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Automated solid phase extraction and quantitative analysis of human milk for 13 phthalate metabolites

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Abstract

While the demonstrated benefits associated with breastfeeding are well recognized, breast milk is one possible route of exposure to environmental chemicals, including phthalates, by breastfeeding infants. Because of the potential health impact of phthalates to nursing children, determining whether phthalates are present in breast milk is important. We developed a sensitive method for measuring 13 phthalate metabolites in breast milk using automated solid phase extraction (SPE) coupled to isotope dilution–high-performance liquid chromatography (HPLC)–negative ion electrospray ionization–tandem mass spectrometry. We used D_4-phthalate diesters to unequivocally establish the presence in human breast milk of enzymes capable of hydrolyzing the ubiquitous phthalate diesters to their respective monoesters. The analytical method involves acid-denaturation of the enzymes after collection of the milk to avoid hydrolysis of contaminant phthalate diesters introduced during sampling, storage, and analysis. The method shows good reproducibility (average coefficient of variations range between 4 and 27%) and accuracy (spiked recoveries are ~100%). The detection limits are in the low ng/ml range in 1 ml of breast milk. We detected several phthalate metabolites in pooled human breast milk samples, suggesting that phthalates can be incorporated into breast milk and transferred to the nursing child.

Keyword: Phthalates

1. Introduction

Diesters of phthalic acid, commonly known as phthalates, are a group of industrial chemicals with many commercial uses such as solvents, additives, and plasticizers. The potential for nonoccupational exposure to phthalates is high given their use in a vast range of consumables such as personal-care products (e.g., perfumes, lotions, cosmetics), paints, industrial plastics, and certain medical devices and pharmaceuticals [1–6]. Human exposure to phthalates can occur via ingestion, inhalation, and dermal routes, and intravenous and parenteral absorption in patients undergoing medical procedures that involve the use of medical devices containing phthalates. Upon exposure, phthalates are rapidly hydrolyzed to their monoesters. These monoesters may be further metabolized by oxidation and/or glucuronidation and excreted in urine and feces [1–4].

Several phthalates are carcinogenic in animal models [4,7,8]. In addition, some phthalates and their metabolic products act functionally as antiandrogens during the prenatal period [9–11] and cause reproductive and developmental toxicities in animals [12–15]. Exposure to high doses of dibutyl phthalate (DBP) results in spontaneous abortion of rat pups, demasculinization of fetal male rats, and testicular atrophy in rats, mice, ferrets, and guinea pigs [3,10]. DBP reduces the production of testosterone by the fetal testis through an antiandrogenic mechanism [16]. Studies in male rodents exposed to high doses of di-2-ethylhexyl phthalate (DEHP) indicate that the testes are a primary target [4]; mono-2-ethylhexyl phthalate (MEHP), a metabolite of DEHP, may be the ultimately active testicular toxicant [17,18]. Long-term exposures of adult female rats to DEHP also appear to have deleterious effects, including hypoestrogenic anovulatory cycles and polycystic ovaries [19]. DEHP appears to suppress estradiol production in the ovary, leading to anovulation [20].

Little information is known about the effects of phthalate exposure on humans. To evaluate the potential adverse

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effects of exposure to phthalates, accurate methods for measuring the amount of phthalates absorbed by the body must be developed. The measurement of monoester phthalate metabolites in urine and serum has been used for assessment of exposure to phthalates [21–30]. These studies suggest that exposure to phthalates is widespread.

Interest is increasing in monitoring breast milk for environmental contaminants because breast milk is the major route of exposure to these contaminants by the breastfeeding infant. Data on the levels of phthalates in milk are necessary for assessing the potential impact of phthalates to nursing mothers and their children. Trace levels of the phthalate diesters DBP (14 ng/ml) and DEHP (19 ng/ml) were detected in the milk of one person [31]. Because of the ubiquitous presence of phthalate diesters in the environment and hence the likelihood of contamination during the collection, storage, and measurement processes, measuring phthalate metabolites would be preferable [32]. However, measurements of phthalate metabolites in human milk are susceptible to contamination from the ubiquitous phthalate diesters that can be hydrolyzed to their respective monoesters by milk esterases [33,34]. Therefore, if the enzyme activity is not eliminated, the milk phthalate monoester measurements will be artificially high.

