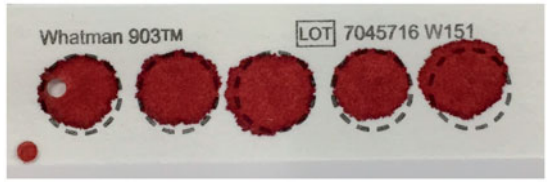


Figure 2. Whatman 903 Protein Saver card typically permits the collection of up to five drops of blood using a minimally-invasive heel or finger prick to obtain blood samples. When drops of blood fill the dotted guidelines they contain approximately 50 µL of whole blood. Here, we report a sensitive and specific DBS assay for quantifying cotinine in a single 3.2 mm punch of blood (shown above).

### Haematocrit effects

Haematocrit effects have been reported as a source of variance for some DBS assay (Wilde *et al.* 2008, O'mara et al. 2011). To evaluate haematocrit effects, additional reconstituted DBS samples were prepared by mixing washed red blood cells from a nonsmoker (as described above) with saline solutions spiked with 50 ng/mL of cotinine and 5% BSA, varying the relative volume of red blood cells from 30 to 60%. Reconstituted whole blood was pipetted onto Whatman 903 Protein Saver cards in 50 µL aliquots, and DBS with resulting 30%, 35%, 40%, 45%, 50%, 55% and 60% haematocrit were assayed in triplicate.

Downloaded by [Northwestern University] at 09:29 18 September 2017

### Partitioning of cotinine in blood between plasma and red blood cells

A possible explanation for reported differences between cotinine levels in plasma and DBS samples is differential partitioning of cotinine across red blood cell membranes in circulating blood (i.e. higher or lower cotinine concentrations in red blood cells compared to plasma). To investigate the effects of cotinine partitioning across red blood cells, whole blood was collected from a male nonsmoker in EDTA and spiked with cotinine at two different concentrations – 6.22 ng/mL and 64.8 ng/mL. Spiked whole blood samples were mixed by inversion and incubated at 4 °C with periodic manual mixing. Aliquots were removed after 0.5, 1, 2, 4, 6 and 8 hours and centrifuged at 1000×*g* for eight minutes to isolate plasma samples. Both plasma and whole blood aliquots were stored at –80 °C until analysed.

### Assay validation and application to DBS samples

When developing new assays using alternative biospecimens (e.g. DBS samples versus plasma or serum), it is important to compare biomarkers in matched samples to evaluate differences in analyte concentrations across sample matrixes. To compare cotinine levels between DBS samples and plasma (i.e. the gold standard for cotinine measurements in blood), we analysed 35 pooled plasma samples and matched reconstituted DBS samples collected from smokers and nonsmokers (Lin *et al.* 2006). Reported smoking status was compared to smoking status based on plasma cotinine levels using cutoff points previously reported by Yang *et al.* (2013), with smokers as having cotinine concentrations >10 ng/mL for plasma and >6 ng/mL for DBS. All samples that had DBS cotinine concentration above the LOQ were run a second time and duplicate values were averaged. Results from matched DBS and plasma samples were used to determine assay sensitivity and specificity.

We then applied this method to 50 stored finger-prick DBS samples that were collected from infants, children and young adults 1–21 year of age to estimate exposures to tobacco smoke using cotinine blood concentrations to establish smoking status. As stored DBS specimens are commonly available without self-reported smoking status recorded (e.g. state-archived DBS samples collected by newborn screening programs), these results serve as proof-of-concept that cotinine blood levels can be independently measured to estimate exposure to tobacco smoke when other information on smoking status is not available.

## Results

### Cotinine in matched reconstituted DBS and plasma samples

Results comparing cotinine concentrations measured in matched reconstituted DBS and plasma samples are summarized in Table 1. As shown in Figure 3, matched plasma and DBS samples were highly correlated, with an $R^2$=0.94. Smokers were defined as having cotinine concentrations >10 ng/mL for plasma and >6 ng/mL for DBS. Using these criteria, 34 out of 35 samples resulted in classifications that matched reported smoking status in both plasma and DBS samples, indicating an assay sensitivity of 100% and a specificity of 94% for differentiating smokers from nonsmokers. Results from replicate runs confirmed our original classification of all smokers and nonsmokers. The one sample that was classified as a smoker based on cotinine concentration, but was classified as a nonsmoker based on self-report, had cotinine concentrations that were just above the cutoff values: 10.27 ng/mL for DBS and 11.06 ng/mL for plasma.

