# HIGH-THROUGHPUT DETERMINATION OF URINARY METABOLITES OF ORGANOPHOSPHATE FLAME RETARDANTS

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**Introduction**

Organophosphate flame retardants (OPFRs) are being used at an increased rate in consumer products due to the fact that they are perceived by the industry to be more environmentally friendly and thus safer alternatives than the halogen-based flame retardants[1]. However, little in known about the human health effects of OPFRs and levels of human exposure. In the recent years, a few methods have been developed to measure potential urinary metabolites and link their concentrations in urine to the exposure to OPFR parent compounds[2, 3, 4, 5]. The majority of these methods, based on solid phase extraction (SPE), chromatographic separation coupled to mass spectrometry, were applied to measure 2 to 5 urinary OPFR metabolites. We recently have developed a bench SPE-based method for the determination of nine urinary OPFR metabolites, including: diphenyl phosphate (DPHP), di-*o*-cresyl phosphate (D*o*CP), di-*p*-cresyl phosphate (D*p*CP), bis(2-chloropropyl) phosphate (BCIPP), bis(2-chloroethyl) phosphate (BCEP), bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), bis-2(butoxyethyl) phosphate (BBOEP), two hydroxylated metabolites of tris(1-chloro-2-propyl)phosphate: 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (TCIPP-OH) and desbutyl-tris-(2-butoxy-ethyl) phosphate (desbutyl TBOEP). In order to increase throughput and reduce the volume of urine require per analysis, a high-throughput method was developed as well for the measurement of the above OPFR metabolites.

**Materials and methods**

*Standards and Reagents*

BDCIPP and BDCIPP-d10 were purchased from Wellington Laboratories (Guelph, Ontario, Canada). BBOEP, BBOEP-$d_4$, TCIPP-OH, BCEP and desbutyl TBOEP standards were provided by Dr. Adrian Covaci. D*o*CP, D*p*CP, D*o*CP-$d_{14}$, DpCP-$d_{14}$ DPP, DPP-$d_{10}$ and BDCIPP were obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Methanol GC-grade and HPLC-grade water were purchased from EMD Chemicals, Inc. (Gibbstown, NJ, USA). Synthetic urine was purchased from Alltech (State College, PA, USA) and prepared with HPLC-grade water as per manufacturer instructions. Quality control normal urine (pool of 10 individuals) was obtained from Lee Bio Solutions (St. Louis, Missouri, USA).

*Automated solid phase extraction*

Extraction was performed on Freedom EVO 150 automatic liquid handling robot (Tecan US, Inc, Morisville, NC, USA) equipped with 96 well SPE vacuum manifold and microplate shaker. Upon manually transferring 1 mL of urine to a 3 mL glass disposable culture tube the automation was started. The robot would first add 1mL of 4% phosphoric acid in water and mix by pipetting up and down in the solution. Next, samples were fortified with 10 µL of 500 pg/µL of internal standard solution in methanol and again individual robot fingers would mix by pipetting samples up and down. The 96 well SPE plates (30 mg, Waters, Milford, MA, USA) were used for extraction. Each well was automatically conditioned with acetonitrile, methanol and HPLC-grade water, 400 µL was used for each solvent. Acidified samples, 1 mL each, were loaded to the conditioned SPE plates and percolated at approximately 5 mL/min. Then, wells were washed with 400 µL of 2% formic acid and dried under vacuum for 30 seconds. Elution was achieved with 600 µL of 2% ammonium hydroxide in methanol and extracts collected in 96 well collection microplates (Waters, Milford, MA, USA). Samples were evaporated to dryness with nitrogen on a 96 well microplate evaporator (TurboVap 96, Biotage, Charlotte, NC, USA). Following the evaporation samples were taken back to the Freedom EVO 150, and then automatically reconstituted with 100 µL of 5% methanol and mixed by plate shaking. Plates containing the extracts were manually sealed with an adhesive seal film (Waters, Milford, MA, USA) and analyzed using UPLC-MS/MS.

