



Determination of dialkyl phosphates in human hair for the biomonitoring of exposure to organophosphate pesticides[☆]

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ABSTRACT

A new, simple, fast and sensitive method that enables the measurement of four dialkyl phosphates (DAPs) in human head hair is presented in the current study. The dialkyl phosphates, dimethyl phosphate (DMP), diethyl phosphate (DEP), diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP) are non-selective metabolites of the organophosphate pesticides (OPs). The extraction of DAPs from hair matrix was achieved by one step methanolic extraction. Head hair samples from general population and population occupationally exposed to OPs were analysed using gas chromatography–mass spectrometry (GC–MS) after derivatization with pentafluorobenzylbromide. The recovery of the target compounds was estimated at 84.3% for DMP, 116.1% for DEP, 109.0% for DETP and 91.5% for DEDTP. The limit of quantitation (LOQ) and detection (LOD) was 20 and 6 pg/mg for DMP, 10 and 5 pg/mg for DEP and DETP and 5 and 3 pg/mg for DEDTP, respectively. With-run and between-run precision as well as accuracy was estimated. The percentage of positive hair samples for DMP, DEP, DETP and DEDTP for the group of general population was 63.0%, 96.3%, 66.7%, and 70.4% respectively. The samples from the group with occupational exposure were positive for all dialkyl phosphates analysed. The median concentrations for DMP were 165.0 and 181.7 pg/mg, for DEP were 51.2 and 812.9 pg/mg, for DETP were 54.0 and 660.1 pg/mg, and for DEDTP were 40.0 and 60.6 pg/mg for the general population group and the group with occupational exposure respectively. Significant differences in the levels of the total dialkyl phosphates amongst exposed and not exposed groups were observed ($p < 0.001$). More specifically, the total ethyl phosphate (DEPs) and DAPs median concentrations were 119.5 and 301.5 pg/mg for the general population group and 1498.8 and 1694.4 pg/mg for the group with occupational exposure.

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1. Introduction

Hair is a matrix that has been extensively used for the analysis of drugs of abuse in routine forensic or clinical practice. In some post mortem cases, hair is the only available biological sample for the assessment of drugs or pharmaceuticals' exposure [1,2,3]. Hair analysis has also been successfully used to assess chronic exposure to metals [4], various xenobiotics [5], environmental pollutants, organochlorine pesticides [6–8] and recently, for the assessment of exposure to organophosphate pesticides (OPs) [9]. Pesticides and particularly OPs were, and still are, intensively used in Crete, but their use has side effects both in the humans and in the environ-

ment. Rural population in Crete is exposed to OPs via the food chain and environmental pollution [10], yet, severe exposure is mainly attributed to occupational use, e.g. exposure of pesticides sprayers and applicators.

The frequencies of detection of OPs or the OPs specific metabolites in hair were lower compared to those observed in other groups of pesticides like organochlorines [11]. This is because OPs are rapidly metabolized to non-specific dialkylphosphate metabolites (DAPs) (Table 1). Consequently, DAPs are considered as biomarkers for a great number of pesticides and are used for the monitoring of organophosphate cumulative exposure [12,13].

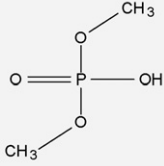
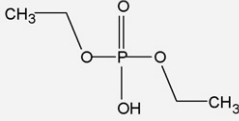
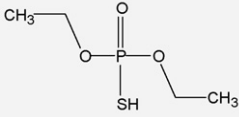
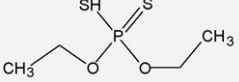
DAPs have been detected in various samples such as urine, blood, amniotic fluid and meconium samples from the general population, from the occupationally exposed population and also from the poisoning cases [14–17]. Recently, it was shown that DAPs can also be detected in the hair of laboratory rabbits treated with organophosphate pesticides via their drinking water [18] while further studies confirm the detection of DAPs in human hair of rural population in Crete [19].

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Table 1
Studied dialkyl phosphate metabolites, their structures and the parent organophosphate pesticides.

