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Anna Fretheim Date

Using Meconium as an Alternative Matrix to Measure Cumulative Fetal Exposure to Phthalates

By

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Bachelor of Science (Environmental Health)
University of Washington
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Thesis Committee Chair: Dana Boyd Barr, Ph.D.

An abstract of
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Rollins School of Public Health of Emory University
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Abstract

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By Anna Fretheim

Phthalate diesters are a class of synthetic chemicals that are widely used in industrial and consumer products. Phthalates are known endocrine disruptors and exposure in all age groups has been linked to negative health outcomes. Developing fetuses are considered most vulnerable to their potential effects. The determination of prenatal exposure to phthalates and other compounds is difficult, with maternal exposure commonly used as a proxy. This is a preliminary study aimed to characterize the validity of using meconium as an alternative matrix for determining cumulative exposure to phthalates over half of pregnancy. We have developed a novel method for analyzing ten phthalate metabolites in meconium. 40 meconium samples underwent solid phase extraction and were analyzed via high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). All ten metabolites were present at detectable levels in the majority of the meconium samples. Nine of the metabolites were compared to NHANES urinary concentrations in the general population and were found to be significantly higher in the meconium. We conclude that phthalate concentrations in meconium likely represent cumulative fetal exposure for over half of pregnancy.

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Acknowledgements

Most of all, I would like to thank Dr. Dana Boyd Barr for her unending support and encouragement from the beginning of this project to the end, for offering me access to the meconium samples, and for providing me the opportunity to work and learn in her laboratory. The opportunity to work closely with her has proven to be my most valuable experience during my program, and she has been and will continue to be an inspiration to me. Thank you to Dr. Parinya Panuwet and Anthony Murphy for their invaluable dedication to the project's method development, to Priya D'Souza for her support throughout the project, and to Elizabeth Marder for her often-warranted inspirational encouragement. Thank you as well to my family and friends, without whom I would not have made it this far. I would also like to thank and acknowledge the NIH for financially supporting this project with grant 5R00ES020346-05 (PI: Dr. Joe Braun).

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1. Introduction

1.1. Phthalate Diesters

Phthalate diesters (phthalates) are a class of synthetic chemicals that are widely used in a variety of industrial and consumer products. Phthalates have been in use since the 1920s, and are contained in products such as those made from soft polyvinyl chloride (PVC), personal care products, scented products, and pharmaceuticals [2]. Phthalates are widely used as additives to PVC products to increase softness and improve flexibility and resilience, and can constitute up to 40% of the mass of PVC products [3]. Millions of tons of phthalates are produced and used commercially worldwide each year [4]. Because of their widespread use, exposure of the population to phthalates is ubiquitous and occurs with high frequency in every age group.

Phthalates consist of dialkyl and alkyl aryl esters of the compound o-phthalic acid. They can be classified as either low or high molecular weight depending on their alkyl chain length, which can range between three and ten carbon atoms [2]. The molecular weight categories indicate their toxic effects and usage. Low molecular weight (LMW) phthalates have short alkyl chains between three and six carbon atoms, and include di-methyl phthalate (DMP), di-ethyl phthalate (DEP), and di-butyl phthalate (DBP). LMW phthalates are mainly utilized as aerosol delivery agents, scent holders, and cosmetic emollients. They are present in colognes, perfumes, lotions, shampoos, cosmetics, and a variety of other personal care products [5]. High molecular weight (HMW) phthalates have chain lengths of seven to ten carbon atoms and include di-2-ethylhexyl phthalate (DEHP), butyl benzyl phthalate (BBzP), di-n-octyl phthalate (DnOP), di-isononyl phthalate (DiNP), and di-isoecyl phthalate (DiDP). They

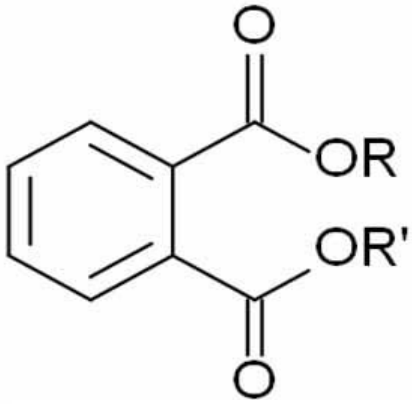


Figure 1. Basic Molecular Structure of Phthalate Diesters [1]

are used as plasticizers in PVC products and are present in adhesives, food packaging, and vinyl products [5]. Phthalates have been shown to leach from products over time because they are not strongly bound to the materials.

Exposure to phthalates is widespread among the population and occurs dermally, orally, and through inhalation. While they are not environmentally persistent chemicals due to their short half-lives in the environment and the body, phthalates have been deemed "pseudo-persistent". While it is a misnomer, it is used because of the nearly continuous or continual nature of the population's exposure to the compounds [2]. The major route of exposure of the general adult population to HMW phthalates is ingestion from widespread contamination of food through its processing (e.g., PVC gloves) and storage (e.g., plastic packaging materials) [6-8]. Phthalates are also present at high levels in indoor dust, which can be inhaled or ingested through hand-to-mouth contact; this is a particularly significant route of exposure for young children [7]. Dermal contact is an important source of exposure to LMW phthalates because of their presence in personal care products. As a result, exposure to LMW phthalates follows a cyclical or continual pattern with use of personal care products, and women are more highly exposed to LMW phthalates than are men [8]. Knowledge about the levels of phthalates that developing fetuses are directly exposed to *in utero* is limited likely because of inadequate frequency of prenatal sampling and exposure variability. Maternal levels of phthalates and other contaminants are often used as a proxy for fetal exposure. For example, maternal urinary levels of phthalates, BPA, and pesticides have been used to approximate fetal exposure to these compounds [9-11].