*Bench solid phase extraction*
Bench SPE method used a sample volume was 5 mL per analysis and the SPE procedure identical to the automated method performed manually. Solid phase extraction was performed using Oasis Wax (3cc, 60mg) SPE cartridges from Waters (Milford, MA, USA).

*UPLC-MS/MS*
Analysis of urine extracts was carried out on a UPLC System from Waters (Milford, MA, USA) coupled to a Waters Xevo TQD MS/MS **(**Milford, MA, USA) operated in electrospray (ES) positive or negative mode. Separation of metabolites was performed at 40°C using an Acquity UPLC BEH $C_{18}$ column from Waters (1.7 µm, 2.1 mm x 50 mm) attached to a Waters Van Guard BEH $C_{18}$ pre-column (1.7 µm, 2.1 x 5 mm). The mobile phase consisted of (A): 10 mM ammonium acetate in water and (B): methanol. The gradient programming was as follows: initial gradient 5% (B) to 90% (B) in 2.5 minutes, to 95% (B) in 1.75 minutes, hold for 4.2 minutes, and 4.08 minutes equilibrate to 5% (B). Flow rate was set at 0.22 mL/min. Bench and automated methods instrumental analysis differed in injection modes. To accommodate the analysis of 96 well format UPLC injection mode used for automated method was Partial Loop With Needle Overfill (PLNO) and 3 µL of samples were injected, while bench method UPLC injection mode was partial loop mode and 1.5 µL of samples were injected. Quantifiers and qualifiers of multiple reaction monitoring (MRM) transitions of the target analytes and internal standards used as well as associated collision energies are presented in Table 1. Source temperature, desolvation temperature, and desolvation gas flow were set at 150°C, 350°C and 650 L/hour, respectively.

**Table 1**. MRM transitions, collision energies and ionization mode

| Compound | Quantifier | CE (eV)* | Qualifier | CE (eV)* | Ionization mode |
|---|---|---|---|---|---|
| **BDCIPP** | 316.71 → 34.73 | 8 | 318.83 → 34.81 | 8 | ES- |
| **BDCIPP $d_{10}$** | 328.80 → 34.74 | 8 | 326.7 → 34.48 | 8 | ES- |
| **DPHP** | 248.97 → 92.87 | 26 | 248.97 → 154.89 | 22 | ES- |
| **DPHP $d_{10}$** | 259.03 → 97.93 | 26 | 259.03 → 158.86 | 22 | ES- |
| **BBOEP** | 296.88 → 78.96 | 50 | 296.88 → 196.6 | 20 | ES- |
| **BBOEP $d_4$** | 300.98 → 198.73 | 20 | 300.98 → 78.52 | 50 | ES- |
| **TCIPP OH** | 308.78 → 98.88 | 20 | 250.72 → 98.82 | 20 | ES+ |
| **BCIPP** | 250.8 → 98.81 | 20 | 252 → 98.81 | 20 | ES+ |
| **BCHP or BCEP** | 222.78 → 98.72 | 16 | 222.78 → 160.76 | 12 | ES+ |
| **D*p*CP/D*o*CP** | 278.97 → 90.91 | 30 | 278.97 → 165.67 | 24 | ES+ |
| **D*p*CP $d_{14}$/D*o*CP $d_{14}$** | 292.98 → 97.15 | 30 | 292.98 → 69.54 | 52 | ES+ |
| **desbutyl TBOEP** | 343.02 → 242.92 | 12 | 343.02 → 100.92 | 14 | ES+ |

*CE = collision energy

*Method performance*
The method detection limit (MDL) and the limit of quantitation (LOQ) were determined according to the EPA Regulation 40 CFR part 136 (Appendix B) method[6]. The relative percent recoveries were based on the internal standards recoveries. For compounds for which no labelled internal standards were available (i.e., TCIPP-OH, BCIPP, BCEP and desbutyl TBOEP), D*o*CP $d_{14}$ /D*p*CP $d_{14}$ was used as a surrogate internal standard.
To minimize the matrix effect, an extracted calibration curve was prepared in a surrogate blank matrix, which contained no detectable levels of any of the target analytes. The best-suited matrix was found in the form of the synthetic urine obtained from Alltech (State College, PA, USA). The matrix-matched calibration curve was linear over a concentration range from 0.5 ng/mL to 100 ng/mL with a coefficient of correlation ($r^2$) greater than 0.998 for all of the compounds of interest in both the automated and the bench methods.

