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Phthalates and their metabolites in human breast milk, blood and urine as measures for monitoring exposure in human risk groups

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1. Svensk sammanfattning

För att undersöka halter av ftalater i svenskar och vilken matris som bäst lämpar sig för hälsorelaterad miljöövervakning har ftalater och ftalatmetaboliter analyserats i en grupp kvinnor som nyligen fött barn. I samband med förlossning på Universitetssjukhuset i Lund tillfrågades förstföderskor om medverkan och 42 kvinnor kom att ingå i studien. När barnet var 2-3 veckor gammalt pumpade mamman ut 50 mL bröstmjölk. Blod- och urinprov togs en vecka senare. Omfattande förändringar av standardmetoder för provtagning av mjölk och blod gjordes för att minimera risken för kontaminering av proverna. För mjölkprovtagningen användes en specialkonstruerad manuell pump av polykarbonat med ftalatfri packning. Blodprov togs med hjälp av endast kanyl och provrör (eftersom propparna i vaccutainrör innehåller ftalater). Proverna förvarades i värmebehandlade glasbehållare och fosforsyra tillsattes för att motverka metabolism av ftalater i mjölk- och blodprover. Analyserna av bröstmjölk visade värden nära eller under detektionsgränsen (LOD) för flertalet ftalater eller deras metaboliter. Även i blod och serum var nivåerna vanligtvis nära eller under LOD. I urin analyserades endast metaboliter och dessa kunde kvantifieras i 53-100 % av proverna. Nivåerna av ftalatmetaboliter i urin hos de svenska kvinnorna var i paritet med nivåerna hos en allmänbefolkning i USA och Tyskland. Några klara korrelationer mellan nivåer i t ex urin och bröstmjölk respektive blod påvisades inte. Resultaten av studien anger att för närvarande är analys av ftalatmetaboliter i urin den mest framkomliga vägen för skattning av ftalat-exponering hos människa. Provtagning och analys av mjölk och blod innebar betydligt större svårigheter. Framför allt framstår risken för kontaminering vid provtagning som betydande och en stor del av ftalaterna och dess metaboliter uppvisade låga halter, vid eller under LOD. Dessutom kan ftalater brytas ned i blod och mjölk. I flertalet internationella publicerade studier av ftalatexponering används urinmetabolit-analyser som ett mått på exponering för ftalater. I en nyligen publicerad amerikansk studie av ett 80-tal nyfödda pojkar sågs ett samband mellan kort ano-genitalt avstånd och nivåer av ftalatmetaboliter i urin hos deras

mammor under graviditeten. Den amerikanska studien behöver bekräftas, men metaboliterna var desamma som i vår studie och en jämförelse visar att mediannivåerna var lägre för vissa men högre för andra metaboliter. Vår studie indikerar att svenska kvinnor i fertil ålder inte sällan exponeras för ftalater i nivåer som satts i samband med fosterpåverkan.

2. Introduction

Phthalates are a group of chemicals used in large quantities as softeners in many plastic products. Phthalates are also used in paint, glue, putty, pharmaceutical products and cosmetics. About 5000 – 6000 tons of phthalates, including di(2-ethylhexyl) phthalate (DEHP), are used per year in Sweden. Typical for many products are that phthalates are not chemically bound to the product matrix and may thus migrate and permit extensive exposure. People may be exposed in the work environment, via food from plastic containers, via inhalation of dust e.g. in domestic environments. Small groups of the population may be exposed via medical equipment. For example, DEHP migrating from plastic tubings used for treatment of prematurely born children can give rise to very high exposures[1]. Dermal exposure via cloths, cosmetics etc. may also occur. More extensive use of cosmetics by women may explain at least some of the high levels of urinary phthalate metabolites observed in women in a US population[2].

Several toxicological effects have been ascribed to phthalates. Previously, many studies focused on hepatocarcinogenic effects seen in laboratory animals[3]. This carcinogenic effect has been related to the activation of PPAR , a nuclear receptor which is activated by lipid ligands and may transactivate lipid metabolizing genes. Most environmentally occurring phthalates exhibit carcinogenic potentials at high doses only. Phthalates are nongenotoxic in most test systems and the carcinogenic effect is generally ascribed to a nongenotoxic mode of action. Indeed, mice lacking the PPAR receptor are resistant to the heptocarcinogenic effect of e.g. DEHP. There are no conclusive evidences for carcinogenic effects in humans.

Recently, studies have reported reproductive health effects, including e.g. reduced sperm production in laboratory animals, and there is concern that phthalates may induce anti-androgenic and or estrogenic effects in humans. Developmental defects in male rat pups, similar to those seen in a syndrome termed testicular dysgenesis syndrome (TDS), have been documented after dosing pregnant rat dams with certain phthalates (di-n-butyl phthalate (DBP), DEHP or butyl benzyl phthalate (BBzP)), albeit at high doses[4]. Mechanistic studies implicate alterations in insulin growth factor 3 expression in the development of phthalate induced effects, and it has been shown that this growth factor is down-regulated in foetal rat testis by *in utero* exposure to DBP[5].

In a Swedish epidemiological study, exposure to phthalates via house dust has been related to asthma and allergic symptoms in children[6]. Human studies also include a recent US study in which high levels of phthalate metabolites in urine samples from pregnant women correlated to a relatively short ano-genital distance (AGD) in their male babies[7]. This endpoint has previously been coupled to TDS in animals, and a longer AGD has been used as a measure of virilization[4]. Pregnant women were recruited from an ongoing larger multi-centre study of couples attending prenatal clinics, and after delivery 85 newborn boys were included in the study[7,8]. Prenatal urinary levels of four phthalate metabolites (mEP, mBP, mBzP, and miBP) were inversely related to AGD. Of the 10 boys with the highest score for phthalates, 9 had

AGD below the mean. The median metabolite levels associated with short AGD, or other signs associated with TDS, did not exceed those found among 25 % of the female US population[7].

Phthalates are metabolised in the body and most studies of human exposure report levels of monoester metabolites in urine. In serum or blood the parent compounds may be metabolized to monoesters by esterases during handling of the samples[9], and contamination problems may prevail. Monoesters can be further metabolised via oxidation and glucuronidation. The enzyme, uridine 5´-diphosphate glucoronosyltransferase (UDP-GT) is polymorphic, so urine levels might be affected by individual differences in rates of metabolism. Furthermore, possible interactions between phthalates and PCBs have been discussed in terms of competition for UDP-GT[10]. Biological samples are easily contaminated by phthalates during sampling and by analytical equipment and airborne contaminants. Thus, oxidized metabolites are considered important in studies like the one presented here as they cannot easily be ascribed to contamination. A newly detected oxidized metabolite of DEHP in urine, not included in our study, might be responsible for the anti-androgenic/estrogenic effects of DEHP[11].

Recent US studies summarize data from over 2500 individuals participating in the National Health and Nutrition Examination Survey (NHANES) [2 12]. In general, higher levels of urinary metabolites were found in women than in men. This pattern was also seen in a German study [13]. There were also ethnic differences[2]. Based on urinary metabolite levels, daily intakes of phthalates have been calculated for a group of Germans (n = 85) and the data suggest that the tolerable daily intake (TDI) for DEHP[14] (which is based on reproductive toxicity) is exceeded by 12 % of the general German population[15].