We report here a method for the quantitative detection of 13 phthalate metabolites in human milk. The method involves the initial acid denaturation of the milk enzymes to eliminate the esterase activity. The phthalate metabolites are extracted from breast milk using an automated solid phase extraction (SPE) procedure, separated from other extracted components in the eluate by reverse phase high-performance liquid chromatography (HPLC), and detected by isotope dilution–negative ion electrospray ionization–tandem mass spectrometry. We also report the use of D_4-phthalate diesters to unequivocally establish the presence in human breast milk of esterases.

2. Experimental

2.1. Analytical Standards and Reagents

Monomethyl phthalate (mMP), monoethyl phthalate (mEP), monocyclohexyl phthalate (mCHP), monobenzyl phthalate (mBzP), mono-*n*-butyl phthalate (mBP), mono-2-ethylhexyl phthalate (mEHP), mono-2-ethyl-5-oxohexyl phthalate (mEOHP), mono-2-ethyl-5-hydroxyhexyl phthalate (mEHHP), mono-*n*-octyl phthalate (mOP), and mono-3-methyl-5-dimethylhexyl phthalate (isononyl, mNP) (>99.9%), their $^{13}C_4$-labeled internal standards (>99.9%), $^{13}C_4$-4-methyl-umbelliferone, and ring-deuterium-labeled phthalate diesters, dibutyl-D_4 phthalate (D_4-DBP), benzyl-butyl-D_4 phthalate (D_4-BzBP), and di-2-ethylhexyl-D_4 phthalate (D_4-DEHP) (>98%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Monoisobutyl phthalate (miBP, 97%) and the ring-deuterium labeled miBP (95%, 98% isotopic purity) were provided by Prof. Jürgen Angerer (University of Erlangen-Nuremberg, Germany). Mono-3-carboxypropyl phthalate (mCPP) and $^{13}C_4$-mCPP were obtained from Los Alamos National Laboratory (Los Alamos, NM, USA) and from Cambridge Isotope Laboratories, Inc. Acetonitrile (HPLC grade), water (HPLC grade), methanol (99.8%, HPLC grade), and ethanol were purchased from Caledon (Ontario, Canada). Phthalic acid (PA) and $^{13}C_2$-PA were purchased from Sigma–Aldrich (Milwaukee, WI, USA); phosphoric acid (85%) was purchased from Fisher Scientific (Pittsburg, PA, USA); and glacial acetic acid (99.9%) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid (98% minimum, GR) and trichloroacetic acid (TCAA, >99%) were purchased from EM Science (Gibbstown, NJ, USA) and Sigma–Aldrich, respectively. 4-Methylumbelliferone and its glucuronide, and ammonium acetate (>98%) were purchased from Sigma (St. Louis, MO, USA). β-Glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical (Mannheim, Germany). Breast milk used for method development and quality control (QC) characterization was purchased from Mothers' Milk Bank (San Jose, CA, USA).

2.2. Preparation of standard solutions and QC materials

Reagent solutions were prepared in acetonitrile and water using standard laboratory procedures. The procedure for the preparation and storage of the standard solutions of phthalate monoesters is explained elsewhere [32]. A solution of 4-methylumbelliferone glucuronide, prepared in water as described before [32], was added to all samples to monitor β-glucuronidase enzyme activity; β-glucuronidase enzyme was used to hydrolyze the glucuronidated phthalate metabolites. The ammonium acetate buffer was prepared by dissolving ammonium acetate (38.6 g) in HPLC-grade water (200 ml), the pH of this solution was adjusted to 5.5 with glacial acetic acid.

A stock solution of D_4-labeled phthalate diesters was prepared by mixing 1 ml of each D_4-labeled phthalate diester solution in isooctane (D_4-DBP, 559 mg/l; D_4-BBzP, 1207 mg/l; D_4-DEHP, 546 mg/l). This combined D_4-labeled phthalate diester stock solution was evaporated to dryness using a Turbovap (Zymark Corporation, Hopkinton, MA, USA) and reconstituted in 10 ml of ethanol to afford a spiking solution of D_4-phthalate diesters (D_4-DBP, 55.9 mg/l; D_4-BBzP, 120.7 mg/l; and D_4-DEHP, 54.6 mg/l). The spiking D_4-phthalate diesters solution was stored at 4 °C in a Teflon-capped glass bottle until use.