Following intake into the body, phthalates immediately undergo rapid hydrolysis into their respective hydrolytic monoesters. This is the dominant metabolic process for LMW phthalates [12]. The hydrolytic monoesters of some phthalates—mainly HMW phthalates—will then also

undergo Phase 1 oxidative and/or elimination metabolism. Both hydrolytic monoesters and oxidative/elimination metabolites then undergo Phase 2 metabolism to become glucuronidated and are ultimately excreted in urine. As a result, HMW phthalates are more present in the urine as glucuronidated oxidative metabolites while LMW phthalates are mainly present as glucuronidated hydrolytic metabolites. The biological half-lives of phthalates in the body are short, and they are rapidly excreted in the urine less than 24 hours post exposure [13].

1.2. Health Effects

Exposure to phthalates has been linked to a variety of adverse health effects during both the prenatal and postnatal exposure periods. Phthalates are endocrine disruptors (or endocrine modulators) that exhibit antiandrogenic effects. The introduction of phthalates and other environmental chemicals in the maternal-fetal compartment contributes to an altered *in utero* environment [14]. While the developing brain is thought to be relatively elastic and resistant to low-dose chemical exposures, if exposures are chronic or high enough in acute doses then they may lead to irreversible changes [15]. The prenatal stage is considered to be the most vulnerable life stage to negative effects from exposure to phthalates because organogenesis and neurodevelopment occurs during this period [16]. Disruption of organogenesis by environmental chemicals can lead to abnormal development and negative health effects either immediately or later in life [16]. Organogenesis occurs most rapidly during early pregnancy, and most organ-forming processes—including neuronal and gland differentiation—commence within the first several weeks of fertilization. These processes are mediated by complex hormone signaling systems. The systems that have been demonstrated to be the most sensitive to the influences of environmental chemicals include the pituitary gland, sex organ, and neurological systems [16].

Any disruption of these intricate processes by exposure to phthalates or other environmental chemicals can cause irreversible changes.

Prenatal exposure to phthalates has been shown to induce germ cell loss, impact reproductive hormone levels, reduce anogenital distance and penile size, and affect pubertal development through reduced uterus size and decreased bone age-chronological age ratio [17-20]. Prenatal phthalate exposure has also been linked to inhibited neurodevelopment, as demonstrated by the increased occurrence of behavioral problems and evidence of reduced mental and psychomotor development [21-26]. Preterm and low-birth-weight infants have been found to have higher exposures to phthalates than full-term and full-weight infants, and women exposed to higher levels of phthalates during pregnancy have higher odds of delivering preterm [27-29]. Prenatal exposure has also been shown to increase the risk of asthma symptoms and respiratory tract infections throughout childhood [9].

Postnatal exposure of children to phthalates has been linked to altered reproductive hormone levels, altered thyroid function, increased blood pressure, and insulin resistance [30-34]. Exposure has also been strongly linked to multiple respiratory conditions including airway inflammation, asthma, and allergic symptoms [35-38]. In addition, an association between childhood exposure to phthalates and both the diagnosis and symptom severity of ADHD has been reported [39, 40]. Adult exposure to phthalates has also been linked to a variety of effects including altered hormone levels, allergies, breast cancer, infertility, and reduced sperm count and quality [41-51].

Because of the extensive hazard and risk characterization of LMW phthalates, restrictions have been adopted in Europe, and to a lesser extent in the United states, on the use of selected phthalates in toys and childcare articles [6]. In the United States, the manufacture, import, or sale

of children's toys or childcare articles containing more than 0.1% of DEHP, DBP, or BBP is prohibited; childcare articles and toys that can be placed in a child's mouth are also prohibited from containing more than 0.1% DINP, DIDP, or DnOP [52].

1.3. Biomonitoring

Biomonitoring is the measurement of environmental chemicals, their metabolites, or reaction products in human biological matrices. Biomonitoring provides a valuable snapshot of the amount of chemicals that enter the body from the surrounding environment, including water, air, dust, soil, consumer products, and food. Unlike external measurements of chemicals in the environment that people are potentially exposed to (ambient environmental monitoring), biomonitoring provides an indication of the amount of a chemical that has actually entered the body—the internal dose [12]. Levels of chemicals measured within the body represent an integrated measure of exposure from all sources and routes [12]. Biomonitoring can be used to indicate what chemicals are entering people's bodies and the levels at which they are found, and can demonstrate how exposure trends differ spatially and temporally [4]. It can be used to determine the amount of people with chemical levels above known toxicity levels, as well as identify groups who are at higher risk for chemical exposure. Data from biomonitoring can fuel further studies on exposures to toxic chemicals and their potential health effects, as well as influence and demonstrate the impact of environmental policies, regulations, and interventions.

A variety of biological matrices can be used for biomonitoring. Matrices such as urine, blood, breast milk, saliva, adipose tissue, stool, bone, hair, and placenta have all been successfully utilized. Advances in analytical chemistry have made it possible for highly sensitive and selective

techniques to accurately measure increasingly lower levels of chemicals in these biologic tissues [12]. The type of environmental chemical determines how it will be metabolized and distributed in the body, and consequently which tissues and organs it or its metabolites will be most present in. The matrices differ significantly in the ease and availability of their collection and analysis [12]. Many biological matrices have low sample availability, with only small amounts being able to be collected from each individual [53]. Blood is a commonly used matrix for measuring biomarkers and is ideal for determining current exposure of many chemicals as it is in equilibrium with the tissues where chemicals are deposited, but its relatively invasive nature limits it in several ways. Blood samples are normally small in volume, they often cannot be collected from children and infants, and its requirement significantly lowers participation rates [2, 54]. Hair and nails can be well representative of some exposures, but external sample contamination is a major issue [54]. Urine is the most frequently used matrix because of the relative ease and noninvasiveness of its collection in comparison with other matrices. Its main disadvantage is its variable dilution, but this can be corrected for in some instances via creatinine adjustment if the population tested is mostly homogenous (e.g., all children in a narrow age range). With heterogeneous populations, creatinine adjustment is more problematic.