The current toxicological profile of phthalates, and perhaps also a more prevalent high exposure of women, suggests that pregnant women, foetuses and newborns, could be highly sensitive risk groups for phthalates. Furthermore, little data are available on levels of phthalates in human milk[16-18,19]. In the present pilot study, performed at the request of the Swedish Environmental Protection Agency, common phthalates and their metabolites were analyzed in human milk, blood and urine. Special precautions were taken to avoid contamination. Our pilot study was undertaken in an effort to evaluate whether multi-matrix bio-monitoring of phthalates in women of childbearing ages is feasible, and which matrix is optimal for health related environmental monitoring of risk groups in the general population.

3. Methods

3.1 Study group

All contacts with the participating mothers and handling of samples before analysis was performed by the Department of Occupational and Environmental Medicine, Lund University, Sweden. Ethical permits were obtained from the Ethical Comity at University of Lund and from Institutional Review Board at CDC

3.1.1 Recruitment of participants and general instructions for sample collection

Women delivering their first child were contacted at the University Hospital, Lund, Sweden. They were asked to participate in the study and gave their informed consent. Women born in Sweden, who had a normal delivery, were included in the study. Totally, about 400-500 women were asked to participate. Of these, 68 mothers agreed to participate. When leaving the hospital they were given a questionnaire (see below) to fill in at home. A nurse contacted the women and visited those who wanted to participate (42 mothers). The mothers were given a special breast milk pump and other equipment to collect and store the milk (see below). They were given instructions not to use skin care products before sampling, how to collect 50-100 mL milk and to store it at 4 °C (see below). It was collected for transport to the laboratory within a couple of hours when phosphoric acid was added (see below). The milk was stored in the freezer (-20 °C). At the visit, the nurse interviewed the mothers about dietary habits and life style (see below). One week later the nurse came back to collect the sampling equipment and to take samples of blood (non-fasting) and urine (see below). After analysis the mothers were informed about the levels of contaminants in their milk, blood and urine. 42 mothers gave milk samples.

3.1.2 Interview

At the first home visit, the mothers were interviewed about factors that may influence or interact with the exposure and levels of phthalates and their metabolites in milk, blood and urine, specifically, about the delivery (including medication), body weights of the mother and the baby, the mother's height, age, education, family, occupation, place of residence, renovation of the home, car, exercise, life style habits (coffee, tea, smoking, snuff), use and brands of cosmetic and hygiene products (perfume, antiperspirant, skin lotion, tooth paste, washing and washing-up detergents), health, and medication.

3.1.3 Descriptors of the selected study group

The study group consisted of 42 mothers all giving one breast milk sample. 38 of the mothers gave urine samples and 37 blood. The age of the mothers was between 23 and 39 years (median 29 years). 35 mothers were non-smokers, five ex-smokers. Six mothers reported a chronic disease (allergy, eczema, thyroid disorder). Nine mothers had suffered some kind of pregnancy problems. 27 of the mothers had > 3 years of university education, 5 had 1-3 years of university education, 8 had 3-4 years of high school education and the remaining two mothers had 1-2 years of high school education. 24 mothers lived in a private house and 18 in apartments. 26 of the mothers reported that their home had been renovated since they moved there. The babies were 25 girls and 17 boys. Their birth weight ranged from 2,874 to 4,620 g (median 3,540 g). At the time of breast milk sampling the babies were 14-20 days old (median 17 days).

3.2 Sampling

3.2.1 Blood

Blood samples were collected in glass test tubes (heated to 400 ^{o}C). The samples were mixed with either heparin or EDTA and finally acidified with phosphoric acid. The tube openings were covered by clean aluminium foil to protect the sample from contacts with the PTEF screw caps. The samples were stored in a freezer (-20 °C) until analysed.

Venous blood was taken by stainless steel needle from the left arm cubital vein and allowed to drop into the tubes. 10 000 IE heparin was mixed with 5 mL blood by turning the tube upside down several times. Aluminium foil separated the blood from the finger. Phosphoric acid was added, the foil was kept around the stopping and they were stored frozen.

3.2.2 Serum

Four tubes are filled with 10 mL blood were centrifuged. Serum was collected by using phthalate-free tubings and a heated pipette. Serum was mixed with phosphoric acid.

3.2.3 Breast milk

The milk samples were collected by the aid of a pump made of polycarbonate. The pump consisted of a piston in cylinder housing. The piston was originally equipped with a rubber seal, found to contain high concentrations of DEHP. Even if the seal was exchanged to a phthalate free one, high levels of DEHP appeared in the blank samples. This finding made it clear that it was not possible to clean the piston. New pumps, never used with a rubber seal but equipped with special seal made from tubing of fluoroelastomer (black Viton) were used. The pump was rinsed with ethanol before use. After collection the milk was transferred to preheated (400 °C) laboratory glass bottles with glass stopper. The samples were stored in a freezer (-20 °C) until analysed.

The mother was asked to collect 50 mL of milk by using breast pump. Within a few hours the sample was transferred to a glass beaker that had been heated to 400 °C. Phosphoric acid was added and the sample.

3.2.4 Urine

100 mL urine was collected in a heated (400 °C) 100 mL beaker. 5 mL were frozen (-80 °C) in in a tube and transferred to the Centre for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. All equipment used had been preheated to 400 °C.

3.2.5 Additional comments

Chemicals added to the samples, phosphoric acid (added to all samples except urine), heparin and EDTA, were checked by GC-MS before use. Nitrile plastic gloves, containing negligible amounts of phthalates (controlled by GC-MS) were used in this study e.g. when collecting body fluids. The samples were acidified with phosphoric acid (1 M; 125 µl/mL) about one hr after the collection [16,20] in order to avoid enzymatic hydrolysis of the phthalate esters. The samples were collected and stored in a freezer at the University Hospital of Lund (Section of Occupational and Environmental Medicine).

Samples were transported to the IVL laboratory in Stockholm on dry ice for analysis of parent compounds in blood and milk, and to the CDC laboratory in Atlanta, USA, for analysis of metabolites in serum, milk and urine.

3.3 Analysis of parent compounds in blood and milk

Analysis of parent compounds was performed at IVL Swedish Environmental Research Institute Ltd, Stockholm, Sweden.

Table 1. Limits of detection (LOD) of **parent phthalates analysed.**

Analyte	**LOD (ng/mL)**		**abbreviation**
	Blood	Milk	
diethyl phthalate	0.13	0.4	DEP
dibutyl phthalate	0.43	2.97	DBP
butyl-benzyl phthalate	0.1	0.1	BBzP
bis-(2-etylhexyl) phthalate	1.0	0.9	DEHP
di-octyl phthalate	1.4	0.5	DOP
di-iso-nonyl phthalate	<100	?	DINP
di-iso-decyl phthalate	<100	?	DIDP

3.3.1 Chemicals and standards

All solvents used were of the highest quality available and were used as received. Hexane, acetonitrile, methanol, 2-propanol and *tert*-butyl methyl ether (TBME) were delivered from Rathburn (Chemical Ltd., Peeblesshire, Scotland). Before use all solvents were checked for contaminants. This was carried out by concentrating solvents by nitrogen blowdown at room temperature prior to GC-MS analysis.

The solid phase extraction columns containing aminopropylen phase were delivered from International Sorbent Technology, Ltd (IST), UK.

To calibrate the GC-MS instrument a certified standard mixture of six phthalate esters (Ultra Scientific; J.T. Baker) was used. The mixture contained DMP, DEP, DBP, BBzP, DEHP and DOP. The commercial products DINP and DIDP in isomeric mixtures were kindly supplied by Neste (Finland).