DAPs	DAPs structure	Parent pesticides
DMP		Azinphos-methyl, dichlorvos, dicrotophos, dimethoate, fenitrothion, fenthion, malathion, methyl parathion, trichlorfon, chlorpyrifos-methyl, methidathion, mevinphos, oxydemeton-methyl, phosmet, primiphos-methyl, temephos, tetrachlorvinphos, trichorfon, isazofos-methyl, naled, dicrotophos
DEP		Chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, sulfotepp, terbufos, tribufos
DETP		Chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, ethyl parathion, phorate, phosalone, sulfotepp, terbufos, tribufos
DEDTP		Disulfoton, ethion, phorate, phosalone, terbufos

The aim of this study was to develop a simple, precise, efficient and less time consuming method, compared to a previously published one [18], for the detection and the quantitation of DAPs in head hair samples. We further investigated the DAPs levels in head hair of the general population and population occupationally exposed to OPs in order to identify differences in the exposure levels, for risk assessment purposes.

2. Experimental

2.1. Sample collection

A total of 33 head hair samples were collected and analysed. Approximately 200–500 mg of hair was cut from the root, at the back of the head and used for the analysis. Twenty-seven hair samples were collected from 10 males and 17 females working in the Department of Morphology, Medical School, University of Crete, who were not occupationally exposed to OPs and were classified as general population samples. The length of the hair samples from the participants varied from 3 cm to 24 cm, which corresponds to a testing period up to 24 months [11].

Six samples were collected from pesticide male applicators during the spraying period (total hair length 3–5 cm). The subjects were asked to fill up a questionnaire regarding the duration and intensity of their exposure to pesticides via their occupation. Two of them complained for symptoms of mild intoxication several times during applications (headache and vomiting). The hair's length in these subjects was 3–5 cm. All hair samples were stored in paper envelopes, at ambient temperature until analysis.

In both cases (real samples), we analysed the whole lock of hair in order to estimate the total exposure of populations to OPs.

2.2. Materials

Dimethylphosphate (DMP; 98%) was purchased from Acros Organics (Geel, Belgium; New Jersey, USA), diethyl phosphate (DEP; 98.9%) was from Chem Service (West Chester,

New York, USA), *O,O*-diethylthiophosphate potassium salt (98%) (DETP) and diethyldithiophosphate salt (95%) (DEDTP) were from Sigma–Aldrich (3050 Spruce Street, St. Louis, USA). Toluene (99.5%) and potassium carbonate (K_2CO_3) were obtained from Merck (Darmstadt, Germany). Acetonitrile and dibutyl phosphate (DBP) (97%) were purchased from Roth (Karlsruhe, Germany) and Fluka (Steinheim, Germany) respectively. The derivative agent 2,3,4,5,6-pentafluorobenzylbromide (PFBBBr) (99%) was obtained from Sigma–Aldrich (Steinheim, Germany).

2.3. Gas chromatography and mass spectrometry conditions

Electron ionisation mass spectrometric analysis was performed on a GC–MS QP-2010 Shimadzu system equipped with a BPX5 (30 m × 0.25 mm × 0.25 μm) capillary column (SGE, Argent Place, Ringwood, Victoria, Australia). Pure helium (99.999%) with a column flow of 1 ml/min was used as a carrier gas. 2 μl of the solution was injected into the system in the splitless mode and analysed under the following conditions: the column temperature was initially held at 60 °C for 1 min, raised to 180 °C at 20 °C/min, held for 1 min, raised to 250 °C at 4 °C/min, held for 1 min and was finally raised to 300 °C, at 25 °C/min, where it remained stable for 2 min. The injector temperature was 270 °C. The interface temperature was set at 300 °C. The ion source temperature was 230 °C.

An auto-tune of the mass spectrometer using perfluorotributylamine (PFTBA, tuning standard) was performed before the analysis of every set of samples. Quantitative analysis was achieved in selected ion monitoring (SIM) mode with a scan time of 0.2 s, using one target ion for quantitation and two qualifier ions for the confirmation of each compound. The chromatogram was divided into five different segments for DAPs and IS. The first segment was from 17.0 to 18.0 min (m/z 110, 306, DMP), the second from 19.5 to 20.5 min (m/z 258, 334, DEP), the third from 23.0 to 24.0 min (m/z 350, 274, DETP), the fourth from 24.6 to 25.6 min (m/z 366, 185, DEDTP) and the fifth from 26.6 to 27.6 min (m/z 335, DBP-IS) (Fig. 1). The column temperature was programmed in this way in order to avoid any interference from the matrix (hair) and the derivatizing agent.

