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Rapid LC-MS/MS quantification of Organophosphate non-specific Metabolites in Hair Using Alkaline Extraction Approach

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Abstract

Assessing human exposure to commonly used, highly toxic, but non-persistent organophosphates (OPs) is challenging because these toxicants are readily biotransformed into dialkyl phosphates (DAPs) and other metabolites. Growing hair accumulates toxicants and their metabolites, which makes hair a valuable non-invasively sampled matrix that can be used to retrospectively examine chemical exposure. However, the efficient quantification of hydrophilic DAP compounds in hair is challenging due to complex hair matrix effects. To improve upon existing methods, we first examined the acid dissociation constants (pKa) of DAPs and amino acids (major components in hair) and identified the best pH conditions for minimizing matrix effects. We hypothesized that under basic pH conditions DAPs and amino acids would be negatively charged and have weak interactions favorable to DAP dissociation from the matrix. To test this, we compared the efficiency of various pH conditions for optimal use of solvents to extract six DAPs from hair samples, and we quantified these DAPs using liquid chromatography-tandem mass spectroscopy (LC-MS/MS). As expected, a basic extraction (methanol with 2% NH_4OH) approach had the highest extraction efficiency and yielded satisfactory recoveries for all six DAPs (72%−152%) without matrix effects. Additionally, the alkaline extract can be directly injected into the LC-MS/MS. This relatively rapid and simple procedure allowed us to process up to 90 samples per week with reproducible results. To our knowledge, this is the first method to quantify all six DAPs simultaneously in hair using LC-MS/MS with electrospray ionization (ESI) in negative ion mode. Finally, we demonstrated the feasibility of measuring DAP levels in hair from patients affected with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease potentially linked to OP exposure. Due to our optimized solvent extraction process, the method we have developed

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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is compatible with the rapidity and sensitivity needed for hair analysis applied to population biomonitoring.

Keywords

Organophosphate pesticides; dialkyl phosphates; Hair matrix; Solvent pH; ElectroSpray Ionization; LC-MS/MS

1. Introduction

Organophosphate (OP) pesticides are widely used in agriculture to control insects. These pesticides are often hand-sprayed directly onto crops, leading to significant human exposure. Previous publications have firmly established an association between OP exposures and various adverse health outcomes, including respiratory disease, reproductive disruption, and neurological illness [1],[2],[3]. This previous research primarily examines acute or very severe high-level occupational exposures [4],[5],[6]; however, low-level chronic exposure remains relatively unexamined because it can be challenging to monitor low-level exposures to chemicals that do not persist in the environment or body. Thus, improved exposure assessment methods are necessary to better investigate adverse health outcomes associated with chronic low-level OP pesticide exposure.

Measuring OP concentrations provides an inaccurate assessment of human exposure because these chemicals undergo rapid biotransformation. After absorption, OPs are quickly transformed into various metabolites, including six non-specific dialkyl phosphates: dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP, diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP). Previous studies have identified these metabolites in biological matrices such as urine, blood, tissue, and hair [4],[7], and prior studies have also reported correlations between metabolites in these matrices [8] [9], [10], [11]. While urine and blood can provide a cross-sectional snapshot of exposure, pesticide exposure is typically sporadic and therefore cannot be accurately assessed or represented by single-time-point measurements. Three main characteristics make hair a particularly promising biomarker for OP exposure: hair can be collected non-invasively, hair is easy to collect/transport/store, and hair's chemical/metabolite levels reflect long-term exposure. Additionally, with a growth rate of approximately 1 cm/month for head hair, hair samples can reveal the profile of chemical exposures accumulated over weeks, months, or years [12]. Moreover, previous publications have found significant associations between pesticide levels in urine and hair [9], and plasma and hair [8], [13], which further suggests hair as an alternative matrix for biomonitoring research. Several cohort studies have reported on OP levels in hair, including studies of pregnant women [9], occupational and general populations [4], [14], and children [10]; however, these findings are limited by low detection rates and imprecise measurements for many non-specific metabolites, including DMP, DMDTP, and DEDTP [15], [16].

Although hair is easy to collect and store, sample preparation is challenging for DAP's hydrophobic analytes. Hair samples are processed through decontamination, extraction, purification, and concentration procedures before analytes are measured via gas