Table 1. Cotinine in pooled plasma and reconstituted DBS samples (pg/μL).

| Sample type | *N* | Max | Min | Mean | 90th percentile | SD |
|---|---|---|---|---|---|---|
| Smoker plasma | 19 | 140.40 | 29.29 | 79.86 | 131.06 | 38.07 |
| Smoker DBS | 19* | 105.20 | 18.74 | 64.98 | 97.26 | 27.51 |
| Nonsmoker plasma | 16 | 10.27 | 0.00 | 1.29 | 4.71 | 2.88 |
| Nonsmoker DBS | 16 | 10.95 | 0.00 | 0.80 | 0.94 | 2.75 |

*Averages are reported for DBS run in duplicate.

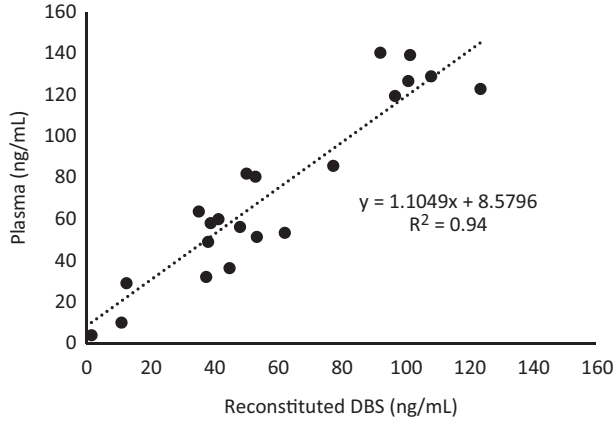


Figure 3. Scatter plot of cotinine in matched reconstituted DBS samples and plasma collected from smokers and nonsmokers. The straight line indicates a perfect fit.

### Quantification of cotinine in finger prick DBS samples

Levels of cotinine measured in finger prick DBS samples collected from infants, children and young adults are summarized in Table 2. The majority of the DBS samples were below the 6 ng/mL cutoff point used to differentiate smokers from nonsmokers. However, one sample from a 13-year-old was found to have a DBS cotinine value just slightly above the cutoff point for smokers, with a cotinine blood level of 6.2 ng/mL. While information on smoking status or exposures to ETS was not obtained from any of the participants, this cotinine value is consistent with light smoking or exposure to second hand smoke. In total, seven samples out of the 50 samples had cotinine concentrations greater than our LOQ of <0.25 ng/mL.

### Evaluation of assay parameters

While DBS extracts were initially centrifuged and filtered using an online 0.50 μm filter prior to analysis, insoluble particulates in the DBS extracts were found to gradually increase the HPLC column backpressure resulting in eventual LC

Table 2. Cotinine in finger prick DBS samples (pg/μL).

| Age group | *n* | Max | Min | Mean | 90th percentile | SD |
|---|---|---|---|---|---|---|
| All | 50* | 6.17 | 0.00 | 0.31 | 0.78 | 1.03 |
| Male | 18 | 6.17 | 0.00 | 0.69 | 2.02 | 1.64 |
| Female | 31 | 0.94 | 0.00 | 0.08 | 0.16 | 0.22 |
| Ages 1–5 | 9 | 3.60 | 0.00 | 0.46 | 0.91 | 1.18 |
| Ages 6–12 | 22 | 1.63 | 0.00 | 0.18 | 0.86 | 0.45 |
| Ages 13–18 | 10 | 6.17 | 0.00 | 0.65 | 0.91 | 1.94 |
| Ages 18–21 | 8 | 0.77 | 0.00 | 0.11 | 0.29 | 0.27 |

*Sex and age were not recorded for one participant.

nano-chip failure when analysing multiple DBS samples. To circumvent this issue, Millex-LG 0.02 μm Hydrophilic PTFE syringe filters were used to remove particulates from DBS extracts. While this additional clean up step increased the time required for sample preparation, it was found to be necessary to increase the longevity of the LC nano-chips with no noticeable decline in MS signal.

The assay LOQ was determined to be <0.25 ng/mL, as defined by the lowest cotinine concentration where calibration standard measured in triplicate resulted in a signal higher than the unspiked blank calibrator measured in triplicate.