The general cleaning procedure for all equipment of glass and metal was carried out using the following steps: (i) detergent washed, rinsed with tap water and distilled water in a dish washing machine and (ii) all glassware was wrapped in aluminium foil and heated in an oven at 400 °C in 4 hours. The aluminium foil was used to prevented adsorption of phthalates from the air in the laboratory.

The extraction and working up procedure involves many solvents transferring steps. Usually Pasteur pipettes are used for this work. To prevent contamination from plasticizers in the pipette filler an air filter (activated charcoal between glass wool plugs) was used.

Equipment made of Teflon and metal was carefully detergent cleaned, rinsed with distilled water and methanol or 2-propanol. Finally, after drying, the material was rinsed with hexane and wrapped in preheated (400 °C) aluminium foil.

Ultrapure water (> 18 M Ω x cm) used for standards and different solutions was produced by a Milli-Q water cleaning equipment (Millipore Corporation, Bedford, MA, USA) equipped with a CPMQ004R1 cartridge for RO or distilled feed water without final filter unit. Every batch of water was checked by GC analysis before use.

Sodium chloride and sodium sulphate (Merck, Darmstadt, Germany) was calcined in an oven at 400 °C overnight.

3.3.2 Analytical methods

3.3.2.1 Blood samples

The samples (10 mL) were thawed and diluted with ultra pure water (10 mL). Surrogate standard was added and the samples were extracted twice with hexane : MTBE (1:1; 5 mL) for 30 min. The final extraction was made with hexane (3 mL) in 15 min. The combined extracts were concentrated with a gentle stream of activated charcoal cleaned nitrogen. The extracts were cleaned up on an amino propylene column. The columns were first eluted with hexane followed by hexane:MTBE (9:1; 5 mL). The latter fractions contained the phthalates and were concentrated, spiked with internal standard (pentachlorobenzene) and used for GC-MS analysis. At a minimum, two blanks were analyzed along with each batch of samples using the same analytical protocol as for the samples.

Some of the test tubes containing blood samples were broken during storage in the freezer. Contamination of the samples was unlikely since the test tubes were, before they were put in the freezer, wrapped in clean aluminium foil. However, special arrangements were taken in order to minimize the contamination risk. The test tubes were carefully washed with acetone. Washed tubes were left to thaw in clean beaker covered by aluminium foil. The blood samples were left to thaw out and were subsequently transferred to new test tube.

3.3.2.2 Milk samples

The samples (10 mL) were thawed and extracted according to a method described earlier [20]. Briefly, sodium chloride (1 g) and ultra pure water (13 mL) were extracted with (a) pentane:acetone (25 + 10 mL) and hexane : MTBE (1:1; 5 mL) in both cases for 30 min. Acetone was removed by shaking with water. A gentle stream of cleaned nitrogen concentrated the extracts. The extracts were dried over sodium sulphate, the solvent evaporated and the lipid content determined by weighing. Two blanks for each batch of samples were analysed by exactly the same procedure as used for the samples. The extracts were subjected to clean up using gel permeation chromatography (GPC; see below) and (b) aminopropylene column (see blood samples) prior GC-MS analysis.

3.3.3 Instruments

3.3.3.1 GC-MS analysis

The extracts were analyzed on a 6890N gas chromatograph with a 5973 mass selective detector (Agilent). The injection was done in pulsed splitless mode at 250°C. The fused silica capillary column (VF-5MS 30 m x 0.25 mm i.d. x 0.25 µm film thickness, Varian) was held at 45 °C for 2 min., 35 °C/min to 100°C, 10 °C/min to 295 °C and held for 10 min. Helium was used as carrier gas (1 mL/min; 36 cm /s). The mass spectrometer transfer line temperature was 290 °C. The detector was used in selected ion monitoring mode with electron ionisation at an energy of 70 eV. The analytes were identified by their characteristic retention time for one quantification ion and in most cases one supporting ion used to increase specificity. Quantification was based on the internal standard methodology. The reported analyte concentrations were corrected according to the determined surrogate standard.

3.3.3.2 HPLC-GPC

The extracts were cleaned up on a high performance liquid chromatography instrument (HPLC) consisting of a pump (CostaMetric 4100. Thermo Sparation Products, Florida, USA) and an UV-detector (LDC Milton Roy SM 4000 Florida, USA.). The GPC-column was a PL-

gel column (300 x 25 mm; 10 µm, 50 Å, Polymer Laboratories Ltd.). UV detection at 254 nm was used to monitor the effluent. The mobile phase was pentane:MTBE (1:1) at 5 mL/min flow rate, and each run lasted 65 min. The phthalates eluted between 20 and 54 min. Two fractions were collected (a) between 19,5-24,5 min and (b) 24,5-54 min. The former, containing some lipids and was therefore re-chromatographed. One fraction from the second run was collected (19.5-24.5 min) and was combined with fraction 24.5-54 min in the first run.

3.3.4 Quality control

The recovery rate determined by the surrogate standards in the analysed samples was 66 % (±11%) for the milk samples and 58 % (± 15%) blood samples.

The spiked control samples were analysed in parallel with the samples and the relative recoveries are presented in table 1.The recovery rate of the spiked samples is adjusted according to the surrogate standard. The measures taken in order to minimise the blank values are presented under the headings “materials” and “sampling”.

Table 2: **Relative recovery (in % of added amount) of phthalates spiked in milk and blood samples.** The results are adjusted according to the surrogate standard

	DEP	DBP	BBzP	DEHP	DOP
Milk	104	86	88	97	77
Blood	95	98	108	103	100

Parallel samples were used to evaluate the precision of the used method. The precision was determined to 20 % at the concentrations detected in the samples. A calculation of the uncertainty in the measurement according to Eurachem/CITAC Guide results in an uncertainty of 19 % in the measurement.

3.4. Analysis of metabolites in serum, milk and urine

Analysis of phthalate metabolites was performed at CDC, Atlanta, GA, USA. The measurement of phthalate monoesters in urine, breast milk and serum involved the enzymatic deconjugation of the phthalate metabolites from their glucuronidated form, automated solid-phase extraction, separation with high performance liquid chromatography, and detection by isotope-dilution tandem mass spectrometry [9,16,21]. Limits of detection (LODs) were in the low nanogram per milliliter (ng/mL) range; 1 mL of sample was used for the analysis. Each analytical run consisted of 50 samples (2 QC materials of high concentration, 2 QC materials of low concentration, 5 reagent blanks, and 41 unknown samples). The QC samples were analyzed along with unknown samples to monitor for accuracy and precision. QC samples were evaluated according to statistical probability rules. If the QC samples failed the statistical evaluation, all of the samples in the run were re-extracted. The CDC laboratory is certified by the Health Care Financing Administration to comply with the requirements set forth in the Clinical Laboratory Improvement Act of 1988 (CLIA ’88) (Certification # 11D0668290) and is recertified annually. As part of the certification, there are semi-annual tests on samples whose concentrations are unknown to the analysts (blinded samples); the analytical results are evaluated by a quality assurance (QA) officer to ensure that the lab is

performing quality analyses. To date, the laboratory has performed well on these analyses which have allowed yearly CLIA '88 recertifications.