chromatography (GC) or liquid chromatography (LC) combined with mass spectrometry (MS). To successfully execute this protocol, care must be taken to clean the samples thoroughly and to efficiently extract analytes, since hair is a protein matrix that tends to strongly bind analytes. Previously published methods for extracting analytes from hair have utilized water, methanol, acetonitrile, and an 80:20 mixture of acetonitrile and water (80:20 acetonitrile: water) [15],[16], [17], [18], [19]. These solvent options were adapted from exploratory research examining a more diverse metabolomic profile. While these solvents are effective for OPs, they often also extract lipids and other undesirable compounds from hair, which can shorten columns' lifetimes and require additional sample processing or instrument maintenance. Several published methods use GC-MS to screen for a wide range of compounds [20] and pesticides [21], but are not well suited to the extraction and sensitive detection of non-specific OP metabolites. An alternate widely used GC-MS based method [18] reports good recoveries for four non-specific metabolites, DMP, DEP, DETP and DEDTP. This method, though useful, does not quantify DMTP and DMDTP. Similarly, other extraction methods provided data only for 3–5 non-specific metabolites instead of the six commonly reported in urine and blood matrices [22], [23]. A full profile of the six DAPs in hair is critical for understanding the body burden of OPs. Furthermore, these previously used GC-MS analysis protocols require derivatization, which is costly, time-consuming, and results in analyte loss and variable recovery rates [15],[18], [16], [24]. This paper presents our group's research into a suitable solvent for DAP extraction from hair matrix for direct LC-MS/MS analysis with satisfactory recoveries. The ideal solvent would efficiently extract DAPs from hair and could also be directly injected into the LC-MS/MS to minimize each sample's processing time and the chromatographic analysis's matrix effect. The ideal solvent would also be sufficiently sensitive to measure the six major DAP metabolites, found in widely variable concentrations. Here, we report that our experiments have achieved these goals. This is the first published LC-MS/MS method that determines six DAPs in hair simultaneously with high recoveries due to an uncomplicated and effective sample processing protocol. To validate this method, we present preliminary data obtained from 50 hair samples donated by patients with amyotrophic lateral sclerosis (ALS) , a neurodegenerative disease potentially linked to chronic OP exposure [2],[3].

2. Material and methods

2.1. Human hair samples

The work described here was reviewed by the Columbia University Human Research Protection Office and exempted from continuing Institutional Review Board (IRB) for the Protection of Human Subjects. The hair samples were acquired from the National ALS Biorepository. Samples were originally collected from ALS patients as part of the U.S. National ALS Biorepository Pilot Study between 2012 and 2015. Hair was collected in the participant's home by a trained specialist. Approximately 50–100 strands of hair were clipped in the occipital area at the rear base of the head. For bald male participants, an equivalent amount of hair was clipped from the torso. There are 21 female participants and 29 male participants in this study. The mean age of participants is 59 years and the mean age at ALS diagnosis is 58 years. To minimize sample waste during protocol optimization and

to provide internal control as part of our quality control procedures (see method below), hair samples were also obtained and pooled from a group of anonymous donors.

2.2. Chemicals and solvents

Standard mixtures were purchased from Cambridge Isotope Laboratories (Tewksbury, MA), including; dialkyl phosphate and phosphorothioate native unlabelled in methanol; and dialkyl phosphates and phosphorothioate cocktail (D, 98%) in methanol. The stock solutions were stored in the dark at −20°C. Multianalyte substandard solutions were prepared by diluting the stock solutions with methanol. Deuterated compounds were used as internal standard (IS), and the intermediate solution was prepared in methanol.

Acetic acid (99.7%) was supplied by Sigma Aldrich (St. Louis, MO). Ammonia solution 25% was obtained from Merck (Darmstadt, Germany). We used Optima grade methanol, acetonitrile, and water supplied by Fisher Scientific (Waltham, MA, USA).

2.3. Sample preparation

Hair samples were stored in glass vials in the dark. Samples were decontaminated by washing twice with water (Optima grade), at the ratio of 20mL/50mg of hair, in a wrist action shaker for 5 minutes (Burrell, Pittsburgh, PA, USA). Samples were washed in 20ml of methanol, with 1 minute of shaking. Samples were air-dried at room temperature overnight.

Dried hair was cut to approximately 1mm snippets. 50mg of hair was weighed in a 1.5ml reinforced plastic vial (Biospec, OK, USA). 4–5 stainless-steel beads were added. The resulting samples were homogenized using the ball mill (Biospec, OK, USA) for 6 minutes, which ground the hair into small particles with sufficient surface area for extraction.

We tested (a) methanol, (b) acetonitrile, (c) acetonitrile at high pH (2% NH_4OH), and (d) methanol at high pH (2% NH_4OH) as solvents for analyte extraction. In detail, 1mL solvent extraction was added to the vial containing 50mg milled hair. The samples were agitated overnight (16–18 hours) using an end-over-end rotator (Gilson, model 818, Automix, Middleton, WI, USA) and then were sonicated in a water bath (Branson 2510, Brookfield, CT, USA) for 1 hour [25],[26]. The samples were centrifuged at 12,000 rpm for 15 min (Mini spin, Eppendorf SE, Germany) at 35°C. The supernatant was transferred to a glass vial using glass Pasteur pipettes and concentrated to near dryness with a nitrogen stream at 3–5 psi. The samples were then resuspended with 80:20 water: acetonitrile/methanol. The extracts were transferred to centrifuge tubes and centrifuged for 15 minutes. The supernatant was collected in filter vials pore size 0.2μm, PTFE membrane (Thompson, CA, USA) and a 5 μL filtered solution was directly injected into LC-MS/MS.