Because of the widespread presence of phthalates in the environment, sample contamination is a major concern. Therefore, their metabolites are measured in place of the parent phthalates to control for the potential contamination. Phthalates are hydrolyzed rapidly upon entering the body, but their hydrolytic metabolites have very short half-lives (70% are excreted within 24 hours) [2]. However, their oxidative metabolites are considered to be longer-lasting within the body as they exhibit approximately 2-7% of the excretion rate, and are therefore considered stronger biomarkers before elimination. Phthalate metabolites are most commonly

measured in urine as they are present at roughly ten times the level normally found in serum; in addition, levels in urine and serum have been demonstrated to be highly correlated [2]. On the contrary, levels in breast milk have not been found to be correlated with levels in urine, serum, and saliva [55]. It is unclear how well levels correlate between the commonly collected matrices and less common ones such as placenta and cord blood.

Although biomonitoring is useful and beneficial, the field has several limitations in addition to the frequent difficulty of matrix collection. Biomonitoring provides a snapshot of the concentration of chemicals in the chosen matrix at the time of collection—it may be representative of cumulative exposure to a chemical over an individual's lifetime or it may only represent recent exposure, depending on the chemical's half-life in the matrix. For phthalates, several studies have suggested that single spot urine samples may be valid to estimate longer-term exposure, potentially up to several months [56-59]. Despite this, it is widely agreed that spot urine samples can lead to a high degree of exposure misclassification, so the inclusion of single urine samples in studies should be used sparingly, while multiple (potentially pooled) samples are preferred [2]. In addition, first morning samples are ideal since they potentially represent the accumulation of metabolites in urine overnight, preventing complications from diurnal intrapersonal variation in exposure. Biomonitoring measurements also cannot provide information on the frequency or magnitude of exposure unless repeat longitudinal samples are collected. In addition, post-collection sample contamination is a major issue in biomonitoring. For example, it is possible for esterases in some matrices such as blood to continue to hydrolyze phthalates exogenously; therefore, in these matrices the oxidative metabolites are more likely to accurately represent exposure than the hydrolytic metabolites [2].

Little is understood about the types and quantities of environmental chemicals such as phthalates that the fetus is exposed to during pregnancy. The majority of biomonitoring of fetal exposure to chemicals is performed by using concentrations of chemicals or their metabolites in maternal matrices such as urine or blood as proxies. However, maternal urine may reflect only recent, short-term exposures to various chemicals instead of total exposure throughout pregnancy. Urine samples must be taken at multiple time points in order to best approximate long-term exposure, although this would prove expensive in terms of the cost of analysis and participant burden.

In addition, it is unclear from the levels of chemicals in maternal urine and other maternal matrices what the exposure to the fetus is. There is little knowledge of how indicative maternal exposures are of fetal exposures, including what quantity of the chemical passes from the mother to the fetus through the fetal placental barrier. It is problematic to accurately determine fetal exposure to a chemical without directly measuring levels in fetal matrices. However, fetal matrices are normally difficult to obtain and measure due to the inaccessibility of the fetus. Potential fetal matrices include cord blood, cord tissue, placenta, fetal liver (in stillborns), and amniotic fluid [16, 60]. Levels of contaminants in cord blood and cord tissue have been found to be significantly correlated with levels in maternal blood when the measurements were taken during late gestation or at term [16]. Placenta has been utilized as a matrix; however, because it has both maternal and fetal origins, it represents a mixed exposure. Additionally, it is a large and non-homogenous matrix, making representative sampling difficult. Removal of the maternal section before analysis is desired, yet complex and difficult [61]. An additional matrix, meconium, has the potential to be a reliable, easily accessible matrix that may indicate cumulative fetal exposure to chemicals throughout half of pregnancy.

1.4. Meconium

Meconium is the first bowel movement of an infant. It begins to accumulate in the gastrointestinal tract of the fetus between the 12^{th} and 16^{th} week of gestation. Chemicals that are present in the mother's body can pass through the semipermeable membrane of the placenta via passive and active transport, entering the exposure zone of the fetus. Thus, any chemical that the mother is exposed to that crosses the placental barrier may also expose the fetus. These chemicals may then be present in meconium, which forms through bile secretion and the swallowing of amniotic fluid by the fetus. It is normally expelled postnatally within one to three days after birth. After it is expelled, meconium can be collected from the diaper and analyzed for a variety of environmental chemicals and their metabolites.

Because it accumulates over approximately half of pregnancy, meconium has the potential to indicate total cumulative fetal exposure to toxicants during that time period if it acts as a "sink" for the toxicants. Several studies have demonstrated that levels of certain chemicals are present in higher levels in meconium than in other matrices, suggesting that it does act as a sink. For example, meconium has been demonstrated to contain comparatively high, dose-dependent levels of nicotine, cotinine, methadone, and pesticides [62-65]. Meconium has often been used as a matrix to determine fetal exposure to drugs of abuse and other xenobiotics. This implies that it can be valid and useful as a dosimeter for prenatal exposure to these and other environmental chemicals [66]. Ramirez *et al.* detected mercury in meconium samples and found that it correlated significantly with the level of mercury in maternal blood [67].

As a biological matrix, meconium presents numerous advantages. It is very easy to collect from the infant's diaper and it is a matrix that would otherwise be discarded, so its collection is

noninvasive. It is generally present in relatively large masses (e.g., several grams), so sample mass is not as much of an issue as with some other matrices.