QC materials were made using pooled human breast milk spiked with phthalate metabolites. Two pools were enriched with phthalate metabolites as needed to afford low (QCL) and high (QCH) level pools. Phosphoric acid (1 M, 125 μl/ml milk) was added to each pool. Then, the pools were uniformly mixed, dispensed into small aliquots (ca. 2.5 ml) into prerinsed glass vials and stored at −20 °C until used.

2.3. Sample preparation

Human milk (1.0 ml) was thawed, treated with 1 M H_3PO_4 (125 μl), vortex mixed, sonicated (5 min) and spiked with isotope-labeled internal standards (100 μl, 0.24–1.0 ng/μl), 4-methylumbelliferone glucuronide (50 μl, 0.8 ng/ml), ammonium acetate buffer (1.75 ml), and β-glucuronidase enzyme (5 μl in 250 μl ammonium acetate buffer). The sample was incubated at 37 °C for 90 min to allow for the deglucuronidation of the phthalate metabolites. After enzymatic deconjugation, the breast milk sample was placed on the Zymark RapidTrace Station (Zymark Corporation, Hopkinton, MA, USA) for the automated SPE procedure. A 60 mg/3 ml Oasis-HLB column (Waters Corporation, Milford, MA, USA) was conditioned with 2 ml of HPLC-grade methanol and 2 ml of water. The milk was diluted with 3 ml of 0.1 M formic acid, and loaded onto the SPE cartridge at a rate of 1 ml/min. The cartridge was washed with 1 ml of water and 2 ml of 40% methanol in water at 1 ml/min. The phthalate metabolites were eluted with 0.5 ml of acetonitrile at 0.5 ml/min. The eluate was evaporated to dryness under a stream of dry nitrogen in a Turbovap evaporator at 55 °C, and the residue was resuspended in 85% methanol in water (200 μl) for HPLC–MS/MS analysis.

2.4. Instrumental analysis

Twenty microliters of the reconstituted SPE extract were injected into a ThermoFinnigan Surveyor liquid chromatograph (ThermoFinnigan, San Jose, CA, USA) coupled with a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer. The phthalate metabolites were separated from the rest of the breast milk extracted components on a Betasil phenyl column (3 μm, 100 mm × 2.1 mm, ThermoFinnigan-Keystone, Bellefonte, PA, USA), which was preceded in-line by 2 and 0.5 μm filters (Upchurch Scientific, Oak Harbor, WA, USA). The chromatographic separation was achieved using a nonlinear solvent gradient from 100% aqueous mobile phase (0.1% acetic acid in water) to 100% organic mobile phase (0.1% acetic acid in acetonitrile) at 0.35 ml/min. At the end of each run, the organic mobile phase was held at 100% for 0.5 min followed by equilibration with the aqueous mobile phase for 1.0 min at 0.35 ml/min. Electrospray ionization in negative ion mode was used to form negatively charged analyte ions. The following instrumental settings were used: electrospray voltage (3000 V), nitrogen sheath gas pressure (35), nitrogen auxiliary gas pressure (10), and capillary temperature (270 °C). For MS/MS collision-induced dissociation, the collision energy was optimized for each analyte, and argon was used as the collision gas (2.0 mTorr). The precursor/product ion transitions, measured in multiple reaction monitoring mode at unit resolution, and used for quantification are given in Table 1. The scan time for each monitored transition was 200 ms.

Table 1
Multiple reaction monitoring analysis of phthalate metabolites

Analyte	Native	^{13}C-labeled	D_4-labeled
Phthalic acid	165/77	167/77	
Monomethyl phthalate	179/77	183/79	
Monoethyl phthalate	193/77	197/79	
Mono-*n*-butyl phthalate	221/77	225/79	225/81
Monoisobutyl phthalate	221/77	N/A	225/81
Mono-2-ethyl-5-hydroxyhexyl phthalate	293/121	297/124	
Mono-2-ethyl-5-oxohexyl phthalate	291/121	295/124	
Monobenzyl phthalate	255/183	259/186	259/187
Monocyclohexyl phthalate	247/77	251/79	
Mono-2-ethylhexyl phthalate	277/134	281/137	281/138
Mono-*n*-octyl phthalate	277/125	281/127	
Mono-3-carboxypropyl phthalate	251/103	255/103	
Mono-3-methyl-5-dimethylhexyl phthalate	291/247	295/250	

2.5. Data analysis

The identity of the analyte peaks was confirmed by matching the retention times with those of their respective isotope-labeled internal standards. Data were acquired and analyzed using the Xcalibur® software (ThermoFinnigan, San Jose, CA, USA) on a PC-based data system. A calibration curve of peak area ratio of each analyte to its isotope-labeled internal standard (after compensating for isotopic impurity of the labeled internal standard) versus the concentration of each analyte concentration was used for quantification.