Table 3. **Limits of Detection - Phthalate metabolites**

Analyte	**LOD (ng/mL)**	**abbreviation**
Mono-n-butyl phthalate	1.071	mBP
Mono-benzyl phthalate	1.000	mBzP
Mono-cyclohexyl phthalate	0.500	mCHP
Mono-ethyl phthalate	1.000	mEP
Mono-(2-ethylhexyl) phthalate	0.981	mEHP
Mono-methyl phthalate	1.000	mMP
Mono-isononyl phthalate	1.000	miNP
Mono-n-octyl phthalate	1.002	mnOP
Mono-(2-ethyl-5-hydroxyhexyl) phthalate	0.954	mEHHP
Mono-(2-ethyl-5-oxohexyl) phthalate	1.068	mEOHP
Mono-iso-butyl phthalate	1.038	miBP
Mono-3-carboxypropyl phthalate	1.000	mCPP

4. Results

Figure 1 summarizes the phthalates and phthalate metabolites that were measured, and in which media (blood, breast milk, urine), as well as the number of samples giving results above LOD (in percent of total number). Several blood, serum and milk samples had phthalate and phthalate metabolite concentrations below LOD, but all urine samples had detectable concentrations of most metabolites. No blood or milk sample contained levels above the LOD for DIDP or DINP.

Concentrations of phthalates and metabolites measured in milk, blood, serum and urine are summarised in table 4.

Concentrations of phthalate metabolites in urine (n=38) are shown in Figure 2 (A and B: ng/mL urine and B displays concentrations below 240 ng/mL urine; C and D: µg/g creatinine and D displays concentrations below 200 µg/g creatinine). The levels of oxidized metabolites of DEHP (mEOHP, mEHHP) correlated with the monoester metabolite mEHP, suggesting that all these metabolites reflect DEHP exposure. The inter-individual variation of DEHP metabolites was low. A larger inter-individual variation was found for mBP and mEP. Median concentrations in urine were in the same range as previously reported by Silva et al [2] for the US population.

Concentrations of phthalates in blood and phthalate metabolites in serum (n=36) are shown in Figure 3 (A and B; B displays concentrations below 25 ng/mL). Except for one sample exhibiting a high level of DEHP in blood (Figure 3A), most concentrations were close to or below LOD (Figure 1). The high level of DEHP found in blood was not reflected by a high level of mEHP in serum from the same woman, suggesting that contamination may have affected this analysis. Our results on levels in blood or serum is in line with what has been reported previously[22].

Table 4. Concentrations of phthalates and metabolites

Milk ng/mL	**DEP**	**DBP**	**BBzP**	**DEHP**	**DOP**	**mBP**	**mBzP**	**mEHP**	**mEP**	**miBP**
Number of samples	37	37	37	37	37	37	37	37	32	37
Number >LOD	8	12	42	42	10	11	3	16	1	2
Min	0,22	1,5	0,064	0,45	0,24	0,54	0,50	0,49		0,29
Max	42	42	42	305	42	42	42	42		42
Median	0,22	1,5	0,49	11	0,24	0,54	0,50	0,49		0,52
75-percentile	0,24	3,0	1,0	21	1,9	1,2	0,50	1,6	0,50	0,52
Mean	1,6	3,8	3,0	30	3,0	2,4	1,8	2,6		1,7
Standard deviation	6,9	7,3	9,5	68	7,3	7,0	6,8	7,2		6,8
Limit of detection (LOD)	0,44	3,0	0,12	0,90	0,47	1,1	1,0	0,98	1,0	1,0

Blood and serum ng/mL	**Blood**			**Serum**					
	DEP	**DBP**	**BBzP**	**DEHP**	**DOP**	**mBP**	**mEHP**	**mEP**	**miBP**
Number of samples	36	36	36	36	36	36	36	36	36
Number >LOD	29	25	29	17	7	17	6	7	3
Min	0,066	0,21	0,050	0,50	0,70	0,54	0,49	0,50	0,50
Max	1,1	9,1	1,4	129	10,0	20	4,5	14	11
Median	0,24	0,78	0,25	0,50	0,70	0,54	0,49	0,50	0,50
75-percentile	0,37	1,3	0,36	2,7	0,70	1,9	0,49	0,50	0,50
Mean	0,31	1,2	0,29	5,9	1,5	1,8	0,77	1,2	0,87
Standard deviation	0,26	1,6	0,27	21	2,1	3,3	0,80	2,3	1,8
Limit of detection (LOD)	0,13	0,43	0,10	1,0	1,4	1,1	0,98	1,0	1,0

Urine ng/mL	mBP	mBzP	mCPP	mEHHP	mEHP	mEOHP	mEP	mMP	miBP
Number of samples	38	38	38	38	38	38	38	38	38
Number >LOD	38	38	25	38	38	37	37	20	34
Min	5,1	2,2	0,5	1,4	2,9	0,54	0,50	0,50	0,52
Max	198	38	9,1	126	57	83	761	15	130
Median	46	13	1,5	15	9	11	35	1,2	16
75-percentile	68	20	2,5	29	17	24	80	2,8	30
Mean	53	16	1,9	25	13	19	84	2,3	21
Standard deviation	45	10	1,7	27	10	19	141	3,0	24
Limit of detection (LOD)	1,1	1,0	1,0	0,95	0,98	1,1	1,0	1,0	1,0

Urine µg/g creatinine	mBP	mBzP	mCPP	mEHHP	mEHP	mEOHP	mEP	mMP	miBP
Number of samples	38	38	38	38	38	38	38	38	38
Min	18	4,5	0,5	5,1	4,6	3,0	5,5	0,3	1,1
Max	191	63	16	86	74	57	862	12	110
Median	50	17	1,6	24	15	15	39	1,9	15
75-percentile	68	27	2,7	33	24	24	119	2,8	23
Mean	56	20	2,4	25	18	18	101	2,5	21
Standard deviation	37	13	2,6	18	14	13	160	2,5	21

Concentrations of phthalates and phthalate metabolites in milk (n=42) are shown in Figure 4 A and B (B is displaying concentrations below 30 ng/mL). As for blood and serum samples, all concentrations in milk were close to or below LOD, except one sample exhibiting a high concentration of DEHP. The individual with high DEHP in milk was not the same as the one with high DEHP in blood, and the metabolite (mEHP) concentration in milk was low, suggesting contamination of the sample. In general, there were lower levels of metabolites than of the corresponding parent compounds in milk. No significant correlations between parent compounds and the corresponding metabolites were detected in milk (DEHP-mEHP: r= 0.02; DBP-mBP: r = - 0.1). In the latter case, the lack of correlation may be due to the low number of samples with concentrations above LOD. If only samples above LOD (n=12) are analyzed, there is a negative significant correlation between DBP and mBP in milk (rs=-0.63; p=0.024).

In general, there was a lack of correlations between individual results obtained in the three matrixes urine, blood/serum and milk. In addition, the individual with exceptionally high DEHP level in blood had quite low levels of DEHP or mEHP in milk and also low levels of DEHP metabolites in urine (there are no data on blood or urine levels from the individual with exceptionally high levels of DEHP in milk).

In a search for correlations between levels in different matrixes several plots were drawn, but no obvious patterns could be discerned. There was an apparent correlation between urine mEP and serum mEP (7 values > LOD) (r= 0.479 $p < 0.003$) (Figure 5), but no correlation with blood DEP (29 values > LOD), so the biological significance of this correlation is uncertain. However, after removal of the apparent outlier, there was still a significant correlation (rs=0.41; p=0.014; n=35). It is possible that this correlation reflect an excretion of un-conjugated mEP, whereas variable conjugation rates (due to polymorphisms) may have eroded correlations for other monoester metabolites.