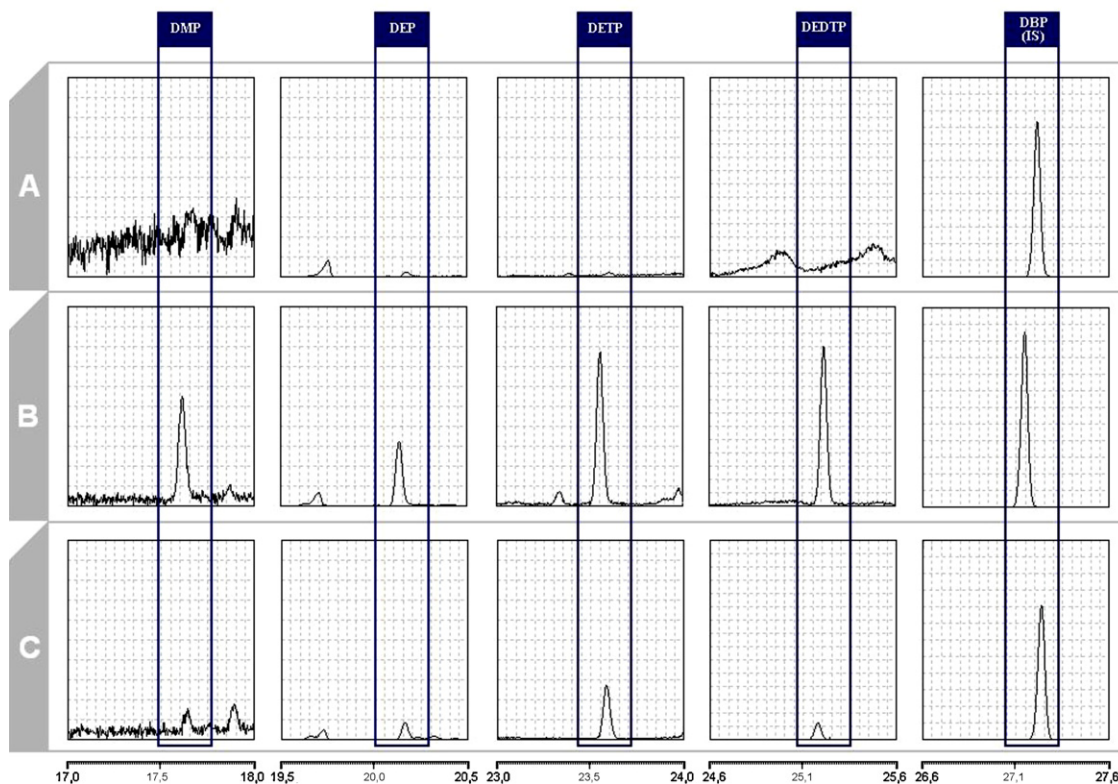


Fig. 1. Chromatogram of (A) blank head hair sample with DAPs levels below the LOQ, (B) spiked head hair sample at the concentration of 100 pg/mg of DMP, DEP, DETP and DEDTP and (C) real case head hair sample. The concentrations of DMP, DEP, DETP and DEDTP in the real case (C) were 33.01, 50.99, 83.21 and 40.30 pg/mg respectively.

The target (quantitation) ions (m/z) for each DAP (derivatives of pentafluorobenzylbromide) were 306 for DMP, 258 for DEP, 350 for DETP and 366 for DEDTP and the qualifier ions were 110 and 306 for DMP, 334 and 258 for DEP, 274 and 350 for DETP, 185 and 366 for DEDTP. The retention times of DMP, DEP, DETP, DEDTP and DBP were 17.34, 19.84, 23.40, 24.97 and 26.98 min respectively.

2.4. Quality controls and spiked hair samples

DAPs are high polar substances. Standards in hair for pesticides and metabolites are not available. We do not expect to obtain such standards in the near future especially due to the fact that pesticides of interest will probably change in coming years and exposure studies will focus on new structures. In this study we abided by the special recommendations for hair testing concerning the quality controls and the preparation of the spiked standards. Spiked samples were prepared following the recommendations of Society of Hair Testing (SoHT) [20]. A pool of human donor's hair with no detectable levels of DAPs was used as blank hair samples and was further used for the preparation of the spiked standards and the quality controls. The pool of hair homogenates with no detectable levels of DAPs were prepared by combining several samples of human donor hair samples in each of which no more than one DAP was detected below the LOQ value. Finally, the pool of hair was prepared by combining at least four different hair samples with only one different DAP detected below the LOQ value. The levels of each one of the DAPs in such homogenized pool was below the LOD value.