### *Partitioning of cotinine across red blood cells*

The primary difference between plasma/serum versus DBS samples is the presence of red blood cells in DBS. To explore potential mechanisms that could cause differences between sample types, we investigated the partitioning of cotinine across the red blood cell membranes in spiked whole blood samples with different incubation times. Levels of cotinine in both plasma and whole blood stabilized after 1 hour, at which time the partitioning of cotinine into the RBC fraction (or binding of cotinine to red blood cell membranes) appeared to achieve steady state. In all cases, the plasma concentrations were found to be consistently higher than the whole blood fraction. The cotinine plasma to whole blood ratio was greater for the 6.22 ng/mL spiked samples than the 64.8 ng/mL spiked samples, with ratios of 1.43 and 1.22, respectively. This finding indicates that the partitioning of cotinine across the red blood cells (or binding of cotinine to the red blood cell membranes) may be influenced by the overall cotinine blood concentration. D3-cotinine was spiked into all samples prior to LCMS analysis and yielded a consistent signal across all samples. This suggests that observed differences between plasma and DBS samples were not due to matrix effects (e.g. differences in instrument response caused by ion suppression).

### *Haematocrit effects*

To examine the effects of haematocrit, reconstituted DBS samples were analysed with haematocrit ranging from 30% to 60%. As shown in Figure 4, peak areas of cotinine were consistent across all percentages of haematocrit. Using Tukey's multiple comparison tests with an $\alpha = 0.05$, no significant differences were observed related to haematocrit.

## Discussion

While previous methods have been reported for measuring cotinine in DBS samples (Spector *et al.* 2007, Sosnoff and Bernert 2008, Yang *et al.* 2012, Murphy et al. 2013a, Spector *et al.* 2014), here we report an new method for quantifying cotinine in DBS samples that has the following improvements: (1) Sample extraction incorporates a Barozyme HT48 pressure barocycler (Pressure Biosciences Inc., South Easton, MA) that provides increased analytical reproducibility, and can extract up to 48 samples at a time, making it amenable to high throughput processing; (2) use of a state-of-the-art Agilent 6490 triple quadruple mass spectrometer coupled with a nano-LC Chip-Cube interface to achieve ultra-high sensitivity using minimal sample volumes (e.g. a single 3.2 mm punch of a DBS), respectively; (3) simplified sample preparation that reduces the complexity of the sample matrix; (4) and quantification performed using DBS-based cotinine calibration standards to more accurately match the DBS sample matrix.

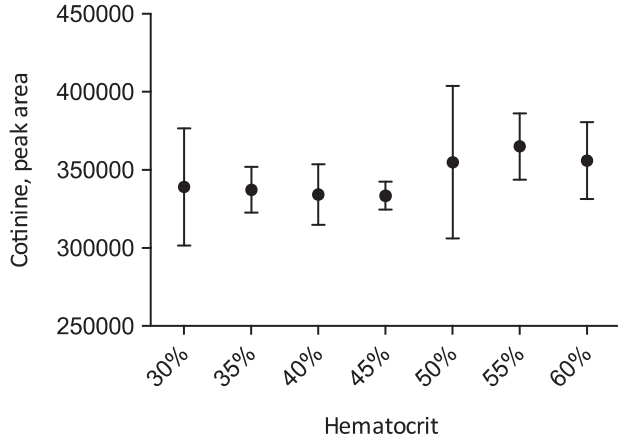


Figure 4. Plot of MS peak area for cotinine in DBS samples with increasing haematocrit analysed in triplicate. Haematocrit effects were not significant in DBS samples ranging from 30% to 60% haematocrit.

Based on self-reported smoking status, we determined an assay sensitivity of 100% and a specificity of 94% for differentiating smokers form nonsmokers. However, smokers and nonsmokers were analysed in pools of 4–6 individuals per group, and the one sample that was incorrectly categorized was just above the cutoff value for smokers. Since smoking status was self-reported, this single discordant result could have been the result of an unreported smoker being included in a nonsmoker pool. As self-reported smoking status is prone to exposure misclassification error, this finding reinforces the importance of obtaining unbiased direct measures of exposures to tobacco smoke, such as biomarker measurements.

