



**HAL**  
open science

# Quantification of the organophosphate flame retardant triphenylphosphate and its main metabolite in whole blood by liquid–liquid micro-extraction and liquid chromatography-tandem mass spectrometry

Barbara Giroud, Audrey Buleté, Loïc Spinner, Julie Deverchère, Léa Payen-Gay, Arnaud Vigneron, Emmanuelle Vulliet

## ► To cite this version:

Barbara Giroud, Audrey Buleté, Loïc Spinner, Julie Deverchère, Léa Payen-Gay, et al.. Quantification of the organophosphate flame retardant triphenylphosphate and its main metabolite in whole blood by liquid–liquid micro-extraction and liquid chromatography-tandem mass spectrometry. *Microchemical Journal*, 2021, 168, pp.106374. 10.1016/j.microc.2021.106374 . hal-03318023

**HAL Id: hal-03318023**

**<https://hal.science/hal-03318023>**

Submitted on 4 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **Quantification of the organophosphate flame retardant triphenylphosphate and its main metabolite in whole blood by liquid-liquid micro-extraction and liquid chromatography-tandem mass spectrometry**

**Barbara Giroud<sup>1</sup>, Audrey Buleté<sup>1</sup>, Loïc Spinner<sup>1</sup>, Julie Deverchère<sup>2</sup>, Léa Payen-Gay<sup>2,3,4</sup>,  
Arnaud Vigneron<sup>2</sup>, Emmanuelle Vulliet<sup>1\*</sup>**

<sup>1</sup>Univ Lyon, CNRS, Université Claude Bernard Lyon 1, Institut des Sciences Analytiques, UMR 5280, 5 rue de la Doua, F-69100 Villeurbanne, France

<sup>2</sup>Inserm UMR-S1052, CNRS UMR5286, Centre Léon Bérard, Centre de Recherche en Cancérologie de Lyon, 69373 Lyon, France.

<sup>3</sup>Laboratoire de Biochimie et Biologie Moléculaire, Groupe Hospitalier Sud, Hospices Civils de Lyon, Lyon, 69495 Pierre Bénite, France.

<sup>4</sup>Hospices Civils de Lyon Cancer institute, CIRculating CANcer (CIRCAN) program, 69495 Pierre Bénite, France.

**\*Corresponding author: [emmanuelle.vulliet@isa-lyon.fr](mailto:emmanuelle.vulliet@isa-lyon.fr)**

## **Abstract**

This work proposes a rapid, simple and accurate analytical method for quantification of the organophosphate flame retardant triphenylphosphate (TPhP) and the biomarker diphenylphosphate (DPhP) in whole blood by a simple and effective sample preparation followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantification. A liquid-liquid microextraction ( $\mu$ LLE) from 250  $\mu$ L of mouse whole blood was developed and validated to quantify TPhP and DPhP. Nature of extraction solvent, grinding for cell lysis and purification step were investigated. The use of distilled solvents proved necessary to reduce and control the contamination of the ubiquitous target compounds throughout the analytical process and to maintain a stable and repeatable signal in the analytical controls.

The validation was performed by matrix-matched calibration with a correction by labelled internal standards: the limit of quantification was 1 ng/mL for DPhP and 2 ng/mL for TPhP with good linearity over the range up to 48 ng/mL for each compound. Accuracy was between 92.5% and 106.7% with relative standard deviation < 20%. The  $\mu$ LLE-LC-MS/MS method

was successfully tested during short exposures of mice to DPhP or TPhP via drinking water or by intravenous injection.

**Keywords:** whole blood; rodent model; microextraction; LC-MS/MS; diphenylphosphate; triphenylphosphate

## 1. Introduction

Among the flame-retardants, the organophosphorus ones are intensively and increasingly present in various products and miscellaneous consumer goods and equipment as chemical additives [1, 2] and especially as brominated flame-retardants alternatives [3, 4]. In terms of exposure, the migration of organophosphorus flame-retardants widely used in packaging for example constitutes a serious concern for human health. Indeed, the polymer packaging are rarely completely inert in contact with the content, the release of these substances into the products may affect their quality or render them toxic [5] and could even be associated with disease as cancer [6].

Triphenylphosphate (TPhP) is an organophosphorus flame-retardant of interest due to its widespread use as both flame retardant and plasticizer and also an ingredient in nail polishes [7]. Due to its high vapour pressure, it may be released from the original material into the air or dust, which results in chronic human exposure. After absorption by organism, TPhP is rapidly metabolized in its main metabolite, diphenylphosphate (DPhP) [8]. DPhP is also the degradation products or metabolites of other additives flame retardants, such as 2-ethylhexyl diphenylphosphate [7, 8].