The homogenates blank hair samples were spiked with known concentrations of DMP, DEP, DETP or DEDTP in a concentration range from 0 to 500 pg/mg (0, 25, 50, 100, 250 and 500 pg/mg) and used as spiked standards for the calibration curves and further determining the unknown hair samples. Quality controls were similarly prepared.

2.5. Stock and working solutions

Stock solutions at the concentration of 1 mg/ml were prepared in methanol for each DAP and stored at -20°C . A mixed working solution of DMP, DEP, DETP and DEDTP was prepared in methanol at the concentration of 10 $\mu\text{g/ml}$. Working solutions of mixed DAPs were prepared by dilutions in methanol weekly in the concentrations of 0, 5, 10, 25, 50, 100, 250 and 500 ng/ml. All working solutions were stored at 4°C in the dark.

2.6. Decontamination of hair

DAPs are high polar and water-soluble substances. For the removal of the external contamination from the hair matrix, hair was washed twice in 5 ml of water (for 10 min) and in 5 ml of methanol (for 1 min) at room temperature. Washed hair samples were dried in the oven at 36°C for 10 min. The last methanol wash was tested for DAPs to confirm that external contamination was eliminated. More specific, 15 mg of K_2CO_3 was added, the methanol was evaporated, derivatized with PFBBr and analysed using GC-MS. The detected level of each DAP was below the LOD.

Subsequently, an amount of hair was weighed out and pulverized in a ball mill homogenizer (Retsch-MM 2000, Bioblock Scientific, B.P.111, F67403 Illkirch Cedex). An amount of 100 mg of powdered hair was transferred in a test-tube with 2 ml of methanol and 25 μl of DBP (10 $\mu\text{g/ml}$) as internal standard was added in the head hair to a final concentration of 2500 pg/mg.

2.7. Hair extraction and derivatization process

Hair was incubated at room temperature in an ultrasonic bath for 4 h and liquid–solid extraction was performed for 30 min by mechanical shaking. The temperature of the ultrasonic water bath was monitored during the ultrasonic extraction not to exceed 40°C .

The mixture was centrifuged at 2576 g for 5 min. The supernatant was transferred through an econofilter 0.2 μm , 25 mm (Agilent Technology) to a test-tube containing 15 mg of K_2CO_3 and methanol was evaporated to dryness under a gentle nitrogen stream at room temperature. Fifteen mg (15 mg) of K_2CO_3 was added to the residue, reconstituted in 1 ml of acetonitrile and 0.1 ml solution of pentafluorobenzylbromide (PFBBBr) in acetonitrile (1:3, v/v) and incubated at 80 °C in a water bath for 30 min with occasional swirling [14]. The mixture was then brought at room temperature and the acetonitrile was evaporated to dryness under nitrogen at 35 °C. The residue was finally dissolved in 50 μl of toluene and 2 μl was injected to GC–MS.

3. Statistical analysis

Levels of non-selective metabolites of OPs were reported as median and interquartile ranges. Differences between the population groups were examined by Mann–Whitney test. Statistical package SPSS 17.0 was used for data analysis and chart creation. A level of significance was set at 0.05. The figure presented is a Box and Whiskers plot. The error bars demonstrate the minimum and maximum values.

4. Results

4.1. Method validation

4.1.1. Linearity

Peak area ratios (DAPs response/internal standard response) were used for quantification. The standard curves obtained from the DAPs working solutions were linear between the concentrations 0 and 500 ng/ml with coefficients of linearity greater than 0.99. Our results also indicate a good linearity in the concentrations range between 25 and 500 pg/mg. The spiked sample curve was linear, $r^2 = 0.9915$ for DMP, $r^2 = 0.9913$ for DEP, $r^2 = 0.9932$ for DETP and $r^2 = 0.9941$ for DEDTP (Table 2).