**Table 2.** MDL, LOQ and recoveries

| **Automated method** | **BDCIPP** | **DPHP** | **BBOEP** | **D*o*CP & D*p*CP** | **TCIPPOH** | **BCIPP** | **BCEP** | **desbutyl TBOEP** |
|---|---|---|---|---|---|---|---|---|
| MDL (ng/mL) | 0.208 | 0.120 | 0.229 | 0.044 | 0.038 | 0.122 | 0.139 | 0.051 |
| LOQ (ng/mL) | 0.660 | 0.382 | 0.762 | 0.140 | 0.128 | 0.406 | 0.443 | 0.169 |
| Recovery (%) | 78.3 | 97.1 | 83.5 | 91.4 | 91.4 | 91.4 | 91.4 | 91.4 |
| **Bench method** | **BDCIPP** | **DPP** | **BBOEP** | **D*o*CP & D*p*CP** | **TCIPPOH** | **BCIPP** | **BCEP** | **desbutyl TBOEP** |
| MDL (ng/mL) | 0.249 | 0.130 | 0.077 | 0.130 | 0.196 | 0.164 | 0.152 | 0.200 |
| LOQ (ng/mL) | 0.829 | 0.432 | 0.256 | 0.414 | 0.583 | 0.488 | 0.451 | 0.596 |
| Recovery (%) | 98.0 | 108.0 | 77.6 | 95.50 | 95.50 | 95.50 | 95.50 | 95.50 |

**Results and Discussion**

The capability of the automated method was evaluated by a triplicate analysis of a commercially pooled urine sample from Lee Biosolutions, using the two sample preparation methods and their results compared (Figure 1).