2.4. LC -/MS analysis

A chromatography column, Hypersil Gold aQ C18 Polar Endcapped (3um, 100 × 4.6 mm), was purchased from Thermo Fisher. Liquid chromatography (Shimadzu) coupled with a Qtrap 6500+ (Sciex, Foster City, CA, USA) was controlled using Analyst software (version 1.6.2, Sciex, Foster City, CA, USA), and data processing was done on the Multiquant (version 3.0.3, Sciex, Foster City, CA, USA). The chromatography separation of each

analyte was done using the column Hypersil Gold aQ C18 Polar Endcapped (3μm, 100 × 4.6 mm) (Thermo Scientific, San Jose, CA, USA) at 40 °C, with a flow rate of 0.7 mL/min. The mobile phase A consisted of 0.1% (v/v) acetic acid in water (optima grade). Mobile phase B contained 1:1 methanol: acetonitrile (optima grade). The gradient program was set up to initially deliver 5% mobile phase B initially, followed by this scheme: 0–6 min: 5– 50% B, 6– 6.5 min: 50–95% B; 6.5–12 min: 95–100%B, 12–12.1: 100–5% B. Before each run, a 3-minute equilibration was initiated.

The mass spectral analysis involved optimizing factors that influenced the ionization and fragmentation of individual compounds. In detail, the standard solutions were directly infused into the mass spectrometer to determine the precursors, de-clustering potential, entrance potential, and collision energy. Compounds were detected by mass spectrometer AB Sciex 6500^{+} using electrospray ionization in negative ion mode, ESI (−), with a set curtain gas pressure of 35 psi, nebulizer gas pressure of 65 psi, turbo gas pressure of 65 psi, source temperature of 500°C, and collision gas pressure of 9 psi.

Quantification of the analytes was based on the peak areas of DAPs normalized by the peak areas of known concentrations of deuterated standards. The concentrations of the analytes in the extracts were determined by using a linear regression calibration curve (weight 1/x) of normalized peak areas in non-hair matrix standard solutions. The final concentrations of the compounds in the hair were determined by dividing the total mass of analytes in the extract by the total mass of the hair sample.

2.5. Quality control/ quality insurance

Pooled control hair samples were externally decontaminated as described above and then were cut into 1mm snippets before being milled into a homogenous powder. The pooled hair mixtures were stored in glass vials until they were processed at the same time as hair samples from a small group of ALS patients with unknown exposure levels (n=50, described below).

For each sample batch (16 samples), we added three quality control samples: one spiked with standards non-hair sample in a solvent solution, one non-spiked pooled hair sample, and one spiked pooled hair sample. Samples were fortified at 2ng of deuterated compounds per 50mg of hair, equal to 40pg/mg of hair. This fortification level is within the common range of concentration of target analytes that have been measured previously [18].

Pooled hair samples spiked with known concentrations of deuterated compounds were extracted in triplicates. The extraction efficiency was calculated using the ratio of the average areas of corresponding deuterated internal standards (as listed in Table 1) of the spiked samples to the areas of the deuterated compounds in standard solution at an equivalent supposed concentration. For instance, the corresponding deuterated compounds of DMP, DMTP, and DMDTP are DMP-d6, DMTP-d6 and DMDTP-d6, respectively. DEP, DETP and DEDTP also have their corresponding deuterated compounds, DEP-d10, DETP-d10 and DEDTP-d10. The accuracy was calculated as the ratios of concentrations of the target analytes spiked to the hair sample (not deuterated compounds) and the actual standard concentration of these analytes. Matrix effect for each hair sample varies depending on the

hair composition. Therefore, we calculate an average hair matrix effect where pooled hair extracts from solid-liquid extraction were filtered through nano-filter vials (pore size 0.2μm, PTFE membrane) and used as a standard matrix to prepare standards in the hair matrix. The matrix effect was calculated as the ratio of peak areas of corresponding deuterated compounds in hair matrix extract standards versus those in methanolic standards.

Inter-day precision was calculated as the relative standard deviation (RSD) of the results from different batches. Intra-day precision was determined as the average RSD of duplicate runs of the sample extracts on the same run day which reflected the instrumental performance.

The instrument limit of detections was determined as the concentrations of analytes corresponding to the peaks that gave a signal-to-noise ratio of 3. The limit of quantification was the concentrations of analytes corresponding to the peaks that gave a signal-to-noise ratio of 10. We estimated the method limit of detections based on the 50 mg mass of hair used for the extraction.