4.1.2. Limit of quantitation

The limit of quantitation (LOQ) and the limit of detection (LOD) were determined in hair by injecting decreasing concentrations. We defined LOQ and LOD as the peaks that gave a signal to noise ratio of 10 and 3 respectively. The LOQ was estimated to be 20 pg/mg for DMP, 10 pg/mg for DEP and DETP and 5 pg/mg for DEDTP. The LOD was estimated to be 6 pg/mg for DMP, 5 pg/mg for DEP and DETP and 3 pg/mg for DEDTP (Table 2).

4.1.3. Recovery

Methanolic standard solutions at concentrations 100 and 500 ng/ml and spiked solutions at concentrations from 25 to 500 pg/mg of each analyte were included in every batch of samples analysed. Positive control samples were used to monitor any possible change to analytical parameters.

We evaluated the recovery of the method on spiked hair by adding DMP, DEP, DETP and DEDTP in blank hair at four different concentrations. These concentrations were 50, 100, 250 and 500 pg/mg representing level close to LOQ, low, medium and high concentration in human head hair. The spiked hair samples were extracted in the same manner as the real samples described above. Each sample was measured in triplicate. Extraction recovery was determined by comparing the ratio of DAPs peak areas/IS peak areas of extracted hair sample with the ratio of methanolic standards at the same concentration. The mean recovery of the target compounds with the employed method was estimated to 84.3% for DMP, 116.1% for DEP, 109.0% for DETP and 91.5% for DETP (Table 2). The high observed recoveries reflect the high extraction ability of methanol to isolate the DAPs form hair matrix.

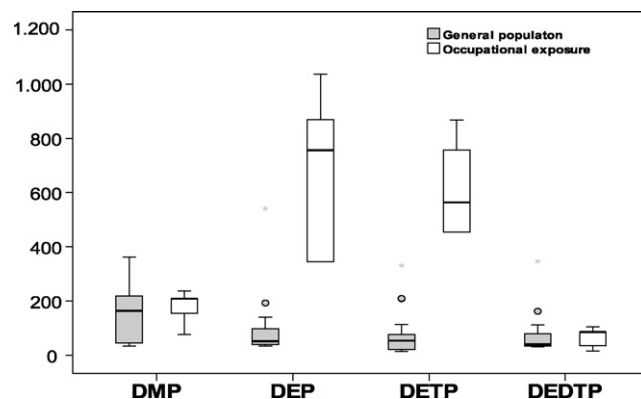


Fig. 2. The median values (pg/mg) of DMP, DEP, DETP and DEDTP in head hair samples for the general population group and the population with occupational exposure. The error bars refer to minimum and maximum values.

4.1.4. Method precision and accuracy

We further evaluated the precision of the method using positive control samples. The within-day relative standard deviation (% RSD) was determined by preparing and extracting four spiked samples at the concentrations of 50, 250 and 500 pg/mg for each analyte and injecting them during one working day into the GC–MS system. The between-day RSD of the procedure was also evaluated by preparing and injecting positive control samples at the concentrations of 50, 250 and 500 pg/mg for three consecutive days. For DMP, DEP, DETP and DEDTP, within-day precision was estimated to be 8.18%, 5.27%, 5.71% and 6.12% respectively at the concentrations of 50 pg/mg; 7.72%, 26.22%, 20.94% and 21.17% respectively at the concentration of 250 pg/mg; and 10.12%, 2.97%, 2.53% and 10.64% respectively at the concentration of 500 pg/mg. For DMP, DEP, DETP and DEDTP, between-day precision was estimated to be 11.76%, 10.03%, 13.57%, and 9.64% respectively at the concentration of 50 pg/mg; 12.50%, 10.08%, 13.57% and 9.56% respectively at the concentration of 250 pg/mg; and 9.99%, 23.94%, 15.59% and 9.72% respectively at the concentration of 500 pg/mg (Table 2).

