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A mixed-mode chromatographic separation method for the analysis of dialkyl phosphates

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ABSTRACT

While reversed-phase (RP) liquid chromatography can separate a wide range of analytes, for strongly acidic compounds such as environmentally relevant dialkyl phosphates (DAPs), this remains a challenge because they have low affinity for standard RP columns or they exhibit inferior peak shapes. Mixed-mode chromatographic (MMC) columns, which contain both RP and ion-exchange functionalities, can address these resolution problems. However, using current MMC separation approaches, analyte peaks are relatively broad as compared to conventional RP chromatography. Herein we present an enhanced MMC-based UHPLC/ESI-MS method for the analysis of DAPs. In contrast to commonly available MMC-based methods, we applied the MMC Luna® Omega PS C18 column that was conditioned by 0.1% formic acid and equilibrated with the initial mobile phase before sample injection. This conditioning step tremendously improved the retention and separation of the DAPs, especially for those with high water solubility and shorter carbon chain lengths. Using water/methanol (95 v/5 v) and ammonium acetate in methanol as the mobile phases, nine DAPs could be baseline resolved with very sharp peaks, including the shorter-chain dimethyl phosphate, diethyl phosphate and bis(2-chloroethyl) phosphate. Other columns were examined to facilitate method optimization, and to identify stationary phases with the ability to separate DAPs as well as to elucidate the retention and separation mechanisms. With this novel UHPLC and post-column dication ion-pairing ESI-MS/MS method, instrumental detection limits as low as 0.01 ng/mL level were achieved. Representing other strongly acidic analytes, the short-chain perfluoroalkyl acid, perfluorobutyl sulfonic acid could also be analyzed with this method.

1. Introduction

Organophosphates (OP) compounds are widely used as insecticides, herbicides, flame retardants and plasticizers [1–3]. Organophosphate (OP) flame retardants constitute one class of extensively used OPs and are being increasingly produced since restriction of the use of polybrominated diphenyl ethers (PBDEs) as flame retardants [3,4]. Because OP flame retardants basically exist in products *via* physical addition, diffusion from host materials results in the continuous release of these compounds to the surrounding environment [1,5]. OP-based pesticides are widely used in the agricultural sector and in residential applications [6]. Given the occurrence of OPs in environmental systems, increasing attention has been devoted to their adverse effects in exposed organisms [7–10]. Previous studies have demonstrated that OPs can be rapidly metabolized through Phase I and Phase II biotransformation pathways to metabolites or hydrolyzed in aqueous systems [11–13], which can result in a wide array of dialkyl phosphates (DAPs), as well as other hydroxylated and carboxylated metabolite isomers [4,14]. DAPs ($(RO)_2P(O)OH$) are major metabolites or degradation products of OPs. Understanding DAPs in the environment is important and including OP pharmacokinetics in exposed organisms. The measurement of DAPs in the urine or blood is also proving useful as biomarkers of OP exposure in humans [5,15–20].

Analyzing trace levels of DAPs in samples is a challenge because of their high polarity and water solubility [14,21–23]. There are presently two main analytical methodologies used for the determination of DAPs in samples: derivatization of the DAPs followed by clean-up and determination by GC–MS or direct determination using LC–MS [15,22–24]. Methods that use GC–MS with derivatization have limited quantitative accuracy because of the poor derivatization efficiency as well as the derivatization process being

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time consuming. LC–MS(MS) methods tend to be very attractive for analyzing DAPs [14,19,21,22]. However, these approaches can result in poor chromatographic separation since conventional reversed-phase (RP) chromatographic columns do not sufficiently retain some of DAPs, and in particular those with short-chain alkyl groups such as dimethyl phosphate (DMP), diethyl phosphate (DEP) and bis(2-chloroethyl) phosphate (BCEP). To address this problem, ion-pairing based (*e.g.*, tetrabutylammonium acetate (TBAA)) chromatographic methods have been developed to enhance the retention factor of DAPs and using RP-LC columns [15,17]. However, ion-pairing additives have been proven to be detrimental for LC–MS determination [25].

Mixed-mode chromatography (MMC) is increasingly popular in analytical applications due to its unique selectivity and retention characteristic for a variety of compounds, especially for polar and charged molecules [26]. MMC stationary phases contain both RP and ion-exchange functionalities. MMCs can be used as an alternative or complementary tool to conventional RP column chromatography and to allow for the use of MS compatible mobile phases [25–28]. A number of new types of MMCs have become commercially available over the past few years. However, while MMCs provide more than one mode of stationary phase interaction with analytes, an analytical challenge is that defining the operation parameters for MMCs can be more critical compared to conventional RP-LC method approaches. Large differences among analytes in MMC retention and separation selectivity using RP-LC indicate that its retention mechanisms may be more complex. There is presently a dearth of knowledge regarding the separation mechanisms of MMCs and there are few published studies [29].

By testing and comparing different conventional RP and MMC columns, the purpose of this study was to develop a UHPLC–MS(/MS) based method for the direct and simultaneous determination of a large suite of DAPs in samples and to achieve high sensitivity by detailed optimization of MMC analysis parameters.