The urinary metabolites were analysed by the same laboratory and according to the same procedure as was used in the US study correlating phthalate levels in urine of pregnant women with a short AGD[7] of their sons, and a comparison of data is of interest. It should however be noticed that prenatal samples were used in the US study, whereas postnatal samples were used in the Swedish study. In Table 5 the 50^{th} (median) and the 75^{th} percentiles for urine levels in the present and the US study are given. It can be seen that levels of some metabolites are higher in the US samples, but that some are higher in the Swedish samples. In particular, the median level of mEP was high in the US samples, while the median level of mBP was high in the Swedish samples. The sums of medians were 181.3 and 148.6 µg/L for the American and the Swedish samples, respectively. Also included are the US 50^{th} and the 75^{th} percentiles for the four phthalate metabolites found to be inversely correlated to AGD. These metabolites are printed in italic.

5. Discussion

The urine data indicate levels of phthalate metabolites in Swedish mothers in a range which might be expected in a population living in a modern society. The correlations seen between the three DEHP metabolites in urine were expected and argue against influences of contaminations in the urine samples. The distribution of the various metabolites may thus reflect common exposures of humans. In a similar German study of the general population the calculated daily intake of DEHP (based on urinary metabolite levels) exceeded the European TDI value for 12 % of the individuals[15].

Table 5. **Comparison of urinary levels in the present study (Sweden), a US study on ADG[7] and a German study**[13]. Metabolites in italic were shown to correlate with AGD[7]

	Sweden		**USA**		**Germany**
Percentile (µg/L)	**50th**	**75th**	**50th**	**75th**	**50th**
Monoester metabolites					
mBP	45,5	68	13,5	30,9	181
mBzP	13,2	20	8,3	23,5	21,0
mCPP	1,5	2,5	2,1	3,6	
mEP	35	80	128,4	437	90,2
miBP	16,4	30	2,5	5,1	
mMP	1,2	2	0,7	3,2	
DEHP metabolites					
mEHHP	14,6	29	11,4	20,1	46,8
mEHP	9	17	3,3	9	10,3
mEOHP	11,3	24	11,1	19	36,5
Sum	148,1	274	181	551	

Perhaps more worrying is the fact that metabolites of phthalates that have been found to produce a TDS-like syndrome in rats (DBP, DEHP, BBzP) are found in slightly higher median levels in Sweden than those found in the study correlating phthalate exposure to a shortened AGD in the US [7]. However, the fact that prenatal/late pregnancy (the US study) and postnatal (the Swedish study) samples were analysed hampers the comparison. Furthermore, the US women in this study had much higher median levels of mEP than the Swedish women, and the 75th percentile indicate about doubled levels among US women. It should also be noted that only levels of mEP, mBP, mBzP and miBP correlated with AGD[7].

The blood and serum data are more problematic and no obvious correlations were seen between these levels and levels in e.g. urine. In particular, there was no correlation to levels of oxidised metabolites in urine, which should not have been affected by contamination[9]. The lack of correlations may partially be explained by many levels below LOD and the fact that the analysis did not permit accurate analysis of prevailing low levels. Other factors that may erode correlations are metabolism during handling the sample and contaminations. Even dust in ambient air might be a factor of importance that is hard to control for in a domestic environment and under conditions employed to collect the samples in our study. Thus, considering the limitations in sensitivity of the analytical procedures, the possible problem of contaminations as well as the discomfort associated with the blood sampling procedure, blood or serum sampling cannot be recommended for surveying phthalate exposure in the general population. Even surveying small targeted groups of susceptible individuals by using blood or serum samples might turn out to be much more complicated than using urine.

Collecting human milk samples was laborious, time consuming and associated with extra contamination problems. It also required special sampling equipment and procedures. Furthermore, the milk samples obviously contained too low levels of phthalates and

metabolites to permit a more comprehensive and detailed discussion of actual levels in this matrix. Due to the fact that milk and urine samples were taken at different time points it is also impossible to evaluate correlations to urine levels. It can thus be concluded that the present study does not support the notion that the mothers´ urine levels can be used for assessing exposure of breast fed children. If such correlations do exist and can be documented, the use of urine samples would greatly facilitate the study of exposure of breast fed children.

Another factor of importance is that the unborn child might be the most urgent target group to investigate further. The recent publication on a short AGD in newborns[7] suggests that foetuses and pregnant women might be the group most sensitive to phthalates, and suggests that health related endpoints can be studied by analysing urine samples.

A recent Danish/Finnish study[19] reports higher levels of some phthalate metabolites in human milk than we found. Thus the levels for mBP were 4.3 and 12 ng/mL, for mBzP were 0.9 and 1.3 ng/mL, for mEHP 9.5 and 13 ng/mL, and for mEP 0.93 and 0.97 ng/mL in Denmark and Finland respectively[19]. Considering the similarities in life style etc between the three Nordic countries these levels are surprisingly high. Only a minority of our samples exhibited values above LOD (about 1.0 ng/mL) for these metabolites, and several of the levels reported[19] should have been possible to detect with our analytical procedure. A closer analysis of factors that might explain these differences is thus urgent. One factor of importance might be that the Danish/Finish samples were collected prospectively and earlier (1997-2001) than our samples. No correlations to anatomical effects such as cryptorchidism were found, but correlations to hormone levels and to levels of hormone binding proteins were noted [19].

6. Conclusions and Recommendations

The results of this pilot study indicate that sampling of breast milk and analysing for phthalates is not a straight forward method that can be recommended for surveillance of small children's exposure to phthalates. The data suggest that breast milk might easily be contaminated with phthalates from skin care products although careful (and labour intensive) sampling techniques were employed. The data also indicate that the analytical methods used were not sensitive enough for adequate quantification of common phthalates and their metabolites in human blood, serum or milk in the selected population. It is also evident that this study cannot answer the question whether urine analysis can reflect exposure of breast fed children. On the other hand, the data indicate phthalate exposure in quantities similar to German and US levels. These levels have been associated with biological effects in some newborn boys in USA that may be interpreted as a sign of undervirilization. It thus seems urgent to study phthalate exposure in Swedish populations and in relation to AGD and other TDS related endpoints. More mechanistically related endpoints are also needed in these studies.

7. References

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Figure legends

Figure 1. Number of samples (in percent of total number) above the limit of detection (LOD) of the phtalates and phthalate metabolites measured in milk, blood, serum and urine.