Accuracy was determined for three concentration levels of spiked hair 50, 100 and 500 pg/mg. The accuracy of the method

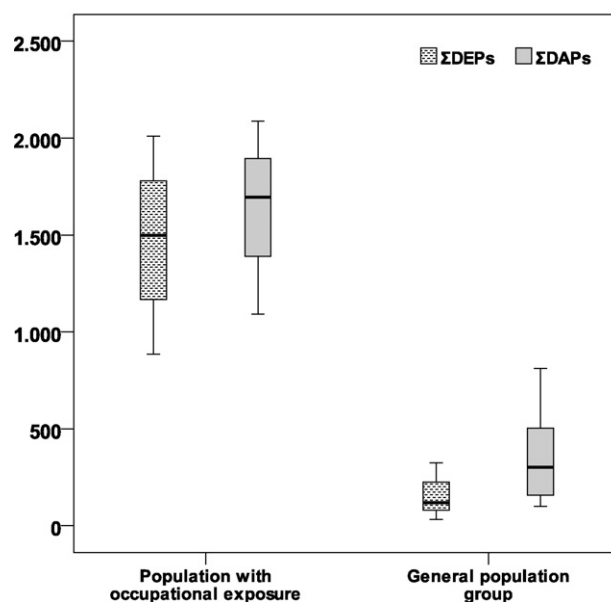


Fig. 3. The SUM median values (pg/mg) of Σ DEPs and Σ DAPs in head hair samples for the general population group and the population with occupational exposure. The error bars refer to minimum and maximum values.

Table 2
Correlation coefficients, limits of quantitation (LOQ), limit of detection (LOD), recoveries, accuracy, precision and % relative standard deviations (% RSD).

	Concentration (pg/mg)	n	DMP	DEP	DETP	DEDTP
r^2 of calibration curves			0.9915	0.9913	0.9932	0.9941
Recovery %			84.3	116.1	109.0	91.5
LOD (pg/mg)			6	5	5	3
LOQ (pg/mg)			20	10	10	5
Accuracy	50	7	51.6	89.5	86.1	98.4
	100	7	107.3	86.2	89.4	78.7
	500	7	74.0	94.5	83.0	62.6
Within-run precision %	50	4	8.18	5.27	5.71	6.12
RSD	250	4	7.72	26.22	20.94	21.17
	500	4	10.12	2.97	2.53	10.64
Between-run precision	50	3	11.76	10.03	13.57	9.64
% RSD	250	3	12.50	10.08	13.57	9.56
	500	3	9.99	23.94	15.59	9.72

ranged from 51.6% to 107.3% for DMP, from 86.2% to 94.5% for DEP, from 83.0% to 89.4% for DETP and from 62.6% to 98.4% for DEDTP (Table 2).

4.2. Levels of dialkyl phosphates in exposed and general population groups

The levels of non-selective metabolites of OPs in hair are shown in Table 3. We did not include DMTP and DMDTP in this study as these DMPs were studied exclusively in detail in two previously published articles [18,19]. The median concentration of DMP in head hair samples was 165.0 pg/mg for the general population and 181.7 pg/mg for the occupational exposure group. The median concentrations of DEP, DETP and DEDTP were 51.2, 54.0 and 40.0 pg/mg for the general population group and 812.9, 660.1 and 60.6 pg/mg for the occupational exposure group (Table 3, Fig. 2). The total DEPs and DAPs median concentrations (Σ DEPs and Σ DAPs) were 119.5 and 301.5 pg/mg for the general population group and 1498.8 and 1694.4 pg/mg for the occupational exposure group (Table 3, Fig. 3).

In each head hair at least one DAP was detected. The percentage of positive hair samples in the group of general population was 63.0% for DMP, 96.3% for DEP, 66.7% for DETP and 70.4% for DEDTP. All hair samples from the population with occupational exposure were found positive for all the DAPs analysed. DEP was detected in higher frequency compared with DMP. This phenomenon is mainly attributed to the kind of OP that the studied subjects were exposed to and less, to the difference of LOQ values between DEP and DMP.

5. Discussion

The study of the efficacy of the extraction was performed with real hair samples obtained from people exposed to OPs. The extraction of DAPs from hair is performed with methanol. Methanol was selected amongst other solvents like ethanol, acetonitrile, mixture (1:1, v/v) of acetonitrile and diethylether as the appropriate extrac-

tion solvent based on the experimental results for the extraction of DAPs. Methanol provided the higher recoveries of DAPs compared to other solvents or mixture of solvents after a 4 h ultrasonic extraction and 30 min liquid–solid extraction.