Each batch consisted of 50 samples (i.e., five modules of the Zymark RapidTrace system) and included 41 unknown, five reagent blanks (one per module), two QCL, and two QCH samples.

2.6. Evaluation of the milk enzymatic activity on the hydrolysis of D_4-labeled phthalate diesters

Aliquots of milk (10 ml) and water were spiked with 0.11 ml of the spiking solution of D_4-ring-labeled phthalate diesters prepared as described above. The D_4-phthalate diester spiked samples were kept at 5 °C until analysis. The same procedure also was performed using milk spiked with D_4-ring-labeled phthalate diesters, but excluding the incubation step. Nonspiked milk and spiked water samples were used as controls. D_4-ring-labeled phthalate monoesters were measured as described above at several time intervals after spiking.

2.7. Evaluation of the effectiveness of phosphoric acid, formic acid, and TCAA as denaturing agents for the milk enzymes

Sixty milliliters of milk were spiked with 800 μl of the D_4-phthalate diesters spiking solution (i.e., ∼50–120 mg/l)

and then separated into four 15 ml aliquots. To determine the best denaturing agent to deactivate the milk enzymes, we added 1875 μmol of phosphoric acid, 1875 μmol of formic acid, and 1875 μmol of TCAA to three separate aliquots; the fourth spiked aliquot was not treated with acid. Another 15 ml aliquot of milk, not spiked with D_4-phthalate diesters, was used as a control. These aliquots were analyzed at several time intervals after spiking for the presence of D_4-ring-labeled phthalate monoesters.

3. Results and discussion

Breast milk is possibly the major route of exposure to certain xenobiotics by the breastfeeding infant. Because of the potential impact of these chemicals to lactating mothers and their children, interest is increasing in monitoring breast milk for environmental contaminants, including phthalates. We have developed a sensitive method to assess human exposure to phthalates through this pathway by measuring phthalate metabolites in breast milk.

We used D_4-phthalate diesters to unequivocally determine the presence of esterases in human milk by measuring the concentration of D_4-phthalate monoesters in spiked milk. D_4-Phthalate monoesters could be produced only by hydrolysis of the spiked D_4-phthalate diesters. We monitored the hydrolysis reaction for 7 days, and detected a rapid increase in D_4-phthalate monoester concentrations after spiking D_4-DBP, D_4-BBzP, and D_4-DEHP into untreated human breast milk (Fig. 1). Four hours after spiking, the concentrations of D_4-mBP, D_4-mBzP and D_4-mEHP were 66, 56 and 85%, respectively of the concentrations after 7 days. These data confirm the presence of active esterases in breast milk which, like in serum, can hydrolyze phthalate diesters into their respective monoesters [33,34]. Therefore, like for serum [26], a special treatment of the milk is required upon sample collection to denature milk enzymes and avoid overestimating the concentrations of phthalate metabolites in milk caused by contamination from the ubiquitous phthalate contaminants that may have been incorporated in the milk during the collection, storage, and measurement processes.

We detected negligible amounts of D_4-phthalate monoesters in water spiked with D_4-phthalate diesters, even after incubating the spiked water samples with β-glucuronidase for 90 min at 37 °C. The levels of D_4-mBP, D_4-mBzP, and D_4-mEHP in water were 2.2, 0.4 and 3.3% of the levels in milk spiked with the same amount of D_4-phthalate diesters (Fig. 1). These findings confirm that the D_4-phthalate diesters are hydrolyzed by endogenous milk components, presumably milk esterases [33,34], and not by the β-glucuronidase added to deglucuronidate the phthalate monoesters [32].