One drawback to using meconium as a matrix for exposure to environmental chemicals is that it cannot indicate exposure during the first trimester of pregnancy because it does not begin to form until between the 12^{th} and 16^{th} weeks of gestation. This misses a critical window of exposure during which organogenesis is most easily disrupted and exposure to environmental chemicals is potentially the most important. However, exposure during the 12^{th} week of pregnancy and on is an important window that can drastically affect fetal development and health. Organogenesis processes continue to occur until close to the end of the second trimester [16]. In addition, disruption of organogenesis is not the only mechanism by which exposure to environmental chemicals could affect developmental health. It is important to characterize exposure during this time period to better understand how fetal, early, and later life health is affected by *in utero* exposure.

There are additional potential drawbacks to using meconium as a matrix. It could become contaminated with urine while it is in the diaper. Phthalate metabolites that are present in urine may then enter the meconium, producing artificially heightened measurements. However, if meconium truly is a "sink" for the chemical contaminants then urine contamination levels should be far below the actual meconium levels. It is also a 'one-shot' matrix, meaning that it can only be collected once from each infant; any loss of sample or laboratory error ends up being irreversible. However, typically a large amount of meconium can be collected, enabling multiple analyses. Similarly, if meconium is expelled by the fetus in the womb, it cannot be analyzed.

Three previous studies have measured levels of phthalates in meconium. Kato *et al.* developed an analytical method for quantifying phthalate metabolites in meconium samples

utilizing on-line solid phase extraction (SPE) and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [66]. They also found detectable levels of metabolites in five meconium samples. Zhang *et al*. performed a nested case-control study on 88 low birth weight cases and 113 controls and found that phthalate levels in meconium were significantly correlated with low birth weight [27]. They also noted that meconium contained a much greater amount of phthalate metabolites than the parent phthalates, as opposed to cord serum. Li *et al*. detected MEHP levels in 135 out of 201 meconium samples and found that levels in meconium were correlated with levels in cord blood, and were much higher in the meconium [68]. The reported methods were far from reproducible in our laboratory, suggesting some limitations in the methodology. Here, we developed a novel analytical method to overcome these limitations and to afford its use in a large-scale epidemiologic study. Our method quantifies 10 phthalate metabolites in meconium. Additionally, analyses were performed on 40 preliminary samples from the Health Outcomes and Measures of the Environment (HOME) Study to validate the use of meconium as a biomarker for cumulative fetal exposure to phthalates during pregnancy.

The specific aims of this research are to: (1) develop and validate a method for measuring 10 phthalate metabolites using solid phase extraction and high performance liquid chromatography-tandem mass spectrometry; and (2) measure 10 phthalate metabolites in 40 preliminary meconium samples and perform descriptive univariate analyses of the measured concentrations. We predict that all ten phthalate metabolites would be found at detectable levels in the majority of meconium samples, and at higher levels than the population baseline levels of urinary phthalates. This thesis will contribute to the field of biomonitoring and improve our understanding of the use of meconium as a biomonitoring matrix.

2. Methods

2.1. Source of Samples

The Health Outcomes and Measures of the Environment (HOME) Study is a NIH-funded (NIH5R00ES020346-05) prospective cohort study following 468 woman-child pairs in Cincinnati, Ohio. Women were recruited from March 2003 – January 2006 between their 13^{th} and 19^{th} weeks of gestation and were followed until the children were 5 years of age. The goal of the HOME Study is to explore the impact of low-level pre- and post-natal environmental chemical exposures on growth and development, particularly neurobehavioral outcomes. The women were interviewed for demographic and social information upon recruitment, and provided spot urine samples at 16 and 26 weeks' gestation and 24 hours after birth. The children's growth and development was recorded at 1 and 5 years of age. 362 infant meconium samples were collected after birth and were provided for the analysis of phthalate and BPA levels, for the future purpose of exploring a potential link with sexually dimorphic characteristics. For this study, phthalate levels were measured in a subset of 40 samples to develop a preliminary determination of meconium's usefulness as a matrix for cumulative exposure over half of pregnancy.

2.2. Basis of Method

One method for the analysis of phthalate metabolites in meconium has been previously published by Kato *et al.* This method consists of off-line solid phase extraction (SPE), followed by on-line SPE and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). In brief, they added H_3PO_4 to 0.5 gram aliquots of meconium to denature enzymes and free adsorbed metabolites, followed by NaOH to adjust the pH, internal standard, and β-

glucuronidase. The samples were incubated for 90 minutes, combined with formic acid, and ultra-centrifuged at 25,000 rpm for 60 minutes. The supernatant was collected and metabolites were extracted using off-line SPE (eluted with acetonitrile), and then evaporated to dryness and reconstituted for analysis using online HPLC-MS/MS. When attempting to repeat this method, we could not appropriately duplicate the results. Thus, we used this method as a baseline for the development of a new method that would allow for the use of lower centrifuge speeds and meconium aliquots of lower mass, while still retaining the steps of off-line SPE and HPLC-MS/MS analysis.

2.3. Standards and Quality Control

Ten standard spiking solutions were prepared for the experiment via serial dilutions of the stock solution containing isotopically labelled internal standard with acetonitrile. Standard solutions containing Standards 1 – 10 were produced for the creation of a standard curve. Concentrations for the standards ranged from 2 or 4 ng/mL to 1000 or 4000 ng/mL depending upon the analyte. Two quality control solutions (QCL with a concentration of 150 ppb and QCH with a concentration of 500 ppb) were prepared via serial dilutions of the stock solution with acetonitrile and were used to prepare a QCL and QCH sample for each run. A blank sample was produced for each experimental run to determine the level of laboratory contamination of the samples. All standards were stored in amber vials and kept at -5°C.