In previous studies liquid–solid extraction with mechanical shaking and sonication was used for the isolation of DMP and DMTP from the hair matrix [18,19]. In these studies, hair sample was sonicated in an ultrasonic water bath for 1 h, followed by horizontal shaking at room temperature for 12 h in order to isolate the OP metabolites from the hair matrix (total extraction time 13 h). In order to reduce the time of hair extraction, in our study hair was sonicated for 4 h followed by liquid–solid extraction with mechanical shaking for 30 min (total extraction time 4.5 h). This extraction method resulted in acceptable recoveries as described in Section 4.1.3.

Differences in DAPs levels in various samples amongst subjects varying in severity of exposure were documented. High concentrations of DEP and DETP (0.2–8.53 μ g/ml for DEP and 0.42–5.07 μ g/ml for DETP) were detected in the plasma of patients (8 cases) poisoned by chlorpyrifos, quinalphos and phosalone immediately after admission to hospital [21]. High blood concentration of DMP has been detected in samples of OP poisoned patients [22]. Differences in the levels of dimethylphosphate metabolites concentrations were observed in urine between children on conventional diets and children on organic diets (0.17 and 0.03 μ mol/l respectively) [23].

The majority of the published studies have demonstrated that DAP metabolites can be detected in biological samples in high frequencies. Ye et al. [24] reported detection frequencies from 81% for DEDTP to 100% for DMTP in urine samples from pregnant women. Bouvier et al. [25] detected 85% positive samples for DMTP, 83% for DMP and 46% for DEP after analysis of urine collected from subjects with occupational or non-occupational exposure in Paris area. Another study has suggested that children in or close to farm areas had higher levels of DMP, DETP, DMTP and DEDTP than those living away from farm areas [26].

Table 3
Median values, 1st and 3rd quartile and sum values of non-selective metabolites (DMP, DEP, DETP, DEDTP, Σ DEPs^a and Σ DAPs^b) (pg/mg) of OPs in hair of the group with occupational exposure and the general population group.

	Population with occupational exposure			General population group			p
	1st quartile	Median	3rd quartile	1st quartile	Median	3rd quartile	
DMP	96.3	181.7	229.5	41.9	165.0	471.3	0.829
DEP	447.8	812.9	995.3	38.9	51.2	97.3	<0.001
DETP	481.4	660.1	839.8	21.3	54.0	84.9	0.003
DEDTP	20.8	60.6	100.3	33.6	40.0	78.6	0.997
Σ DEPs	1025.4	1498.8	1894.7	80.4	119.5	227.0	<0.001
Σ DAPs	1241.0	1694.4	1991.0	153.1	301.5	506.4	<0.001

^a Σ DEPs: diethyl phosphates (DEP, DETP, DEDTP).

^b Σ DAPs: diethyl phosphate and dimethyl phosphates (DMP, DEP, DETP, DEDTP).

Blood and urine samples as well as saliva breath and sweat provide information only of recent exposure, maximum of several days. Past and chronic exposure cannot be recorded using the above mentioned samples while accumulated exposure maybe also underscored or not presented at all. Hair samples on the contrary provide reliable measurement for past exposure, systematic, chronic and accumulated exposure [11,27,28]. Furthermore sectional analysis provides a record of an exposure during a long-term time period. Of course the existence of an appropriate sample is absolutely necessary to perform such a sectional testing of the hair shaft e.g. from the distal segments to the proximal ones.

Organophosphate pesticides have been detected and quantified in head hair samples collected from rural population on Crete [11]. Posecion et al. [29] found 0.35% and 2.84% positive hair samples for the parent chlorpyrifos and malathion respectively ($n=282$) collected from pregnant women. The authors reported very high mean concentration levels for malathion and chlorpyrifos, 4.85 and 4.58 ng/mg respectively which were the detected parent organophosphate pesticides. These pesticide levels were surprisingly much higher than those reported by Tsatsakis et al. [11] where detected concentration levels were in pg/mg scale. The median values for diazinon, malathion and chlorpyrifos were 5.1 pg/mg (2.8% positive samples), 6.1 pg/mg (1.5%) and 7.2 pg/mg (2.4%) respectively. The enormous high levels of malathion and chlorpyrifos in Posecion et al. report maybe attributed to very severe exposure and/or high external contamination of the sampled hair. Anyhow, details on the analytical procedure of the method and information of the decontamination stage were not provided in the article [29].