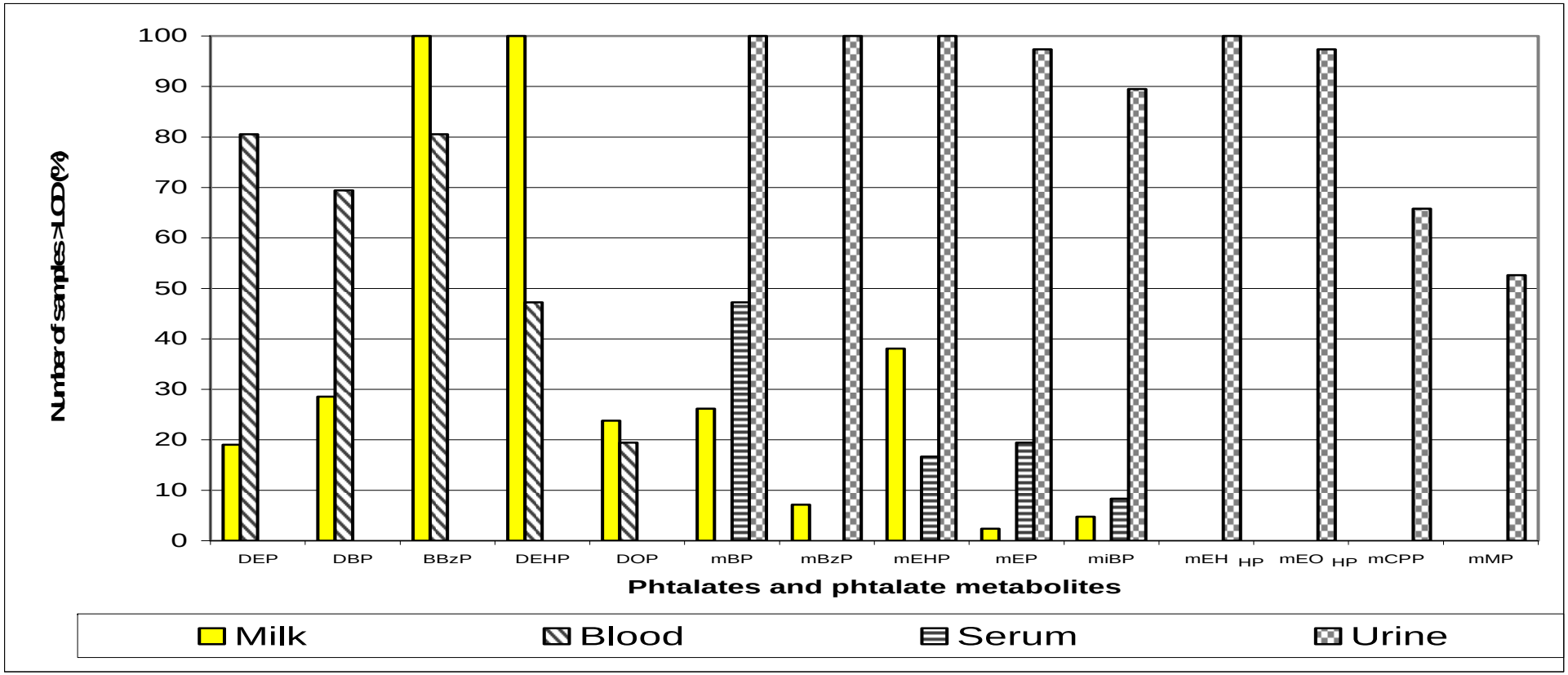
Figure 2. Phtalate metabolites in urine. **A**: all samples (ng/mL) and **B**: samples below 240 ng/mL. **C**: all samples (µg/g creatinine) and **D**: samples below 200 µg/g creatinine. S-DEHPmet = sum of DEHP metabolites.

Figure 3. Phtalates in blood (B-) and serum(S-). **A**: all samples (ng/mL) and **B**: samples below 25 ng/mL.

Figure 4. Phtalates and phtalate metabolites in milk. **A**: all samples (ng/mL) and **B**: samples below 30 ng/mL.

Figure 5. DEP in blood (ng/mL) and mEP in serum (ng/mL) in relation to mEP in urine (µg/g creatinine).

Figure 1.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50
40
30
20
10
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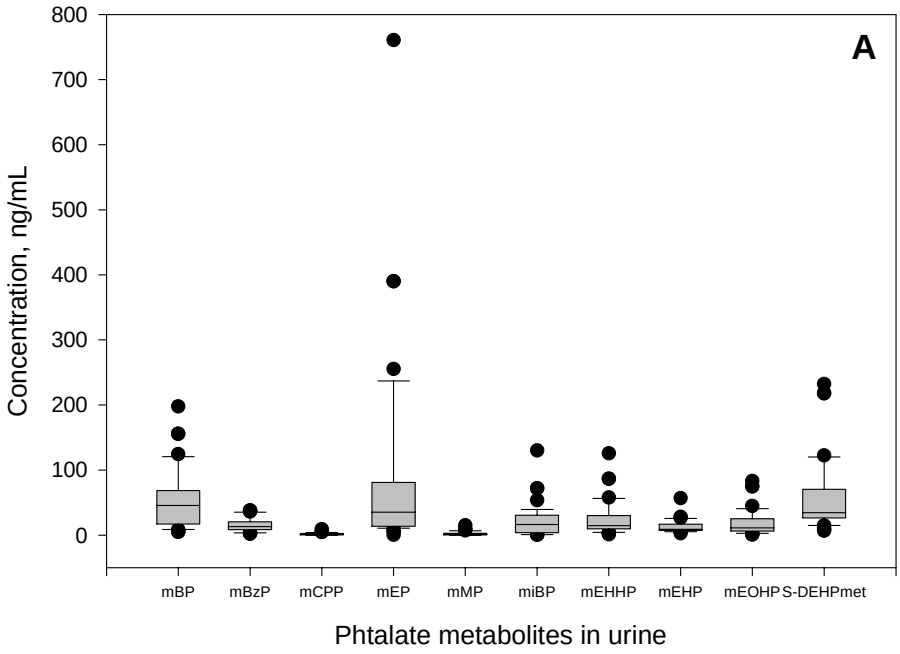
DEP
DBP
BBzP
DEHP
DOP
mBP
mBzP
mEHP
mEP
miBP
mEH HP
mEO HP
mCPP
mMP
Phtalates and phtalate metabolites
Milk
Blood
Serum
Urine