To validate our assay in DBS samples collected from children via finger-prick, we quantified 50 archived DBS samples that were collected from infants, children and young adults ages 1–21. As expected, cotinine concentrations were very low in the majority of the sample, with most below the assay LOQ. This was not surprising given none of the children were confirmed to be smokers, or where known to have significant exposures to ETS. In total, seven samples out of the 50 had cotinine concentrations greater than our LOQ of <0.25 ng/mL, and only one sample collected from a 13-year old had a cotinine level of 6.2 ng/mL that was very close to the cutoff point of 6.0 ng/mL for smoking. While this single

Downloaded by [Northwestern University] at 09:29 18 September 2017

Downloaded by [Northwestern University] at 09:29 18 September 2017

spot measurement cannot differentiate between a more recent modest exposure to tobacco smoke, versus a higher exposure more distant in time, this value is consistent with light smoking or exposure to ETS. While information on smoking status or the potential to be exposed to ETS (e.g. having a smoker in the house) was not obtained, these results confirm that finger-prick blood samples can be used as a minimally-invasive way to obtain blood samples from children to independently estimate exposures to tobacco smoke, without an *a priori* knowledge of smoking status. Methods that can directly detect tobacco smoke exposures using sensitive and specific biomarkers are important in studies involving children because indirect exposure assessment methods such as self-report are subject to misclassification bias, where children and young adults that smoke may under report smoking. In addition, this method can help to differentiate between exposures to tobacco smoke (i.e. samples with elevated cotinine), versus other common sources of environmental toxicants. Since tobacco smoke can be a major confounding factor in studies investigating environmental influences on child health outcomes, cotinine levels in DBS samples should be used in epidemiological studies to control for tobacco smoke exposures.

Comparisons between cotinine levels in plasma/serum and DBS samples must be made with caution. While very strong correlations were observed between match plasma samples and reconstituted DBS samples, reconstituted DBS samples were prepared using washed red blood cells that were collected from a nonsmoker. As a result, red blood cells that were present in these samples would not be expected to contribute to the cotinine levels in these reconstituted DBS samples. However, in DBS samples collected directly via finger or heel prick, the measured levels of cotinine will be influenced by cotinine in and/or bound to red blood cells. For example, cotinine has been previously been observed to bind to sulfhydryl groups on the surface of the red blood cell membrane (Asgary *et al.* 2005). While we found that in blood samples spiked with cotinine the ratio of cotinine between plasma and whole blood stabilized after approximately one hour, the cotinine plasma to whole blood ratio was greater for samples with lower total cotinine levels (6.22 ng/mL) than higher total cotinine levels (64.8 ng/mL), with ratios of 1.43 and 1.22, respectively. These results indicate that partitioning of cotinine into the red blood cells and/or binding of cotinine to the red blood cell membranes is influenced by the overall cotinine concentrations in blood. While further studies are required to confirm these results, these findings suggest that whole blood (e.g. DBS samples) may provide a better estimate of tobacco smoke exposure than plasma/serum, because whole blood captures total blood cotinine levels, and therefore would not be influenced by the effects of differential partitioning of cotinine across blood fractions at different blood cotinine levels.

## Conclusions

We have developed a sensitive, low-cost and minimally-invasive assay for quantifying cotinine in DBS samples to estimate exposure to tobacco smoke. This new assay has a LOQ of $<0.25$ ng/mL using a single 3.2 mm DBS punch, and has high specificity and sensitivity for differentiating smokers from nonsmokers. As this assay requires significantly less than a drop of blood, which can be collected using a minimally-invasive heel or finger prick, it is particularly well suited for estimating exposures to tobacco smoke in epidemiological studies involving infants and children.

## Acknowledgements

The authors thank Pressure Biosciences, Inc. for providing access to the Barozyme HT48 system used in this work. The authors appreciate the assistance of Stephen Rappaport and Hasmik Grigoryan from UC Berkeley who provided the archived pooled plasma samples from smoker and nonsmoker volunteers.

## Disclosure statement

The authors declare no competing financial interests.

## References

Alves, C.A., *et al.*, 2015. Elements and polycyclic aromatic hydrocarbons in exhaust particles emitted by light-duty vehicles. *Environmental science and pollution research*, 22, 11526–11542.

Al-Zoughool, M., *et al.*, 2013. Exposure to environmental tobacco smoke (ETS) and risk of lung cancer in Montreal: a case-control study. *Environmental health*, 12, 112.

Asgary, S., Naderi, G., and Ghannady, A., 2005. Effects of cigarette smoke, nicotine and cotinine on red blood cell hemolysis and their –SH capacity. *Experimental and clinical cardiology*, 10, 116–119.