Moreover, although no significant differences were observed between the two studies in the percentage of the positive samples for organophosphate pesticides, the levels of the detected pesticides were inconsistent. Posecion et al. [29] noted no detection of any pesticides metabolites like malathion monocarboxylic acid (MMA) in hair samples (LOD = 5.88 ng/mg for MMA). Authors suggested that acidic metabolites like MMA tend to partition more towards blood rather than into hair and may require more sensitive methods for their detection. The above remarks support the conclusion that LOD is high (ng/mg) and certainly not adequate to estimate the prevalence of positive samples in such studies. Moreover acidic compound like MMA according to the literature [30] tend to incorporate in the hair shaft via sebaceous and sweat glands besides the incorporation via blood thus making the external contamination an important parameter for the evaluation of the concentration measured.

Ostrea et al. [31] analysed pesticides/herbicides in paired hair and blood samples to determine the most appropriate matrix for the monitoring of pesticides exposure to these compounds. They reported small percentage of maternal hair samples positive for malathion (1.8%) obtained at mid-gestation and chlorpyrifos (0.4%) obtained at birth. MMA was detected in 0.2% of the maternal hair samples. Despite the low percentage of organophosphate present, authors concluded that hair is a more appropriate matrix to analyse exposure for pregnant women to pesticides compared to maternal blood.

The low percentage of detection of organophosphates or their specific metabolites in the above studies [11,29,31], which is attributed to the method's sensitivity, does not allow the biological monitoring of organophosphate pesticides of the population at low levels of exposure. Recently, DAPs were detected in head hair of rural workers with potential occupational exposure to OPs [19]. In the above study, authors reported detectable levels of DEP, DMTP and DMP in 70%, 20% and 40% of the samples respectively at concentration levels from 0.32 to 0.44 ng/mg for DEP, 0.32 to 0.41 ng/mg for DMTP and 0.10 to 0.46 ng/mg for DMP.

The analysis of non-specific metabolites in hair for the biomonitoring of cumulative exposure to OPs was proposed as a more reliable method compared to the analysis of blood or urine. This was proven to be efficient particularly at low levels of exposure.

The percentage of the positive samples ranges from 63.0% (DMP) to 96.3% (DEP) in the present study. DEP was the metabolite with higher frequency of detection. Similar results were presented in the previous study [19]. Generally, DEP metabolites gave detectable levels in higher frequencies than DMP metabolite.

Significant differences were observed in DEP ($p<0.001$) and DETP ($p=0.003$) levels between the examined population groups. The median DEP levels in general population were 51.2 pg/mg and in occupational exposure population were 812.9 pg/mg. Similar results were obtained for DETP (Table 3). Differences amongst the DAPs values detected in the head hair samples are due to the fact that organophosphate pesticides like diazinon, parathion and chlorpyrifos are commonly used in the area of Crete for the protection of vegetables and fruits. They produce DEP and DETP but not DEDTP and DMP. Table 1 presents active ingredients of pesticide formulations that metabolize to DMP, DEP, DETP and DEDTP.

In contrast, the values for DMP and DEDTP between general population and pesticide sprayers did not differ significantly (Table 3). The median levels of DEDTP ($p=0.997$) and DMP ($p=0.829$) were almost stable between the examined population groups (40.0 and 60.6 pg/mg for DEDTP and 165.0 and 181.7 pg/mg for DMP) (Table 3). This fact is associated with the type of the formulations that the sprayers were exposed to and complementary to the lower levels for DEDTP. Our results confirmed that DAP metabolites analysis in hair can be used for the assessment of OPs exposure.

6. Conclusion

In conclusion we developed a simple, fast, precise and highly sensitive (LOQ lower than 20 pg/mg) method for the simultaneously quantitation in hair of four non-specific metabolites of OPs pesticides, the DMP, DEP, DETP and DEDTP, and we applied this method to assess the exposure to OPs in general population and in population with documented exposure to OPs. We observed significant differences between each one of them, the total diethyl phosphates (DEPs) and the total dialkyl phosphates (DAPs) in hair samples between the two examined groups, that of general population and the exposed one. An important outcome of this study is that increased levels of organophosphates metabolites were measured in the hair samples collected from occupationally exposed humans, and detectable levels of these metabolites in hair from general population were also monitored. This biomonitoring of OPs by analysis of hair will gain much attention in future studies for the investigation of health and safety protection issues.

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