2.4. Equipment and Materials

The equipment needed for the protocol included a vortex, sonicator, centrifuge, ultracentrifuge, Tissue Tearor homogenizer (BioSpec, Bartlesville, OK), Turbovap evaporator (Zymark Corporation, Framingham, MA), glass tubes (washed to be BPA-free with 2 mL of methanol and 2 mL of BPA-free water), polypropylene centrifuge tubes (Agilent Technologies, Santa Clara, CA), 5 mL LUER-lock plastic syringes and 1 µm syringe filters (Thermo Scientific Hampton, New Hampshire), Strata-XL 100µm Polymeric Reversed-Phase extraction cartridges (Phenomenex, Torrance, CA), and HPLC glass vials with non-reactive glass inserts (Agilent Technologies, Santa Clara, CA). General laboratory equipment included glassware, pipettes, and a chemical fume hood. Solvents and reagents were all analytical grade and included BPA-free water (prepared using Milli-Q water run through Strata C18-E 10g/60mL Giga Cartridges, Phenomenex, Torrance, CA), β-glucuronidase in 0.2M ammonium acetate, 1.0M ammonium acetate buffer, 0.1M formic acid, acetonitrile, and methanol.

2.5. Procedure

0.25 grams of each meconium sample was aliquotted into a polypropylene centrifuge tube and centrifuged for 10 minutes at 2,000 RPM to collect the meconium at the bottom. Each sample was then spiked with 25 µL of isotopically labeled phthalate internal standards (concentration of 400 mg/mL) for normalization of mass spectrometry signal allowing automatic extraction recovery after analysis. Following this, 3 mL of 1.0M ammonium acetate buffer was added, and the samples were vortex mixed for 1 min. The samples were then homogenized for 1 min, 1 mL of β-glucuronidase buffer was added, and they were further vortex mixed for 2 min. If a meconium

sample was presumed to be less than 0.25 grams, it was not aliquotted and remained in its collection jar; internal standard and ammonium acetate buffer were added directly to the collection jar, it was covered in Parafilm, and was sonicated at 20°C for 5 minutes. After homogenization and addition of β-glucuronidase, the collection jars were vigorously shaken and their contents pipetted into polypropylene centrifuge tubes, after which they adhered to the normal procedure.

Samples were then incubated at 37°C overnight to liberate glucuronide bound phthalate metabolites. To stop the deconjugation reaction, 2 mL of 0.1M formic acid was added to each sample immediately after stopping incubation, followed by brief vortex mixing. Next, the samples were ultra-centrifuged at 13,000 RPM for 60 minutes. The supernatant was then poured into LUER-lock syringes attached to 1 µm syringe filters and injected into new glass tubes.

Polymeric reversed-phase extraction cartridges were then attached to racks and glass manifolds for off-line solid phase extraction. The cartridges were conditioned with 2 mL of methanol and 2 mL of BPA-free water. After conditioning, the supernatant was load into each cartridge and washed with 2 mL of 0.1M formic acid, 3 mL of 40% aqueous methanol, and 3 mL of BPA-free water. Vacuum pressure was then applied for 0.5 minutes to dry the cartridges. Empty glass tubes were placed in the manifolds and the cartridges were eluted with 3 mL of acetonitrile. 1 mL of the acetonitrile was aliquotted into new glass tubes for BPA derivatization, while the remaining 2 mL were evaporated in the TurboVap to almost dryness (50°C at 10-15 psi for 10-15 min), washed with 1 mL of acetonitrile, and further evaporated to total dryness. Next, the samples were reconstituted with 100 µL of BPA-free water, vortex mixed, and transferred to HPLC glass vials with non-reactive glass inserts for analysis using HPLC-MS/MS.

2.6. HPLC-MS/MS Operating Conditions

Chromatographic separation of the samples was completed using Agilent HPLC coupled in tandem to an Agilent triple quadrupole MS with a G1367E autosampler (Agilent Technologies, Waldbronn, Germany). Nebulizer gas flow (nitrogen) was 5 L/min at a temperature of 350°C. The electrospray ion source was used with Agilent's proprietary Jet Stream nebulization. The binary pump on the HPLC was model G1312B and operated at a mobile phase flow of 0.2 mL/min. Mobile phase A was 0.1% acetic acid in water and mobile phase B was 0.1% acetic acid in methanol. The mobile phase was operated using a linear gradient from 100% A to 100% B over 30 min. The thermostated column compartment was model G1316A and had a temperature of 45°C. Mass Hunter Software version B.03.01 (B2065) (Agilent Technologies, Waldbronn, Germany) was used to program the LC and MS modules and for integration of resulting data. Mass spectrometry conditions and specific target chemicals (and their parent phthalates) are shown in Table 1.

Calibration. The ratio of the area of the native standard divided by the area of the internal standard was regressed against the standard concentration. The linear regression line was used to derive the unknown analyte concentration with the exclusion of the slope to eliminate bias from endogenous analyte concentrations.

Quality Assurance/Control Evaluation. Analytes were quantified if they had the appropriate quantification and confirmation ions in predefined ratios, the signal-to-noise ratio was greater than 3, and the analyte peak coeluted with the internal standard. Quality control samples were within ±20% of the spiked value in all runs where data were considered valid. All data were also blank subtracted.

2.7. Method Validation

Several method validation steps were performed to ensure that the method was working properly.

Limits of Detection/Quantification. The methods limits of detection (LODs) and limits of quantification (LOQs) were calculated as the lowest concentration measured that gave a signal to noise ratio of 3 and 10, respectively. All calculated LODs and LOQs were verified by measuring a calibrant at or below the concentration. The LODs and LOQs were calculated as average values over the course of the study.

Accuracy. As no reference materials exist for phthalates, especially in meconium as a matrix, the accuracy (also called relative recovery) was calculated as the ability of our calibration curve to calculate a value spiked into matrix. The values are expressed as an average percentage of 100% with standard deviation.

Precision. Precision was estimated as the relative standard deviation (RSD %) in repeat measurements of QC samples over time.

Extraction Recovery. Extraction recovery was expressed as an average percentage (± RSD) of a spiked amount recovered in the extraction by calculating concentrations in samples spiked both before and after extraction. This is not the same as relative recovery or accuracy. Rather, this value just provides an indication of the extraction efficiency that, in normal analyses, are automatically corrected for using the isotopically labeled internal standards.