Figure 2.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700
600
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400
300
200
100
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mBP
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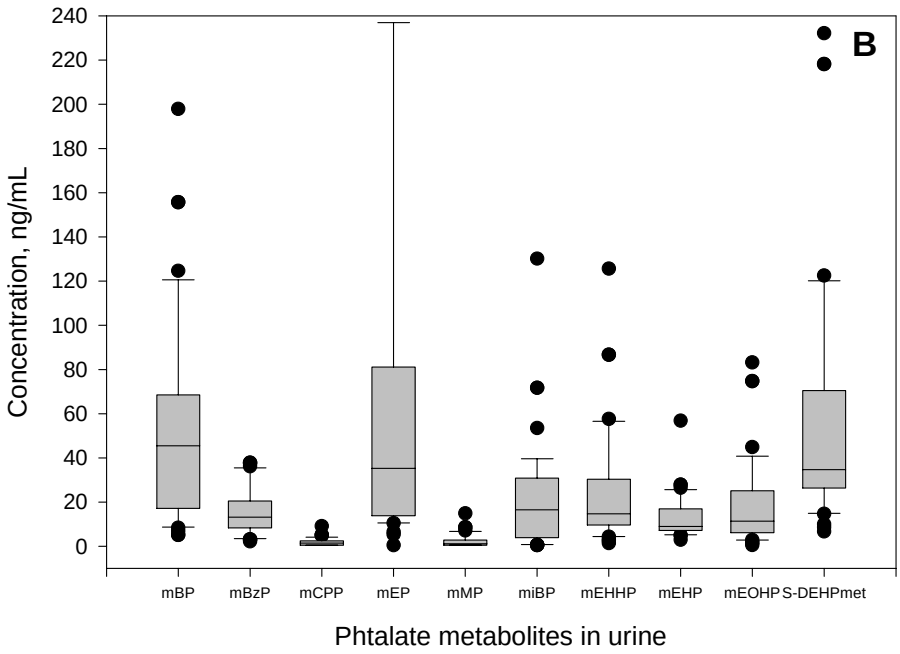
mEHHP
mEHP
mEOHP
S-DEHPmet
Phtalate metabolites in urine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120
100
80
60
40
20
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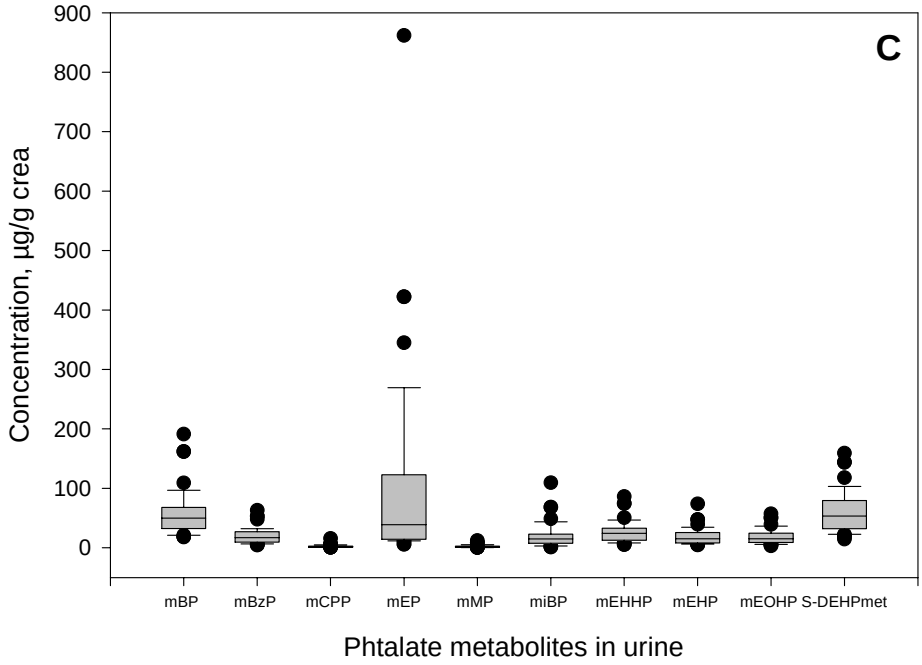
mEOHP
S-DEHPmet
Phtalate metabolites in urine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500
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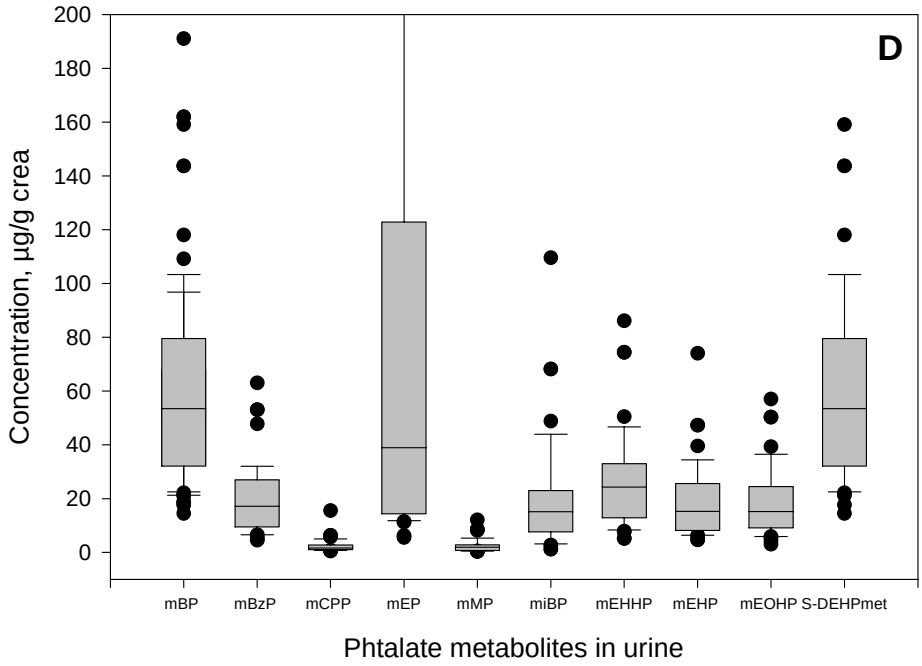
mEOHP
S-DEHPmet
Phtalate metabolites in urine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Concentration, µg/g crea
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160
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120
100
80
60
40
20
0
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miBP
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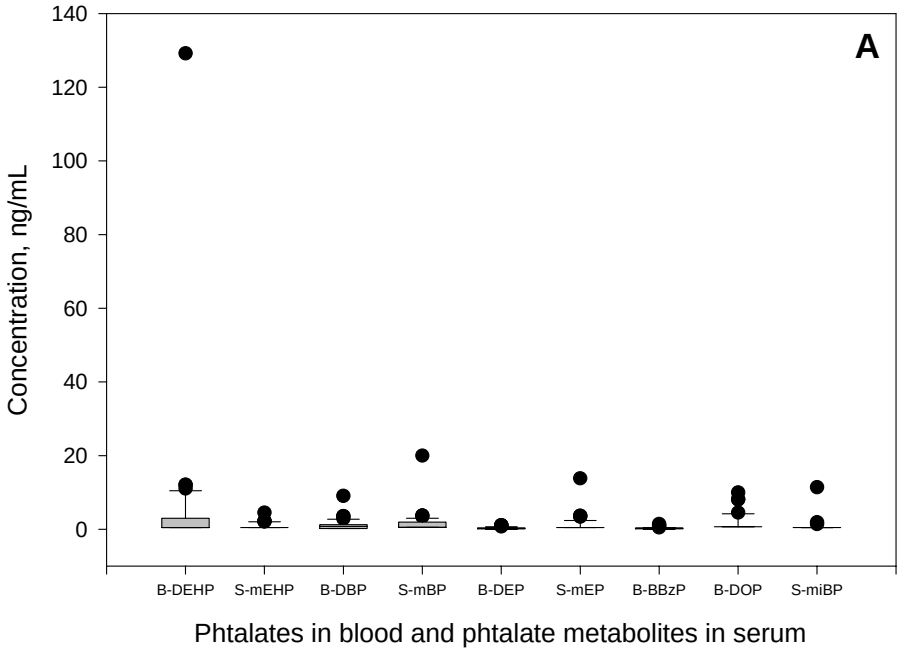
mEHP
mEOHP
S-DEHPmet
Phtalate metabolites in urine