Cai, X., *et al.*, 2014. Untargeted lipidomic profiling of human plasma reveals differences due to race, gender and smoking status. *Metabolomics*, 4, 1–8.

Dinis, M.D. and Fiuza, A., 2011. Exposure assessment to heavy metals in the environment: measures to eliminate or reduce the exposure to critical receptors. In: L.I. Simeonov, *et al.* (eds.), *Environmental heavy metal pollution and effects on child mental development: risk assessment and prevention strategies*. © Springer Science+Business Media B.V., p. 27–50. DOI: 10.1007/978-94-007-0253-0_2

Funk, W., *et al.*, 2015. Use of dried blood spots for estimating children's exposures to heavy metals in epidemiological research. *Journal of environmental & analytical toxicology*, S7, 002.

Joseph, A., *et al.*, 2013. Biomarker evidence of tobacco smoke exposure in children participating in lead screening. *American journal of public health*, 103, e54–e59.

Lewis, S.J., *et al.*, 2003. Cotinine levels and self-reported smoking status in patients attending a bronchoscopy clinic. *Biomarkers*, 8, 218–228.

Lin, Y., *et al.*, 2006. Variability of albumin adducts of 1,4-benzoquinone, a toxic metabolite of benzene, in human volunteers. *Biomarkers*, 11, 14–27.

Lopez, A.D., *et al.*, 2006. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet*, 367, 1747–1757.

Mcdade, T.W., 2014. Development and validation of assay protocols for use with dried blood spot samples. *American journal of human biology*, 26, 1–9.

Mcdade, T.W., Williams, S., and Snodgrass, J.J., 2007. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography*, 44, 899–925.

Murphy, S.E., *et al.*, 2013a. Cotinine and trans 3′-hydroxycotinine in dried blood spots as biomarkers of tobacco exposure and nicotine metabolism. *Journal of exposure science and environmental epidemiology*, 23, 513–518.

Murphy, S.E., *et al*., 2013b. Cotinine and trans 3′-hydroxycotinine in dried blood spots as biomarkers of tobacco exposure and nicotine metabolism. *Journal of exposure science and environmental epidemiology*, 23, 513–518.

Murray, R.P., *et al*., 1993. Error in smoking measures – effects of intervention on relations of cotinine and carbon-monoxide to self-reported smoking. *American journal of public health*, 83, 1251–1257.

O'mara, M., *et al*., 2011. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis*, 3, 2335–2347.

Searles Nielsen, S., *et al*., 2014. Accuracy of prenatal smoking data from Washington State birth certificates in a population-based sample with cotinine measurements. *Annals of epidemiology*, 24, 236–239.

Sosnoff, C.S. and Bernert, J.T., 2008. Analysis of cotinine in dried blood spots by LC APCI tandem mass spectrometry. *Clinica chimica acta; international journal of clinical chemistry*, 388, 228–229.

Spector, L.G., *et al*., 2007. Detection of cotinine in newborn dried blood spots. *Cancer epidemiology, biomarkers & prevention*, 16, 1902–1905.

Spector, L.G., *et al*., 2014. Prenatal tobacco exposure and cotinine in newborn dried blood spots. *Pediatrics*, 133, e1632–e1638.

Taylor, A.E., Johnson, D.C., and Kazemi, H., 1992. Environmental tobacco-smoke and cardiovascular-disease – a position paper from the council on cardiopulmonary and critical care, American-Heart-Association. *Circulation*, 86, 699–702.

Tretzel, L., *et al*., 2016. Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing. *Journal of pharmaceutical and biomedical analysis*, 123, 132–140.

Wilde, H., *et al*., 2008. Influence of hematocrit as a pre-analytical error for dried blood spot analysis. *Clinical chemistry*, 54, A225–A226.

Xiong, J.Y., *et al*., 2016. Comprehensive influence of environmental factors on the emission rate of formaldehyde and VOCs in building materials: correlation development and exposure assessment. *Environmental research*, 151, 734–741.

Yang, J., *et al*., 2012. Cotinine in newborn dried blood spots as a biomarker to measure in utero tobacco smoke exposure at delivery. *American journal of epidemiology*, 175, S81–S81.

Yang, J., *et al*., 2013. Levels of cotinine in dried blood specimens from newborns as a biomarker of maternal smoking close to the time of delivery. *American journal of epidemiology*, 178, 1648–1654.

Downloaded by [Northwestern University] at 09:29 18 September 2017