2.8. Statistical Analysis

Statistical Analysis System version 9.4 (Cary, North Carolina) was used to perform statistical analyses on the quantified results. Univariate statistics were used to analyze calculated metabolite concentrations for descriptive purposes, including mean, median, and distribution percentiles. Correlation analysis was performed to determine correlations between the metabolites. Concentrations below the LOQ were imputed as $\frac{LOQ}{\sqrt{2}}$. Calculated concentrations were then compared to urinary phthalate metabolite concentration values in the general public measured for the 2003-04 National Health and Nutrition Examination Survey (NHANES) to determine if meconium phthalate measurements appear to be an integrated measure over pregnancy or a short-term estimate similar to urine. As concentrations for MBP were not directly measured by NHANES, they were calculated by summing concentrations for MiBP and MnBP.

3. Results

3.1. Method Characteristics

3.1.1. Extraction Recovery Data

Extraction recoveries are shown in Table 2. The recoveries range from 60-116% with wide variations. The significant benefit of the isotope dilution technique used in this analysis is the automatic correction for extraction recovery in each individual sample. We did not correct for this in our recovery experiments because we wanted to understand exactly how much of the target chemical was being extracted out of the meconium.

3.1.2. Precision and Accuracy

Precision and accuracy (relative recoveries) are shown in Table 2. It is important to note that the extraction recoveries presented in the last section are not the same as relative recoveries (also called spiked recoveries or accuracy) presented here. The latter demonstrate the utility of the method in quantifying the target analytes. According to FDA guidelines, the relative recoveries should be within 20% of 100% to be fully quantitative, which our values are. The relative standard deviations (RSDs) were typically less than 15%, indicating good precision.

Table 1. Precursor/Product Ion Pair for quantification of target analytes

Target Chemical	Parent Chemical	Abbreviation	Parent mass	Daughter mass
Mono-(3-carboxypropyl) phthalate	Di-n-octyl phthalate	MCPP	251	103.1
Monoethyl phthalate	Diethyl phthalate	MEP	193	77.1
mono(2-ethyl-5-carboxypentyl) phthalate	Di-2-ethylhexyl phthalate	MECPP	307	159.1
mono(2-ethyl-5-hydroxyhexyl) phthalate	Di-2-ethylhexyl phthalate	MEHHP	293	121
Mono-n-butyl phthalate	Benzylbutyl phthalate; di-n-butyl phthalate	MBP	221	77.1
Mono-isobutyl phthalate	Diisobutyl phthalate	MiBP	221	77.1
mono(2-ethyl-5-oxohexyl) phthalate	Di-2-ethylhexyl phthalate	MEOHP	291	121
mono(2-carboxymethylhexyl) phthalate	Di-2-ethylhexyl phthalate	MCMHP	307	159.1
Monobenzyl phthalate	Benzylbutyl phthalate	MBzP	255	183.1
Mono(2-ethylhexyl) phthalate	Di-2-ethylhexyl phthalate	MEHP	277	134

Table 2. Recoveries and limit of quantification (LOQ) for phthalate ions in meconium

Analyte	Relative Recovery (accuracy) (%)	Extraction Recovery (%)	RSD (%)	LOQ (ng/g)
MCPP	96±6	60±22	18	5
MEP	101±4	82±20	9	5
MECPP	92±8	66±22	14	5
MEHHP	100±2	99±20	5	5
MBP	106±3	86±30	11	5
MiBP	108±3	79±22	9	5
MEOHP	99±5	98±10	6	5
MCMHP	100±4	86±21	15	5
MBzP	99±6	80±21	12	5
MEHP	116±3	116±31	10	5

3.2. Method Application

3.2.1. Meconium Phthalate Levels

Descriptive statistics including geometric mean, median, and 95th percentile for all calculated concentrations of the ten phthalate metabolites and corresponding 2003-2004 NHANES urinary phthalate metabolite concentrations are presented in Table 3 [69]. Because MCMHP was not measured during the NHANES study, comparisons with urinary concentrations could not be made. MEP was present at the highest concentrations in the meconium samples, as well as in the NHANES urine samples. Two MEHHP samples could not be quantified due to the inability to distinguish them from interfering peaks and were treated as missing data. 23 MCPP samples and 9 MEHP samples were below the LOD. As described previously, these samples were imputed as $\frac{LOQ}{\sqrt{2}}$ for analysis purposes. All metabolites excluding MCMHP were present at concentrations significantly greater than the NHANES urinary concentrations. The 95% confidence intervals for

the geometric means and medians did not overlap for any of the metabolites, while the confidence intervals for the 95th percentiles overlapped for two of the metabolites (MCPP and MEHP).

Table 3. Selected descriptors of phthalate metabolite concentrations in meconium and corresponding NHANES urinary concentrations