Figure 3.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S-mEHP
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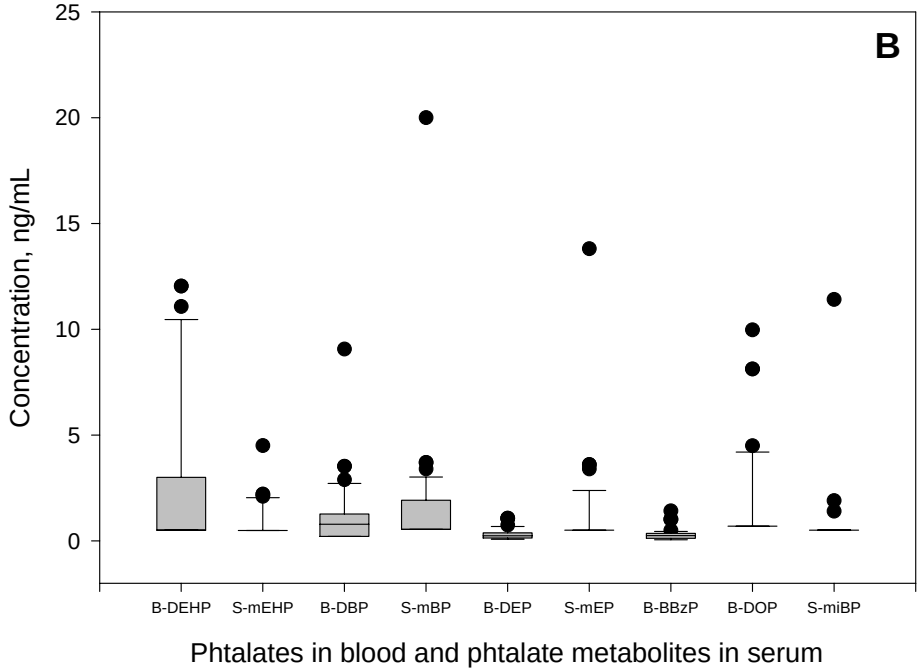
S-mEP
B-BBzP
B-DOP
S-miBP
Phtalates in blood and phtalate metabolites in serum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Concentration, ng/mL
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20
15
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S-mEHP
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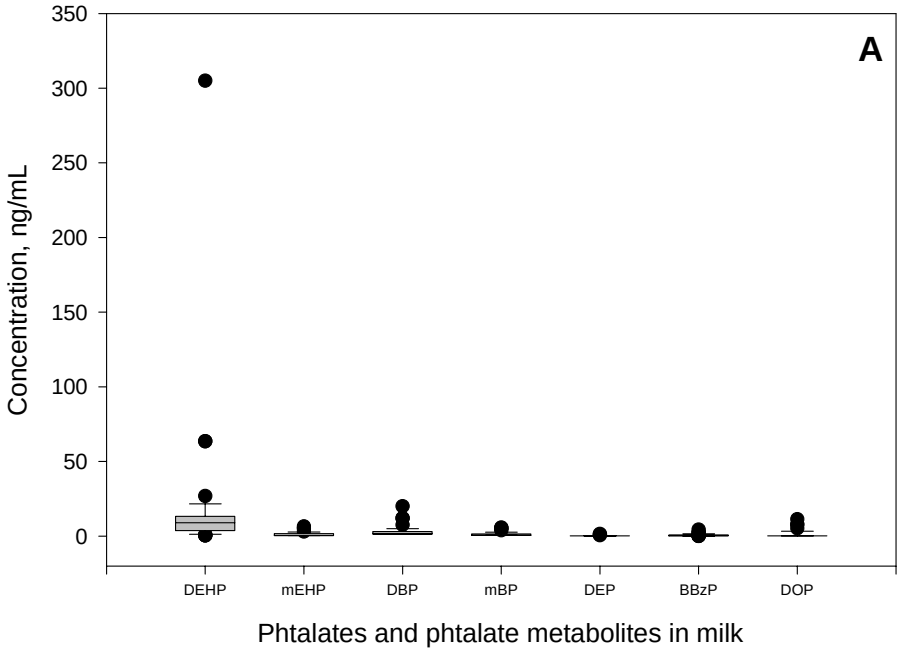
B-BBzP
B-DOP
S-miBP
Phtalates in blood and phtalate metabolites in serum

Figure 4.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mEHP
DBP
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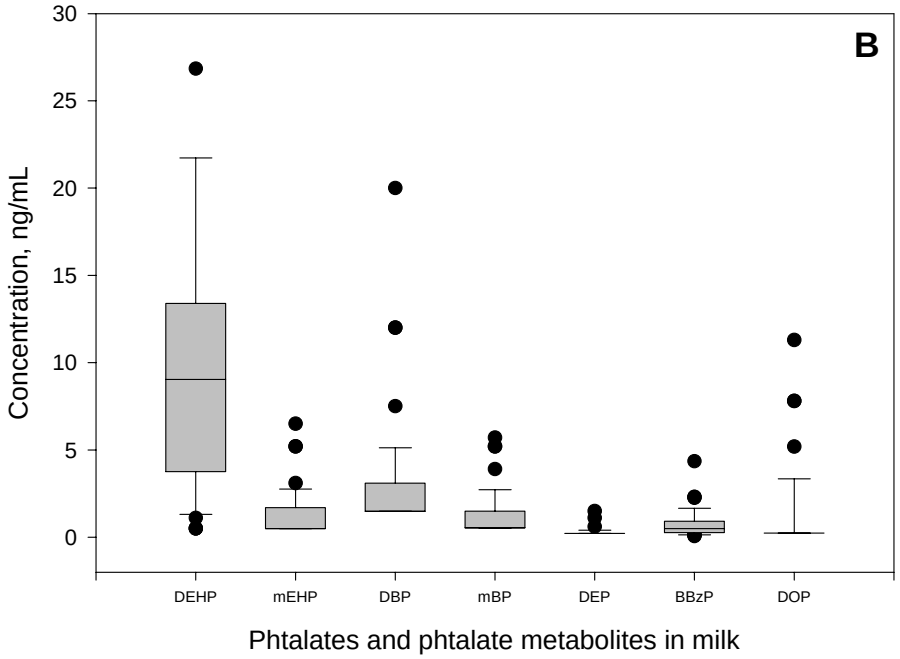
DEP
BBzP
DOP
Phtalates and phtalate metabolites in milk


B
Concentration, ng/mL
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25
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mEHP
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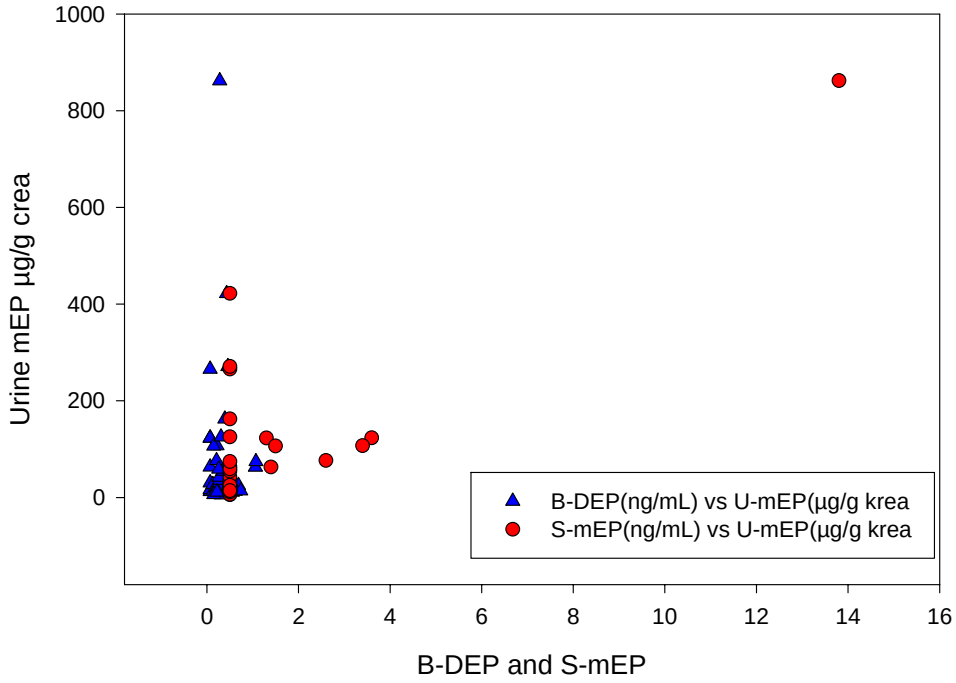
BBzP
DOP
Phtalates and phtalate metabolites in milk

Figure 5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600
400
200
0
Urine mEP µg/g crea
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6
8
10
12
14
16
B-DEP and S-mEP
B-DEP(ng/mL) vs U-mEP(µg/g krea
S-mEP(ng/mL) vs U-mEP(µg/g krea