We tested methanol and acetonitrile as enzyme-deactivating agents [35]. However, these solvents did not eliminate the milk enzymatic activity, at least at a 2:1 ratio of solvent to milk (data not shown). Higher organic solvent to milk ratios could have resulted in deactivation of the milk enzymes, but because methanol and acetonitrile are strong eluting solvents for SPE, we did not attempt to eliminate the enzyme activity by increasing the amounts of these organic solvents. Instead we focused on the acid-deactivation of the milk enzymes (Fig. 2). We have previously shown that treatment with phosphoric acid deactivates the serum enzymes [26]. We found that treatments with formic acid or phosphoric acid also eliminated the milk enzymatic activity; in contrast, treatment with TCAA, did not. Although both formic and phosphoric acid deactivated the milk enzymes, we selected phosphoric acid, like we did for serum (125 μmol of phosphoric acid per milliliter), as the milk postsampling treating agent.

To determine whether the added phosphoric acid promotes the hydrolysis of the phthalate metabolites, milk (1 ml) treated with 125 μmol/ml of phosphoric acid was spiked with a solution containing phthalate monoesters (~100 ng/ml), stored at 4 °C, and analyzed after 24 h. We

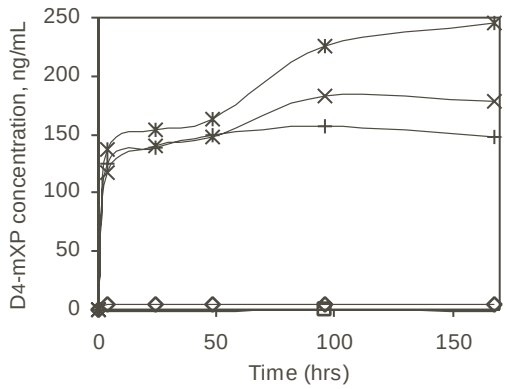

Fig. 1. Hydrolysis of phthalate diesters by milk enzymes. Water spiked with D_4-DEHP (◇); Nonspiked milk (□); milk spiked with D_4-DEHP (+); milk spiked with D_4-DBP (×); milk spiked with D_4-BzBP (*).

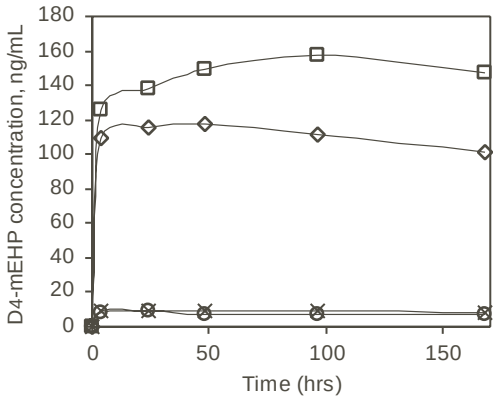

Fig. 2. Effect of acid treatment on the hydrolysis of D_4-DEHP by milk esterases. Milk spiked with D_4-DEHP and untreated (□); milk spiked with D_4-DEHP and treated with TCAA (◇); milk spiked with D_4-DEHP and treated with formic acid (×); milk spiked with D_4-DEHP and treated phosphoric acid (○).

added $^{13}C_4$-phthalate monoester and $^{13}C_2$-PA standards before and after extracting the milk to determine the total and residual amount of phthalate monoesters, respectively. We found that 4.5% of the spiked 11 $^{13}C_4$-phthalate monoesters hydrolyzed to $^{13}C_4$-PA 24 h after treatment. The presence of only residual amounts of $^{13}C_4$-PA and the fact that the amount of native PA did not increase after spiking the milk with the mixture of phthalate monoesters and PA confirmed that 125 mM phosphoric acid does not significantly hydrolyze phthalate monoesters to PA.

Standards and internal standards spiked in milk and water produced calibration curves with slopes not significantly different (data not shown). Therefore, the calibration curves were produced by directly analyzing standards prepared in water.

Milk is a complex matrix with high concentration of lipids, carbohydrates and proteins, and variable concentrations of vitamins and minerals [36]. To extract the phthalate metabolites from milk, we selected the Oasis-HLB cartridges, which contain a synthetic sorbent with high absorbency for a wide array of compounds. We found that the addition of an acidic solution to the milk was important for the retention of the phthalate metabolites to the HLB sorbent; we chose formic acid for its strength and volatile nature. We speculate that the interactions that facilitate the retention of the phthalate metabolites by the sorbent result from some competition between formic acid, the phthalate metabolites, and other milk components for the interaction sites in the sorbent. In addition, formic acid may disrupt the interaction of the phthalate metabolites with other milk components (e.g., proteins) that may not be easily retained by the sorbent.