Metabolite	Geometric Mean (with 95% CI) (ng/g)	Median (with 95% CI) (ng/g)	95th Percentile (with 95% CI)	2003-2004 NHANES Urinary Geometric Mean (with 95% CI) (ng/mL)	2003-2004 NHANES Urinary Median (with 95% CI) (ng/mL)	2003-2004 NHANES Urinary 95th Percentile (With 95% CI) (ng/mL)
MCPP	**8.20** (5.29-11.1)	**3.54** (3.54-5.58)	**30.76** (15.74-44.64)	**2.91** (2.79-3.04)	**3.10** (3.00-3.30)	**15.3** (13.8-16.2)
MEP	**2291** (1255-3327)	**802** (543-1683)	**9657** (7354-14470)	**193** (169-220)	**174** (151-208)	**2700** (2160-3310)
MECPP	**302** (227-378)	**224** (178-298)	**831** (540-1160)	**34.7** (31.0-38.9)	**33.0** (29.1-37.4)	**339** (235-506)
MEHHP	**238** (86.5-390)	**133** (75.7-185)	**756** (389-2848)	**21.7** (19.3-24.4)	**21.2** (18.7-24.1)	**266** (165-383)
MBP	**1338** (903-1774)	**902** (525-1371)	**4229** (3474-5729)	**24.9** (23.2-26.25)	**27.4** (24.9-29.6)	**143.3** (122.6-163)
MiBP	**92.0** (66.4-118)	**65.8** (43.4-106)	**287** (184-359)	**3.80** (3.40-4.25)	**4.20** (3.70-4.80)	**21.3** (18.6-26.0)
MEOHP	**133** (94.8-171)	**91.2** (64.9-141.4)	**407** (294-520)	**14.5** (13.0-16.1)	**14.4** (12.4-16.7)	**157** (106-232)
MCMHP	**42.0** (29.4-54.7)	**28.0** (19.4-45.7)	**128** (82.4-184)	N/a	N/a	N/a
MBzP	**78.7** (47.4-110)	**41.3** (26.4-61.9)	**316** (173-446)	**13.7** (12.7-14.9)	**14.3** (12.8-16.4)	**101** (85.3-125)
MEHP	**12.2** (8.23-16.1)	**7.59** (5.74-12.63)	**35.0** (23.0-68.3)	**2.34** (2.10-2.62)	**1.90** (1.70-2.40)	**31.0** (21.4-42.0)

Table 4. Correlations between phthalate metabolites

	MCPP	MEP	MECPP	MEHHP	MBP	MiBP	MEOHP	MCMHP	MBzP	MEHP
MCPP	-	0.19099 0.2378	0.22287 0.1669	0.18988 0.2535	0.12625 0.4376	0.22967 0.1540	0.23143 0.1508	0.16207 0.3177	**0.97142** **<.0001**	0.27398 0.0871
MEP	0.19099 0.2378	-	-0.12688 0.4353	-0.09509 0.5701	0.33948 0.0321	0.18078 0.2643	-0.10766 0.5084	-0.06931 0.6708	0.18796 0.2455	-0.09554 0.5576
MECPP	0.22287 0.1669	-0.12688 0.4353	-	0.49546 0.0016	0.30592 0.0549	0.31724 0.0461	**0.81845** **<.0001**	**0.86768** **<.0001**	0.27056 0.0913	**0.73059** **<.0001**
MEHHP	0.18988 0.2535	-0.09509 0.5701	0.49546 0.0016	-	0.35852 0.0231	**0.57095** **0.0002**	0.23340 0.1585	**0.62666** **<.0001**	0.34584 0.0334	**0.86922** **<.0001**
MBP	0.12625 0.4376	0.33948 0.0321	0.30592 0.0549	-0.04641 0.7820	-	0.35852 0.0231	0.40494 0.0095	0.31248 0.0496	0.14018 0.3883	0.06930 0.6709
MiBP	0.22967 0.1540	0.18078 0.2643	0.31724 0.0461	**0.57095** **0.0002**	0.40494 0.0095	-	0.22042 0.1717	0.43983 0.0045	0.32655 0.0397	**0.55883** **0.0002**
MEOHP	0.23143 0.1508	-0.10766 0.5084	**0.81845** **<.0001**	0.23340 0.1585	0.40494 0.0095	0.22042 0.1717	-	**0.79452** **<.0001**	0.25938 0.1060	0.47874 0.0018
MCMHP	0.16207 0.3177	-0.06931 0.6708	**0.86768** **<.0001**	**0.62666** **<.0001**	0.31248 0.0496	0.43983 0.0045	**0.79452** **<.0001**	-	0.22436 0.1640	**0.83148** **<.0001**
MBzP	**0.97142** **<.0001**	0.18796 0.2455	0.27056 0.0913	0.34584 0.0334	0.14018 0.3883	0.32655 0.0397	0.25938 0.1060	0.22436 0.1640	-	0.36466 0.0207
MEHP	0.27398 0.0871	-0.09554 0.5576	**0.73059** **<.0001**	**0.86922** **<.0001**	0.06930 0.6709	**0.55883** **0.0002**	0.47874 0.0018	**0.83148** **<.0001**	0.36466 0.0207	

Correlation analysis was performed to determine if the concentrations of any metabolites were associated with one another. Pearson correlation coefficients and p-values are displayed in Table 4. Cells with strong correlations ($r > 0.50$) and p-values below $p=0.05$ are bolded. Several

of the metabolites were significantly correlated with each other. Particularly strong correlations include those between MCPP and MBzP ($r=0.97142$, $p<0.0001$), MECPP and MCMHP ($r=0.867768$, $p<0.0001$), and MEHHP and MEHP ($r=0.86922$, $p<0.0001$).