3. Results

3.1. Optimal extraction solvents for DAPs

This study's objective was to optimize extraction recoveries for all six DAPs in the hair matrix with different solvents at two pH conditions: neutral and alkaline. The best extraction solvent was chosen based on the highest overall extraction efficiency of analytes of interest. Figure 1 shows the extraction efficiency of targeted analytes when several solvents were used for solid-liquid extraction. Satisfactory extraction efficiency rates of most compounds were observed when alkaline methanol (2% NH_4OH added methanol) was used. Extraction efficiency rates for DMP, DMTP, DMDTP, DEP, DETP, and DEDTP were 113%, 89%, 152%, 86%, 92%, and 72%, respectively (Fig.1). In contrast, at neutral pH, extraction efficiency rates ranged from 3–14% for acetonitrile and 10–34% for methanol. Consequently, alkaline methanol was utilized to extract human hair samples.

3.2. Method validation

The response of deuterated standards in hair matrix extracts compared to methanol standards allowed us to assess the matrix effect of hair materials. Little to no matrix effect was observed with DEP-d10, DETP-d10; DEDTP-d10; DMTP-d6; an enhanced matrix effect at 131% was calculated for DMDTP-d6 and a minimal suppressed matrix effect at 75% was observed for DMP-d6. The calibration curves were set with 6 substandard concentrations ranging from 0.3125 ng/ml to 10 ng/ml. The linear regression R^2 for the majority of analytes was greater than 0.994. The accuracy values of the DAPs ranged from 101–116%. Intra-day precision RSD ranged from 0–9% for 25 measurements and inter-day precision RSD was less than 20% for most compounds except 24% for DMP. Limit of detection in hair samples was 0.82 pg/mg for DMP; 0.41 pg/mg for DMTP; 0.30 pg/mg for DMDTP; 0.23 pg/mg for DEDTP and 0.24 pg/mg for DEP and DETP (Table 2).

3.3. DAPs levels in authentic hair sample from ALS patient

Our optimized method was used to measure DAPs concentrations in hair samples from 50 ALS patients from the pilot study of the National ALS Registry. Detection frequencies of DEP and DEDTP are higher at 82% and 90%, respectively compared to DMP and DMTP at 48% and 18%. DMDTP was detected in 2 samples and DEDTP was detected in only one sample, but the values were below limit detection. DMP levels in hair ranged from 0.11–7.49 pg/mg; DMTP ranged from 0.01–1.14 pg/mg; DEP ranged from 0.04–12.7 pg/mg and DEDTP ranged from 0.26–3.55 pg/mg. (Table 3)

The detection frequencies varied sharply depending on the concentration of each analyte, in descending order from 90% to 2%: DETP> DEP>DMP>DMTP>DMDTP>DEDTP. The data show higher detection frequencies of DETP, DEP, and DMP (90 %, 82%, and 48%, respectively) alongside low detection rates of DMTP,DMDTP and DEDTP (18%, 4 %, and 2%, respectively) which is consistent with the literature [24].

4. Discussion

Impact of pH of organic solvents on DAP extraction

Previously published methods have used methanol, acetonitrile, and an 80:20 mixture of acetonitrile and H_2O (80:20 acetonitrile: water) as extraction solvents. [18], [16], [21] These solvents were determined to cover a wide range of hydrophobic and hydrophilic analytes. In published protocols, acetonitrile has been reported as the first-choice solvent for pesticide extractions from blood and urine samples due to its ability to precipitate protein and therefore minimize unwanted matrix interference. Since DAPs have high polarity, methanol has also been a popular extracting solvent. (Table S1)

Direct comparison of extraction efficiency for each solvent combination is not possible for most DAP compounds based on previous methods. Often, a subset of these compounds was analyzed in hair extraction methods either in untargeted metabolomic or for a broad profile of pesticide metabolites, methodologies that depend on relative differences between samples rather than absolute quantification [12], [27]. Previously published methods reported low overall recoveries as low as 12% for DMP when using 80:20 acetonitrile: water [16]; or 51–66% when using water followed by diethyl: acetonitrile (1:1, v/v) [15]; and 51%−107% when extracting hair with methanol [18]. (Table 4). Notably, though good recoveries were presented in [15] and [18] for several DAPs, recovery calculations were based on the high spiked levels ranging from 100–10000 pg/mg of hair and 50–500 pg/mg of hair, ranging from 2–25 times higher than the maximum concentrations found in our set of hair samples. Thus, our sample set could not be directly compared to the existing methods.

In this study, we first used those published methods, and only 10–34% of DAPs were recovered when using methanol as extracting solvent and just 3–14% when using acetonitrile as extracting solvent. With 2% NH_4OH added to the solvents, better extraction efficiency was achieved for both extraction solvents. Basic pH acetonitrile yielded good recoveries for DMDTP and DEDTP at 88% and 96%, respectively; however, DMP and DEP recoveries were deficient (<10%). Satisfactory recoveries (>70%) for most DAPs were

obtained when using basic methanol (added 2% NH_4OH). 2% NH_4OH in methanol thus results in the best extraction efficiency for this suite of six DAPs.