2. Materials and methods

2.1. Chemicals and reagents

Table 1 lists the names, abbreviations, structures of the DAPs studied in this project. DMP, DNBP, DPHP and DEHP were purchased from Sigma-Aldrich (Oakville, ON, Canada). BCEP, BDCIPP, BBOEP, BCIPP, d_8-BCEP, d_{10}-BDCIPP, d_4-BBOEP, and d_{10}-DPHP were purchased from Dr. Belov at the Max Planck Institute for Biophysical Chemistry (Germany). DEP was purchased from AccuStandard (New Haven, CT, USA). d_6-DMP was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Ammonium acetate and formic acid was obtained from Sigma-Aldrich (Oakville, ON, Canada). Decamethonium bromide was purchased from Sigma-Aldrich (Oakville, ON, Canada), and the method to convert this dicationic reagent into its hydroxide form was reported in our previously published paper [21]. Ultrapure water was obtained from a Milli-Q system.

2.2. Liquid chromatographic separation and mass spectrometric determination

Chromatographic separation and determination of DAPs was performed on a Waters XEVO-TQ-S UHPLC-QQQ-MS/MS (Waters Limited, Milford, MA, USA). During the method development process different LC columns were tested and compared; these included the Luna® Omega PS C18, Luna® Omega C18, Kinetex® EVO C18, Luna® HIHLIC and Acclaim™ Mixed-Mode WAX-1 columns. The column suppliers and other information are listed in Table 2. Various isocratic and gradient approaches for chromatographic separation with different mobile phases were used to identify the optimal analyte separation conditions. The optimal mobile phases and gradients for individual column separation are listed in Table 3. An isocratic chromatographic separation method was used for the HILIC and Mixed-Mode WAX-1 columns. During the method optimization process, 10 μL standard solution with a concentration of 100 ng/mL for each analyte was injected into LC system, except when MS/MS was operated in the ESI(+) mode.

The finally optimized chromatographic separation of the suite of DAPs was accomplished on a Luna® Omega PS C18, 1.6 μm, 50 × 2.1 mm (Phenomenex, CA, USA). Column and sampler temperatures were 40 °C and 20 °C, respectively. The mobile phases were A1: formic acid/acetonitrile/water, 0.1 v/5 v/95 v; A2: methanol/water, 5 v/95 v; and B2: 2 mM of ammonium acetate in methanol. The flow rate was 0.5 mL/min. Before injection an "inlet postrun" was performed with A1 for 3 min and then an "inlet prerun" was performed with 100% A2 for 5 min to equilibrate the LC system. The injection volume was 10 μL. The gradient elution started at 100% A2 and was held for 1 min, and then increased to 95% B at 6 min, and held for 2 min. Thereafter, the mobile phase composition was returned to 100% A2 in 0.2 min. As "inlet postrun" was not performed for the first injection in sample sequence, a blank injection sample must be run before real sample analysis if Waters Masslynx V4.1 software is used.

The XEVO-TQ-S QQQ-MS/MS system used an electrospray ionization (ESI) source that was operated in the negative mode (ESI(−)-MS/MS), or in the positive mode (ESI(+)-MS/MS) if the post-column dicationic ion-paring method was used. Nitrogen was used as the nebulizing gas and desolvation gas, and argon was used as the collision gas. The capillary voltage was 0.5 KV or 0.6 KV for the negative and positive modes, respectively. The desolvation temperature and cone temperatures were 500 °C and 150 °C, respectively. Desolvation and cone gas flow rates were 1000 and 150 L/h, respectively. When the system was operated in the ESI(+) mode the dicationic reagent solution (0.1 mM aqueous solution) was introduced into the MS/MS system downstream of the column with a "T" connector at a flow rate of 20 μL/min using a PerkinElmer 200 micro pump. Analyses were performed using the multiple reaction monitoring (MRM) mode, and the transitions, compound dependent operation parameters and retention times are listed in Table 4. The instrument control was *via* Waters Masslynx V4.1 software.

3. Results and discussion

3.1. Chromatographic separation optimization

Previous studies using conventional C18 RP-LC columns in the LC–MS/MS analysis of DAPs resulted in the separation of most DAPs and with generally sharp chromatographic peaks [11,19,21]. However, in the analysis of shorter alkyl chain DAPs, namely DMP, DEP and BCEP, the separation and peak resolution was very poor and the peaks tended to be very broad because of poor RP column retention behavior. In the present study, Luna C18 and Kinetex® EVO C18 conventional RP columns with the optimized UHPLC gradient 3 (described in Table 3) were also tested. The resulting UHPLC-ESI-MS/MS mass chromatograms illustrated in Figs. S-1 and S-2 showed again that most DAPs having long alkyl chains were well separated and with sharp peaks, but for the shorter chain DAPs, DMP, DEP and BCEP, the separation was poor and with broad and tailing peaks. Neither of these RP columns was able to adequately retain DMP and DEP even using 100% water as the mobile phase. The DMP peak tailed and had a peak width ($W_{1/2}$) on the Luna C18 and Kinetex EVOC18 columns of 0.143 min and 0.093 min, respectively, and where DMP and DEP co-eluted.