To determine whether the phthalate metabolites concentrate in the aqueous fraction of the milk (supernatant) or in the solid fraction (pellet), we spiked the milk with native phthalate monoesters (24.0–197.4 ng/ml) and calculated the SPE recoveries of the monoesters from the supernatant obtained after centrifuging the milk at 3000 rpm for 10 min. RF_a, RF_m, and RF_s are the response factors obtained from spiking isotope-labeled standards to the milk supernatant extract after SPE, the milk before centrifuging and extracting the supernatant, and the supernatant after centrifuging and before SPE, respectively. The recoveries of the relatively small phthalate monoesters, such as mEP and mBP, from the supernatant were comparable whether the milk was spiked before ($RF_a/RF_m \pm$ standard deviation [S.D.]: $81.3 \pm 2.4\%$, mEP; $78.3 \pm 0.3\%$, mBP) or after ($RF_a/RF_s \pm$ S.D.: $89.6 \pm 0.3\%$, mEP; $102.6 \pm 0.6\%$, mBP) centrifuging. In contrast, for the larger phthalate monoesters including mEHP and mOP, the recoveries were significantly lower for milk spiked before centrifuging ($12.5 \pm 1.2\%$, mEHP; $14.5 \pm 6.2\%$, mOP) than after centrifuging ($73.8 \pm 2.7\%$, mEHP; $73.8 \pm 13.1\%$, mOP). These data suggest that the larger phthalate monoesters are bound to the less soluble milk components that constitute the pellet formed after centrifuging and do not partition well into the supernatant, while the smaller phthalate monoesters are found mostly in the aqueous fraction of the milk. Therefore, we used the milk not centrifuged for the SPE extraction (vide supra).

The SPE recoveries of the various phthalate metabolites from milk were calculated as RF_a/RF_b, where RF_a and RF_b are the response factor obtained from spiking the milk sample with the isotope-labeled standards after and before the SPE separation, respectively. The SPE recoveries of most phthalate metabolites from milk were good (Table 2). We observed a lower recovery for PA (~60%) compared with the other metabolites, probably because of its increased

Table 2
Solid phase extraction (SPE) recoveries, accuracy of spiked recoveries, linear range, and limits of detection (LODs)[a]

Analyte[b]	LOD	Linear range	SPE recoveries (%)[c]			Spiked recoveries (%)[d]		
PA	0.9	1–800	69	61	58	149	106	102
mMP	0.2	1–800	75	71	69	104	99	103
mEP	1.9	0.75–600	94	92	90	97	99	97
mCPP	0.4	1–800	93	91	85	83	99	98
mBP	1.0	1–800	118	86	76	108	102	105
miBP	0.3	1–800	100	85	92	157	96	98
mEHHP	0.2	0.8–650	94	93	91	101	98	101
mEOHP	0.5	1–800	91	106	91	107	101	103
mBzP	0.5	1–1200	89	88	83	126	98	98
mCHP	0.2	0.5–400	91	86	89	97	98	94
mEHP	0.6	0.75–600	74	75	75	170	100	99
mOP	1.0	1–800	66	75	68	144	105	96
mNP	1.7	1–800	68	63	67	[e]	[e]	109

[a] LODs and linear range reported in ng/ml.

[b] PA (phthalic acid), mMP (monomethyl phthalate), mEP (monoethyl phthalate), mCPP (mono-3-carboxypropyl phthalate), mBP (mono-*n*-butyl phthalate), miBP (monoisobutyl phthalate), mEHHP (mono-2-ethyl-5-hydroxyhexyl phthalate), mEOHP (mono-2-ethyl-5-oxohexyl phthalate), mBzP (monobenzyl phthalate), mCHP (monocyclohexyl phthalate), mEHP (mono-2-ethylhexyl phthalate), mOP (mono-*n*-octyl phthalate), mNP (mono-3-methyl-5-dimethylhexyl phthalate).

[c] SPE recoveries at 20, 50 and 100 ng/ml.

[d] Spiked recoveries for standards 2, 5 and 8.

[e] Not determined at 4 and 32 ng/ml; only at 320 ng/ml.