Ideally, a reference material of hair with known elevated concentrations of target analytes would be used to determine theextraction efficiency. However, this standard was not available for our current study. We spiked pooled hair samples with known concentrations of native standard materials and deuterated standard materials. This step accepted the assumption that if the analytes of interest were disassociated from the hair solid phase, the efficient extracting solvent should keep the analytes soluble in the extracts. The following steps in the protocol should recover the spiked concentrations of DAPs and their deuterated compounds.

The superior recovery rate under alkaline conditions can be explained by DAPs and amino acids' acid dissociation constants (pKa). These DAP metabolites are reasonably strong acids (pKa from 1.25 to 2.86), meaning they are usually dissociated and polar in water (Figure 2). Raising the pH of extracting solvents not only changes analyte charges but also changes the properties of other components in the mixture—most notably the protein groups in hair that bind these compounds. To minimize the matrix effects of hair, the analytes and amino acids should have the same charge, and as Figure 2 illustrates, suitable situations are either strong acidic or basic conditions. Between pH 2 and pH 10, amino acid compounds exist in dipolar ion form with a positively charged amine (–NH2) and a negatively charged carboxylic acid (–COOH). Still, within this pH range, negatively charged DMP can be adsorbed by the positively charged amine.

Eisenbeiss et al 2019 [27] also tested the relative efficiency of solvents with various pH for extracting untargeted metabolites. Their experiments show that protein amino acids were extracted with neutral pH. The presence of amino acids in the final extracts could exacerbate the matrix effect and interfere with the signals and peak shapes, a plausible explanation for the suppressed LC-MS signal reported in previous studies [28]. Theoretically, at low pH (1–2) compounds of interest would have negative charges while the hair surface would have a positive charge, leading to the binding of these compounds to the hair surface. Previous publications have used acidic hydrolysis for hair analysis, however many compounds become unstable during the harsh sample processing step [25]; furthermore, acidic extracts are corrosive and can damage LC-MS machinery. Therefore, we avoided the acidic extraction method.

At the high pH (~11), when 2% NH_4OH is added to the extracting solvent, compounds of interest have negative charges and increased solubility, while the hair surface also has a net negative charge, thus decreasing the affinity to the target analytes. In addition, NH_4OH is evaporative and can easily be removed from the extract by the concentrating step (blowing nitrogen), which is favorable for LC-MS analysis. This results in an efficient extraction with fewer interfering amino acids. We can estimate the lack of matrix effect based on a comparison between the signals from internal standards in methanol relative to those extracted in hair. Indeed, the matrix effect was 75% for DMP, 91% for DMTP, 131% for DMDTP; 93% for DEP; 96% for DETP and 97% for DEDTP, confirming that this extraction method has little matrix effect for most DAPs, except for DMDTP.

Omit purification steps

Initially, we also implemented purification steps to clean up the extract. Unexpectedly, Solid Phase Extraction (SPE) extracts did not show peaks for DMDTP and DEDTP despite using hexane as a strong eluting solvent with several sequential solutions. Therefore, we did not apply the SPE step. We also attempted to exchange the solvent with acetonitrile to precipitate protein. This solvent exchange process was unsuccessful because the solvent exchange also seemed to cause the complete disappearance of DMDTP and DEDTP and their internal standards peaks. Additional clean-up steps are labor-intensive and require more sample handling, potentially increasing the loss of target analytes and resulting bias. Previously published data also showed low recoveries for DMP (10.2 %) and DEP (3.6%) when using SPE and LLE, respectively at the purification step [17]. Therefore, this method only uses the most necessary clean-up steps. The final method merely has three steps: solid-liquid extraction, centrifugation, and filtration of the samples with vial filters to remove the hair from the extracts before LC-MS analysis. The simplicity of the method reduced the time and labor effort required to prepare the samples for analysis.

Sensitivity

Our method LODs of DAPs ranged from 0.2–0.8 pg/mg for the six DAPs (Table 3). These instrumental LODs are comparable to other DMP and DMTP and better for other DAPs, including DEP, DETP, DEDTP, and DMDTP. This suggests that this current method could be used for general population hair analysis. Relatively low LODs were reported by [15], where the LOD ranged from 20–100 pg/mg for DEP, DMP, and DMTP. A method by [18], for comparison, reported even lower LOD for DEP, DETP, DEDTP, and DMP at 5, 5, 3, and 6 pg/mg, respectively. Other methods reported similar LODs values for most DAPs, except DMP, in comparison to this study [16]. (Table S2) These methods differ in their detection limits partly because they use GC-MS rather than LC-MS/MS separation and detection, which is less sensitive and more labor intensive in that it requires derivatization. Figure S1 showed the chromatogram of DAPs of a substandard at level 1.5 ng/mL, their corresponding deuterated compounds at level 100 ng/mL (Fig.S1). With a simple extraction procedure and an effective solvent extraction, we could minimize the matrix effect of interferences from the hair matrix. This allowed us to obtain sensitive quantification of the DAPs measurements.