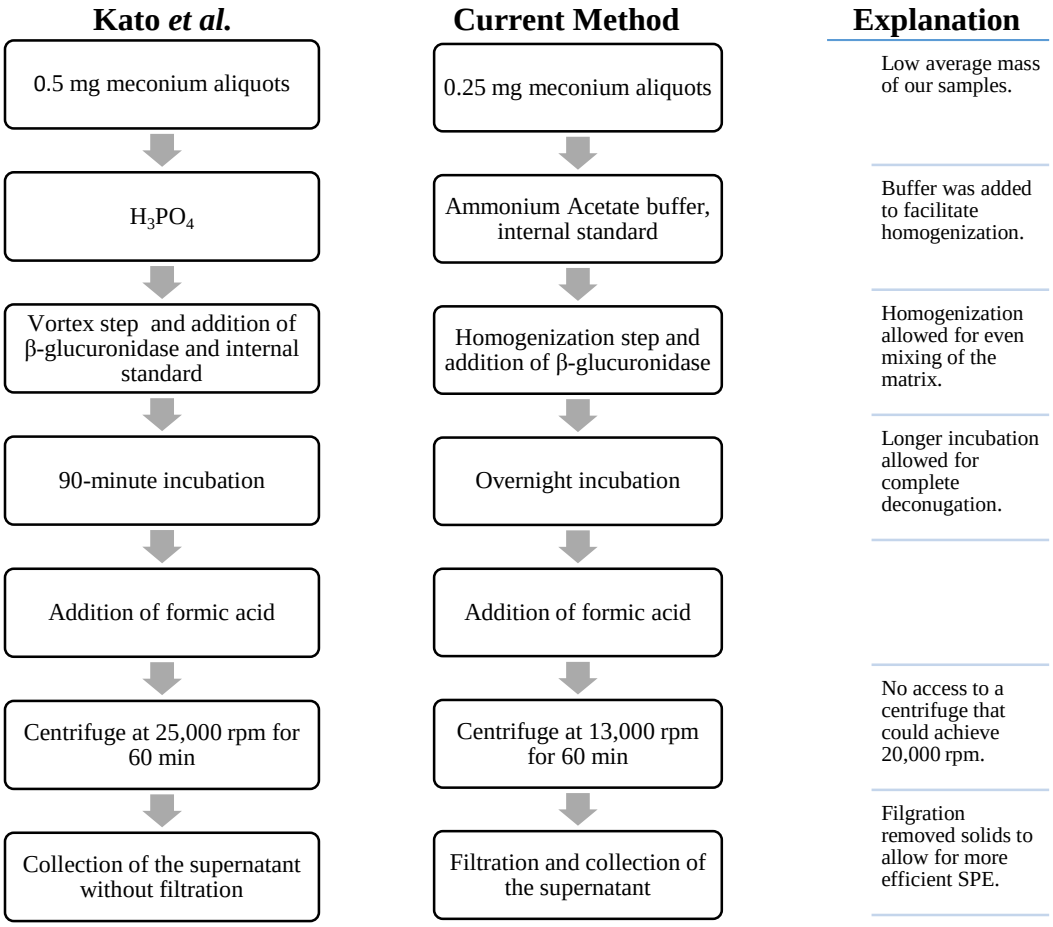
Analysis was also performed for the five DEHP metabolites (MEHP, MEHHP, MEOHP, MECPP, and MCMHP) to determine their relative predominance. MEHP, MEHHP, MEOHP, MECPP, and MCMHP constituted 1.70%, 31.61%, 18.56%, 42.25%, and 5.87% of metabolized DEHP, respectively. This relative order of contribution appears to be similar to the urinary metabolic profile of DEHP metabolites found in the general population [70, 71].

4. Discussion

4.1. Method

As mentioned previously, our method development began with an attempt to adapt the method from Kato *et al.*'s previously published paper on quantifying phthalate metabolites in meconium. We were unable to use their method to successfully extract and analyze our meconium samples, ultimately leading us to alter key parts of their method. Figure 2 outlines the similarities and differences between our method and Kato *et al.*'s method during the incubation step, where they differ the most.

Figure 2. Comparison with Kato *et al.*'s method



4.2. Limitations of using Meconium as a Matrix

Despite its usefulness as a measure of integrative exposure to environmental chemicals over half of pregnancy, meconium offers several potential limitations as a matrix. It is non-homogenous, meaning that its physical properties and concentrations of environmental chemicals are not consistent throughout the matrix. In addition, it is lipid-rich and has a thick mucus-like consistency, making it difficult to break down. Because of these factors, meconium needs to be homogenized during sample preparation. In this study, we used a tissue homogenizer to achieve this. Due to its lipid-rich contents, meconium is likely to clog the pores of the solid phase extraction

cartridges. To counter this, we centrifuged the samples at high speeds to collect solid components at the bottom and then ran the supernatant through 1 µm filters. Due to the additional time, equipment, and labor needed to account for the challenging physical properties of the matrix, the analysis cost for meconium samples is likely to be up to double the cost for urine samples. Additionally, a significant number of the chromatograms for the meconium samples tended to appear "dirty", with jagged boundaries and interfering peaks that prevented quantification. Significant retention time shifts could lead to the potential for peak misidentification.

4.3. Phthalate Metabolite Concentrations

All phthalate metabolites were present at concentrations significantly higher than the corresponding NHANES concentrations (excluding MCMHP, for which comparisons could not be made). Because the meconium concentrations are significantly higher than urine concentrations, potential contamination of the meconium samples with urine in the infant's diaper does not appear to be an issue of concern. The higher phthalate metabolite concentrations measured in meconium suggest that meconium functions as a "sink" for the metabolites, acting as a depository for the metabolites from the start of its formation around the 12^{th} week of gestation to when it is expelled shortly after birth. As a result, concentrations appear to be an integrative measure of exposure to phthalates over two trimesters of pregnancy. It is likely that meconium acts as a "sink" for other environmental chemicals as well and can offer the same integrative measure of exposure for them.

These results demonstrate the usefulness of meconium as a biological matrix for fetal exposure to environmental chemicals. The use of meconium offers a method by which to directly quantify the fetus' exposure to the chemical of interest. Direct exposure measurements overcome

the uncertainties and downsides of estimating fetal exposure by utilizing maternal matrices as proxies (most commonly urine and serum).

4.4. Conclusion and Future Directions

From the results of this study, we conclude that meconium is a valid matrix for the assessment of cumulative exposure to phthalate diesters for over half of pregnancy, and that the same likely holds true for other environmental chemical exposures. The phthalate concentrations of the remaining 322 meconium samples from the Cincinnati HOME Study will continue to be analyzed and described in future publications. Because of the conclusions of this study, it is recommended that future epidemiologic studies utilize meconium as a matrix for fetal exposure to phthalates and other environmental chemicals in order to determine possible associations with negative health outcomes. The findings from this study contribute valuable knowledge to the field of biomonitoring, and to the field of environmental health overall.

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