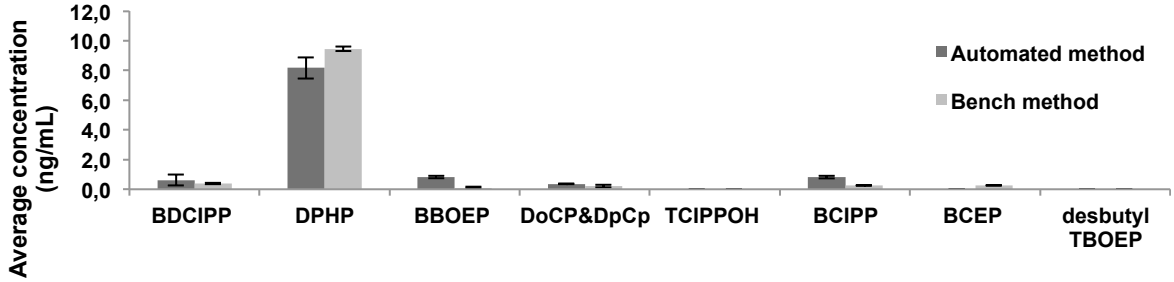


**Figure 1.** Comparison of automated and bench sample preparation methods (n = 3)

Although pooled urine contains majority of the metabolites at the levels below or close to LOQ values, it is obvious that the automated method is an appropriate alternative for the analysis of urinary OPFR metabolites, particularly given its method performance, increased analysis throughput and reduced sample volume required for the analysis. The automated sample preparation method was applied for the measurement of nine urinary OPFR metabolites in 13 paired urine samples obtained from Canadian women at pregnancy as well as at postpartum. The results from the analysis were summarized in Figure 2. While sparse data exist on levels of OPFR metabolites, levels measured here compare well with levels from other studies[5, 7, 8, 9]. All OPFR metabolites were measured without enzymatic deconjugation, including two newly characterized metabolites (i.e., TCIPPOH and desbutyl-TBOEP) which are considered to be mainly present as conjugates in urine [10]. In this study, free TCIPPOH was detected in urine of three postpartum urine samples. Di-cresyl phosphate isomers were not chromatographically resolved; therefore, their concentration was presented as the sum of the two isomers and were detected in 88.5 % of the study participants, with a median concentration of 0.65 and 0.53 ng/mL at pregnancy and postpartum, respectively. DPHP, a non-specific metabolite and BCIPP were the most frequently detected (85 and 100 %, respectively) OPFR metabolites measured in this small group of Canadian women, suggesting potential widespread use of either the metabolite itself (DPHP) or parent compounds in Canada. Median concentrations of DPHP levels observed in the study participants (median 6.49 ng/mL) were higher than those measured in 39 urine samples obtained from 8 pregnant women from the United States (US) (median 1.6 ng/mL)[11]. In contrast, the DPHP levels measured in this study were an order of magnitude lower than the levels recently published from the general Australian populations[7]. In the present study

group, although small (n = 13), only one individual had higher DPHP concentration in her urine after delivery, while other 12 individuals were found to have significantly higher DPHP levels during pregnancy. This observation instigates further investigation on occurrence and metabolic pathways in pregnant women.

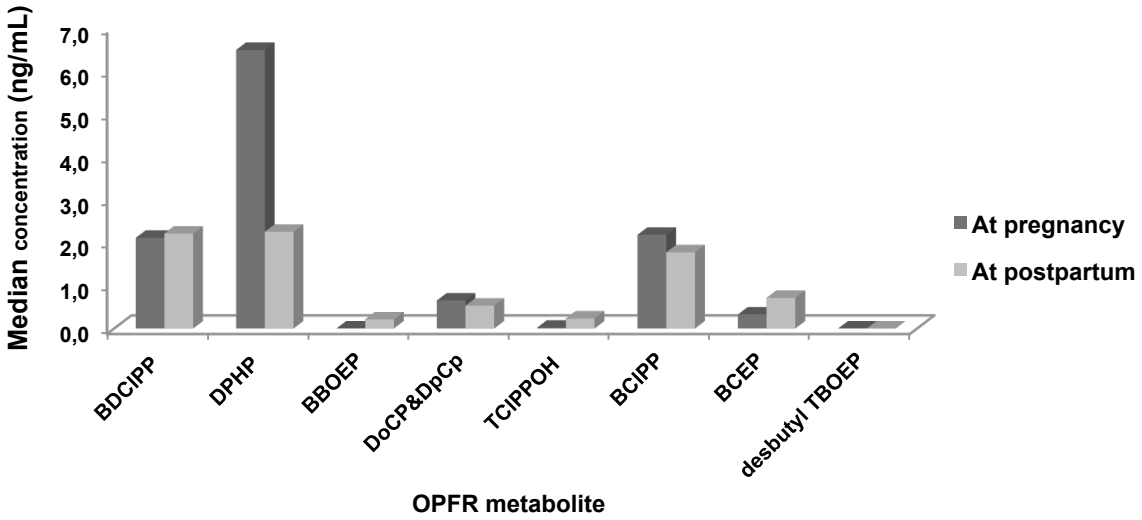


**Figure 2**. Urinary OPFR metabolites from 13 paired samples, at pregnancy and postpartum stages

Even though bench SPE method performed very well it required a sample volume of 5 mL, it was also limited by the number of samples that can be manually processed a day (a maximum of 24 samples), while the automated method on the other hand was much less labour intensive, required only 1mL of the sample and was significantly faster to perform. In addition, the extraction on the EVO 150 robot took only three hours for 96 samples, including the time-consuming step of sample evaporation.

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