Application

DAP accumulation in hair was proposed to be a reliable indicator of long-term exposure to OPs [29]. The modest improvement in detection and quantitation limits that we report here is essential to analyze concentrations in natural hair samples, which are usually much lower than previous methods could measure, particularly for DEP, DEDTP, and DMDTP. In our 50 hair samples from U.S. ALS patients, the range of concentrations of DAPs measured are notably lower than those reported in several previously published studies that sampled hair from pesticide sprayers or agricultural and rural communities in European or Asian countries [18], [30], [31],[32]. For instance, the levels of DMP in our study range from 0.11–7.49 pg/mg for ALS patients, while for rural populations in Crete [18], the median DMP concentration was 181.7 pg/mg. This could be explained by the relatively low representation of agricultural workers and rural communities in our samples. Indeed, while

in the National ALS Registry pilot study ALS cases were recruited to represent all 50 states, overall, they were reported to cluster in large metropolitan areas [33]. Additionally, only ~4.5% of the participants from whom we obtained hair samples reported occupational usage of any types of pesticides. In support of this difference in population representation, the DAP levels that we measured here in U.S. ALS patients is more within the range reported by other studies in general or urbanized European and Asian populations. [9], [14], [34]. For example, in French pregnant women DMP levels were from 0.07–700 pg/mg; P75 = 2.54 pg/mg [9]; in our study they range from 0.11–7.49 pg/mg (P75 = 3.15 pg/mg). In agreement with our data, several studies measuring DAP levels in urine reported overall lower levels in the U.S. population as compared to European and Asian populations, which could be due to differences in pesticide use and food crop regulation stringency [35], [36].In contrast, detection frequency for DETP (90%) and DMP (48%); is high compared to an European population-based study in Luxembourg with 29% and 23%, respectively; however agriculture is very limited in Luxembourg and may not be representative of other European countries [34]. The higher detection frequency of DEP and DETP compared to DMP and DMTP in this set of samples are in line with most of the publications [18].

Similarly, low detection frequencies of DEDTP and DMDTP (0– 4%) are consistent across several studies [9], [34].

Matrix effects

Hair is not an easy matrix to use for exposure assessment due to its complex chemistry and physical structure. Hair is 65–95% coiled protein, 15–35% water, 1–9% lipids, and other minerals, and it is very difficult to separate those protein coils to access bound contaminants [37]. However, these characteristics also make hair a stable matrix for the assessment of retrospective chemical exposures. Hair extracts have complex chemistries that impose a matrix effect in LC-MS/MS analysis [28]. Thus, we tested the intensity response of internal standards and standards in hair extracts. Several extracts of pooled hair samples were combined to make the standard matrix. Spiked internal standards and native standards were prepared similarly to how methanolic standards were made. A pesticide-free hair matrix would be ideal for creating calibration standards to ensure the inclusion of matrix interferences. We observed that matrix effects were not significant for OP metabolites when comparing the deuterated standards responses in pooled hair extracts versus methanolic standards for most DAPs, except DMDTP-d6, which accounted for the 152% extraction effieciency reported. Since a pesticide-free-pooled hair sample was not available for this research, we quantified DAPs based on methanolic DAPs and deuterated standards. Thus, the quantification of DAPs in this study was based on methanolic standards calibration.

However, we found considerable signal suppression when analyzing the same samples for pyrethroids metabolites, 3-PBA and 4F-3PBA. This suppression rendered our attempt to simultaneously determine OPs and pyrethroids metabolites in hair extracts unsuccessful. Although it would be ideal to analyze both OPs and pyrethroids metabolites in health studies, due to their differences in polarity it is challenging to extract both classes of compounds effectively with a single method. Future improvement of the current method

will be geared toward allowing the simultaneous quantification of even more pesticide metabolites in one simple protocol amenable to large biomonitoring studies.

5. Conclusions

Here we report a new and effective extraction procedure enabling the reliable measurements of DAPs in hair samples. Basic pH methanol was chosen as the extraction solvent for optimal recoveries of DAPs from the hair complex matrix. Satisfactory recoveries of six DAPs were obtained and ranged from 72–152%. In addition, this optimized extraction solvent allowed us to reduce the interference materials in the final extract, which minimized the matrix effects. Ultimately, our method provides satisfactory sensitivity and reproducible results along with reduced labor intensiveness and instrument maintenance time. This is also the first report that analyzed pesticides in hair matrix using LC-MS/MS in ESI (−). Finally, to confirm the validity of our method, we report OP metabolite levels in hair samples of an ALS patient cohort. This simple optimized procedure could be applied to other population health studies to determine DAPs levels in human hair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Hair is a challenging matrix to recover six dialkyl phosphates (DAPs) from effectively.
2. pH of extraction solvents affects recoveries of target analytes
3. Alkaline Extraction (methanol +2% NH_4OH) showed satisfactory results both theoretically and practically
4. The final extract can be directly injected into LC-MS/MS with ESI (−)
5. Up to 90 samples can be processed per week.