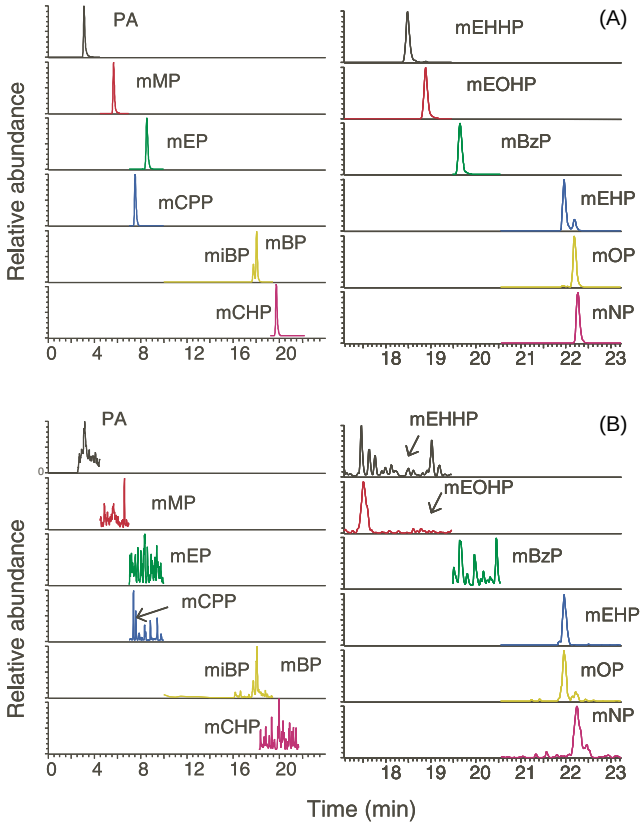

Fig. 3. HPLC–ESI–MS/MS sample chromatograms of a standard mixture of phthalate metabolites spiked in milk (A), and a pooled-breast milk extract (B). We made no attempt to eliminate the lipase activity in the milk. Therefore, the concentrations of hydrolytic phthalate metabolites in the pooled breast milk sample may include an unknown contribution from the metabolites formed from the enzymatic hydrolysis of possible contaminant diesters.

hydrophilicity. Because milk samples vary in their composition [36], we expected significant variability in the recovery of the various phthalate metabolites during SPE and in their extent of ionization. To minimize this confounder, we used the isotope-dilution technique with isotope-labeled phthalate metabolites, which allows for automatic recovery correction for each sample and improves the assay precision and accuracy. The native and labeled phthalate metabolites, being chemically identical, elute at the same time during the SPE and HPLC separations, but they can be differentiated by MS.

Milk spiked with phthalate metabolites and isotope-labeled phthalate metabolites (Fig. 3) was repeatedly analyzed to determine the method recovery, reproducibility, and limit of detection (LOD). The LOD was calculated as $3S_0$, where S_0 is the standard deviation value as the concentration approaches zero [37]. S_0 was determined from the replicate analysis of low-level standards. The calculated method LODs in 1 ml of milk were in the low ng/ml range (Table 2) and compared well with the LODs of phthalate metabolites in both urine [38] and serum [26].

The accuracy was established by determining the recovery of milk samples spiked with both phthalate metabolites and isotope-labeled phthalate metabolites (Table 2). To examine the consistency of the recovery over a range of phthalate metabolite levels, the measurements were taken by quintuplicate at three different concentrations (e.g., low, medium, and high). Because we used the isotope dilution technique, the spiked recoveries should be close to 100%. The mean recoveries of most phthalate metabolites in milk, expressed as a percentage of the expected value, were excellent at the medium (96–106%) and high spiking levels (94–109%). The recoveries at the lower spiking level were higher (97–170%) probably because of variations associated with making measurements near the LOD. The slopes of a linear regression analysis of the calculated versus the expected concentration ranged from 0.95 to 1.06, which confirmed the excellent accuracy of the method.

The precision of the method was determined by calculating the average coefficient of variation (CV) of 18–24 repeated measurements of the QC materials over a 4-week period (Table 3). The average CVs ranged from 4 to 27%. These values, which reflect both the intra- and interday variability of the assay, indicate the good reproducibility of this trace analysis method. Acid-treated QC materials remained stable for at least 3 months. The metabolite concentration associated with diester or monoester hydrolysis did not appear to increase or decrease.