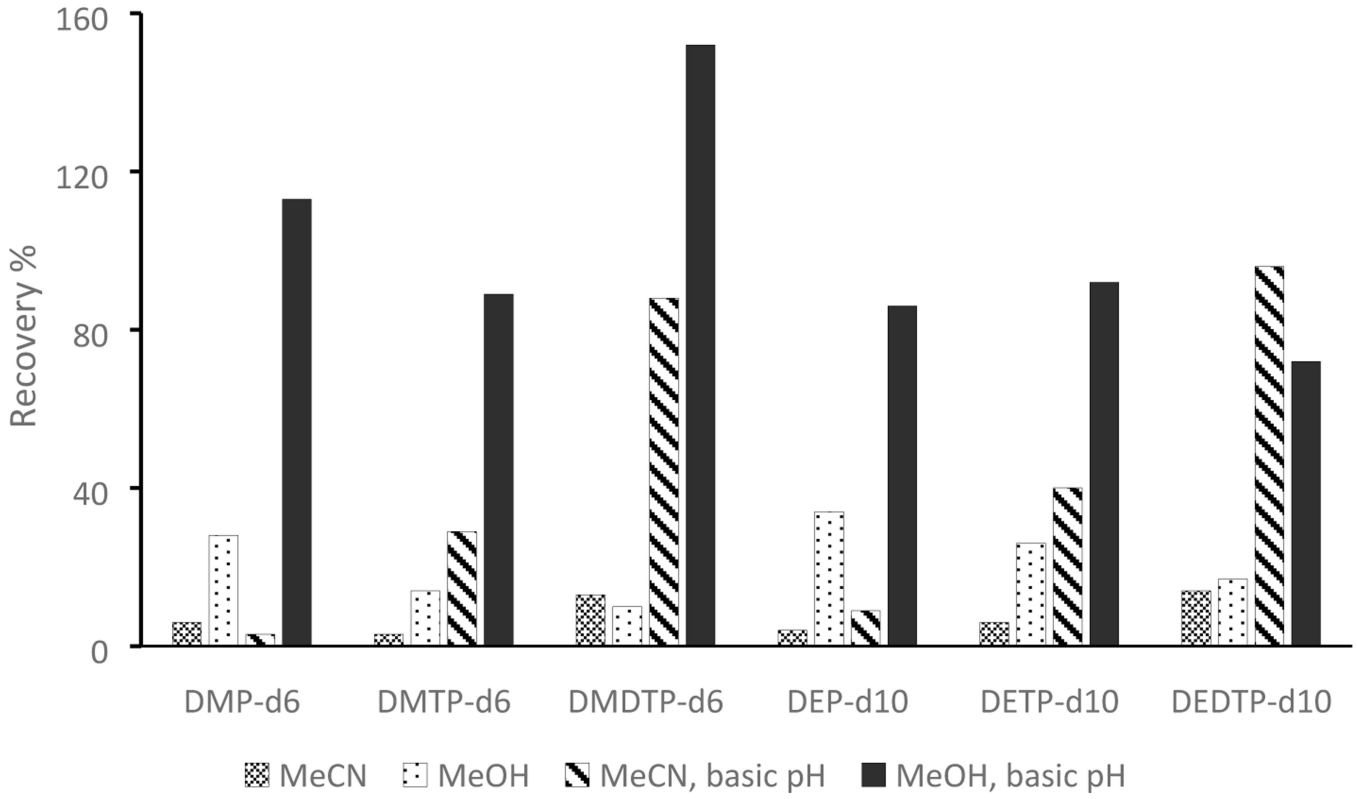

Figure 1:

Extraction efficiency of DAPs internal standards in hair matrix when different solvents were used for the extraction of organophosphate metabolites in hair. Alkaline methanol (2% NH4OH) was selected in this study as the solvent extraction to optimize the recoveries of DAPs metabolites from the hair matrix.

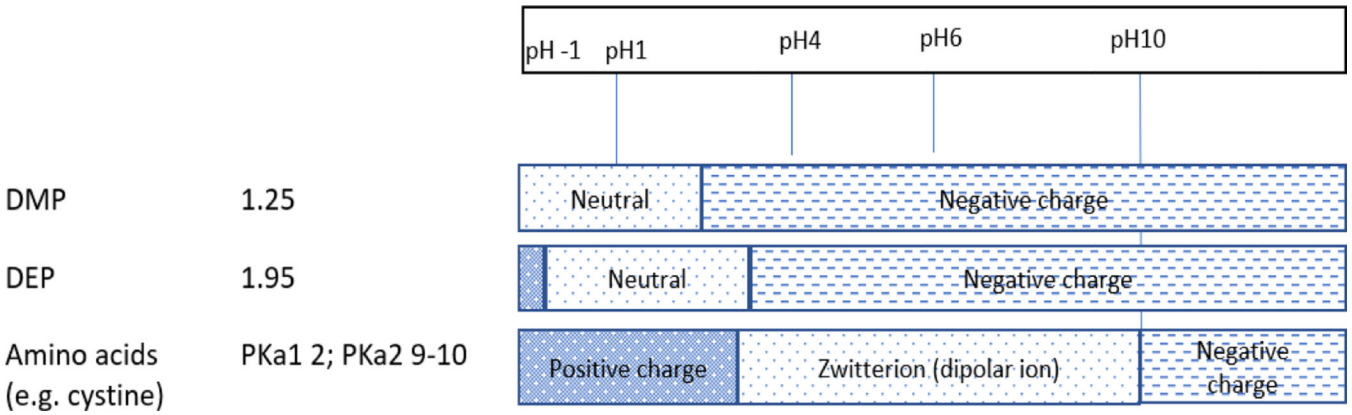

Figure 2:
Net charges of DMP, DEP and amino acids in hair depending on their pKa and pH of the solutions

Table 1:

Name, acronym, log pKa, MRM transitions, collision energy, de-clustering potential and dwell time of dialkyl phosphates (DAPs)

Analyte	Acronym pKa	Precursor	CE (volts)	DP (volts)	Dwell time (ms)
Dimethyl phosphate	DMP 1.25	124.9 – 62.8	23	50	50
		124.9 – 78.9 (*)	31	50	50
Dimethyl thiophosphate	DMTP 2.86	140.8 – 125.9 (*)	21	36	50
		140.8– 95.8	29	36	50
Dimethyl dithiophosphate	DMDTP 1.35	156.8 – 141.9 (*)	22	52	25
		156.8 – 112	30	52	25
Diethyl phosphate	DEP 1.37	152.8 – 125.1 (*)	15	28	50
		152.8 – 79	29	28	50
Diethyl thiophosphate	DETP 1.49	168.9– 140.9	17	31	50
		168.9– 94.9 (*)	27	31	25
Diethyl dithiophosphate	DEDTP 1.35	184.8–110.9 (*)	26	36	25
		184.8 – 94.9	45	36	25
Dimethyl phosphate deuterated d6	DMP-d6	130.9 – 63	25	35	50
Dimethyl thiophosphate deuterated d6	DMTP-d6	146.9 – 96.9	30	35	50
Dimethyl dithiophosphate deuterated d6	DMDTP-d6	162.9 – 145	23	40	25
Diethyl phosphate deuterated d10	DEP-d10	162.9 – 78.9	41	38	50
Diethyl thiophosphate deuterated d10	DETP-d10	178.9 −95	29	38	50
Diethyl dithiophosphate deuterated d10	DEDTP-d10	195.3 – 110.9	27	40	25

Table 2:

Method validation data for DAPs

Analytes	R2 of calibration curve	LOD (Pg/mg) (S/N= 3)	LOQ (Pg/mg) (S/N= 10)	Extraction efficiency	Intra-day Precision (n=2)	Inter-day Precision (n=4)	Accuracy	
							0.1 ng/mg	0.2 ng/mg
DMP	0.998	0.82	2.46	113	5	24	108	111
DMTP	0.994	0.41	1.23	89	7	13	106	111
DMDTP	0.997	0.30	0.9	152	9	10	111	109
DEP	0.999	0.24	0.72	86	6	12	111	115
DETP	0.999	0.24	0.72	92	5	11	111	106
DEDTP	0.999	0.23	0.69	72	0	19	101	108

Table 3:

Organophosphate concentration levels (pg/mg) measured in random 50 hair samples from the ALS cohort (n=50).

Metabolites	Detection frequency	Mean	Std. Dev	Min	P25	P75	Max
DMP	48	2.51	2.17	0.11	1.10	3.13	7.49
DMTP	18	0.33	0.32	0.01	0.19	0.35	1.14
DMDTP	4	0.26	0.05	0.22	0.24	0.28	0.30
DEP	82	2.56	3.06	0.04	0.59	2.78	12.7
DETP	90	0.89	0.72	0.26	0.48	0.9	3.55
DEDTP	2	-	-	-	-	-	0.13

Table 4:

List of recovery rate/ extraction efficiency between existing methods and the current method for simultaneous quantification of pesticide metabolites in hair.

Recovery	Extraction solvent	DMP	DMTP	DMDTP	DEP	DETP	DEDTP
Current study *	MeOH, 2% NH4OH	113	89	152	86	92	72
[15]	Water	55–66	97–107	na	61–99	na	na
[16]	MeCN: water (80:20)	12–16	43–66	19–24	51–69	70–87	na
[18]	MeOH	51	na	na	89	86	98

na: not applicable

*: extraction efficiency