We evaluated the applicability of the method to detect phthalate metabolites in human breast milk by analyzing three pooled breast milk samples (Fig. 3). Because these samples were not treated immediately after collection to eliminate the enzymatic activity that might have falsely elevated the phthalate metabolites levels, the concentrations of phthalate

Table 3
Precision of concentration measurements in spiked QC pools[a]

Analyte[b]	QC high		QC low	
	Mean	CV (%)	Mean	CV (%)
PA	58.4	8.2	12.6	17.8
mMP	125.0	5.0	23.9	9.6
mEP	29.2	10.2	2.7	23.2
mCPP	98.3	9.5	54.9	10.8
mBP	25.8	6.0	5.9	19.4
miBP	44.4	9.1	9.4	23.9
mEHHP	38.3	6.8	6.7	13.3
mEOHP	29.3	6.5	4.2	9.3
mBzP	41.9	7.4	4.0	6.6
mCHP	9.1	3.7	0.8	22.9
mEHP	44.6	7.7	9.2	17.2
mOP	39.1	7.5	4.3	27.0
mNP	41.5	11.6	18.1	18.8

[a] Mean concentrations in ng/ml. CV is the coefficient of variation.
[b] PA (phthalic acid), mMP (monomethyl phthalate), mEP (monoethyl phthalate), mCPP (mono-3-carboxypropyl phthalate), mBP (mono-*n*-butyl phthalate), miBP (monoisobutyl phthalate), mEHHP (mono-2-ethyl-5-hydroxyhexyl phthalate), mEOHP (mono-2-ethyl-5-oxohexyl phthalate), mBzP (monobenzyl phthalate), mCHP (monocyclohexyl phthalate), mEHP (mono-2-ethylhexyl phthalate), mOP (mono-*n*-octyl phthalate), mNP (mono-3-methyl-5-dimethylhexyl phthalate).

metabolites reported here include an unknown contribution from the metabolites formed from the enzyme-induced hydrolysis of possible contaminant diesters incorporated in the specimens during the sampling process. We detected mEHP and mNP in all of the samples, mBP, in two of the pools, and PA, in one. We also detected the oxidative metabolites mCPP, mEHHP, and mEOHP in all three pools.

The total (and free) mean concentrations and standard deviations (in ng/ml) in the three breast milk pools analyzed were 13.0 (11.4) for PA, 1.3 ± 1.5 (1.1 ± 1.4) for mBP, 7.8 ± 6.8 (7.7 ± 6.8) for mEHP, and 15.9 ± 7.7 (16.1 ± 6.9) for mNP; the concentrations of the oxidative metabolites were close to the LODs and below the limit of quantification. Unlike in serum, where the phthalate metabolites were mostly glucuronidated [39], the phthalate metabolites in breast milk were mostly in their free form. Although the concentrations of the free metabolites included an unknown contribution from the enzyme-induced hydrolysis of possible contaminant diesters, the presence of mostly unconjugated phthalate metabolites in breast milk might be attributed to the more lipophilic character of the free species compared with the corresponding glucuronides. The relatively higher lipophilicity of the free metabolites might have favored their transport into the breast milk during the synthesis of the milk in the body [40]. The mBP concentration in breast milk was significantly lower than in urine [24], but similar to the levels in serum [39]. In contrast, the mEHP and mNP levels in breast milk were higher than the levels in serum [39] and urine [24]. No population data exist for the levels of PA in people. We postulate that the higher amounts of mEHP and mNP than of mBP probably result from sampling contamination with DEHP and di-isononyl phthalate (DINP). DEHP and DINP are commonly used plasticizers in polyvinyl chloride materials [4,41], which might have been used during the collection of the breast milk samples (e.g., tubing for breast pumps). However, the detection of the three oxidative metabolites, mCPP, and the DEHP metabolites mEHHP and mEOHP, in the three pooled samples suggests environmental exposure to phthalate diesters, including DEHP, because the oxidative phthalate metabolites cannot be formed from the enzymatic cleavage by the breast milk enzymes of phthalate diesters. Additional studies to determine the contribution of sampling contamination are warranted.

In summary, we have developed a sensitive and high-throughput analytical method based on an automated SPE separation coupled with HPLC–MS/MS for the quantitative determination of 13 phthalate metabolites in human milk. We evaluated the applicability of the method in a small number of pooled human breast milk samples. Regardless of the mechanism by which the phthalates are incorporated into the breast milk (environmental exposure vs. sampling contamination), our data suggest that phthalate metabolites may be present in breast milk, and therefore, can be transferred to the nursing child. Furthermore, the detection of phthalate metabolites in breast milk reflects the high sensitivity of our analytical method and its suitability for future epidemiologic studies to assess exposure to phthalates in nursing women and their children.

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