Given the presence, chronic release and ingestion of organophosphorus flame-retardants, several methods have been developed to detect and quantify them in different environmental and biological matrices. TPhP was detected more in environmental samples than in biological ones [9] whereas DPhP was ubiquitously detected in urine as a biomarker of TPhP exposure [1, 10, 11] with higher concentrations in women anyway [1]. From an analytical point of view, the urinary matrix is by far the most worked for these compounds [11-18], largely due to the ease of collection. However, from a scientific point of view, blood is extremely informative because it transports chemicals to and from all tissues and represents a reservoir of many endogenous and exogenous chemicals in the body [19, 20].

The long-term impact of the organophosphorus flame-retardants has poorly been studied, even if some of plastic additives such as bisphenol A have already been described as endocrine disruptors or involved in some reproductive cancers [21]. Only few studies discussed about their endocrine disruptors' activity [9, 22-24]. On the other hand, there is a lack of toxicological data and a need to assess risks in chronic disease, associated with the presence of TPhP and/or DPhP. The prerequisite of the acquisition of knowledge on pharmacology or pharmacodynamic of these compounds is the disposal of sensitive analytical methods, capable of measuring the presence and evolution of the concentrations of TPhP and DPhP in blood.

Concerning complex matrix derived from blood (serum, plasma) or whole blood, different extraction protocols are reported to extract and/or preconcentrate organophosphorus flame-retardants or other compounds, such as solid phase extraction (SPE), dispersive liquid-liquid micro-extraction (DLLME), QuEChERS (Quick Easy Cheap Efficient Rugged and Safe) [25, 26] or liquid-liquid extraction (LLE) [27-34]. These sample preparations are followed by gas or liquid chromatography separation coupled to mass spectrometry detection. LLE is commonly used to extract various molecules contained in matrices derived from blood because it is simple and fast. However, to the best of our knowledge, no method has been reported to date for analysing TPhP or DPhP, by single LLE in whole blood. Zhao et al. [35], during a study of the role of flame retardants on the disturbance of sphingolipid homeostasis, developed a method to analyse traces of organophosphorous compounds including TPhP. This method, based on LC-MS/MS detection, is highly selective and sensitive, but requires 3 preparation steps (one LLE followed by two purification steps with two different SPE cartridge systems) which makes it very time-consuming and expending of solvents and laboratory materials. Hou et al [36] also developed a lengthy solvent extraction process (3 successive LLE of 12 hours each) followed by a single clean-up on ENVI-18 cartridge, for the quantification of DPhP and TPhP in human whole blood. Despite this purification, a strong residual matrix effect, i.e. a decrease in the MS/MS signal due to the presence of interfering molecules, was observed.

The objective of this paper was to describe the development of a rapid, simple and efficient analytical method for the quantification of TPhP and DPhP in small volume of whole blood (250  $\mu$ L) from rodent used as model. A method based on a miniaturized liquid-liquid extraction ( $\mu$ LLE) followed by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis has been developed, optimized and validated.

To evaluate the sensitivity and suitability of the method, it was successfully applied to blood samples from short experiments carried out on mice exposed to DPhP or TPhP via feeding or intravenous injection.

## **2. Materials and methods**

### **2.1. Chemicals and Reagents**

TPhP (purity 99%, log  $k_{ow}$  4.6, Fig. 1) and DPhP (purity 99%, log  $k_{ow}$  3.05, Fig. 2) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The internal standards (ISs) DPhP-d10 and TPhP-d15 were synthesized by Orga-Link (Magny-Les-Hameaux, France).

Individual stock solutions were prepared at concentration of 1000 mg/L in distilled methanol (MeOH) and stored at  $-20^{\circ}\text{C}$ . Working solutions were prepared monthly by the adequate mixture and dilution of stock solutions.

LC–MS quality grade for MeOH, acetonitrile (ACN), formic acid (FA) (98%), ammonium acetate (AmAc) and HPLC grade for acetone were obtained from Sigma–Aldrich. Ultra-pure water (18.2 M $\Omega$ cm) was obtained from a MilliQ water system device from Millipore (Saint-Quentin-en-Yvelines, France). All the solvents used in the framework of this study were previously distilled.

Dispersive phase (PSA) containing 50 mg primary secondary amine and 300 mg of MgSO<sub>4</sub> have been purchased from Macherey Nagel (Hoerdt, France).

Fig. 1: Full scan mass spectra in the  $m/z$  50-500 range of TPhP in positive (A) and negative (B) ionization mode, MS/MS mass spectrum of the  $[M + H]^+$  ion ( $m/z$  327) of TPhP (C) with 50 V collision energy.

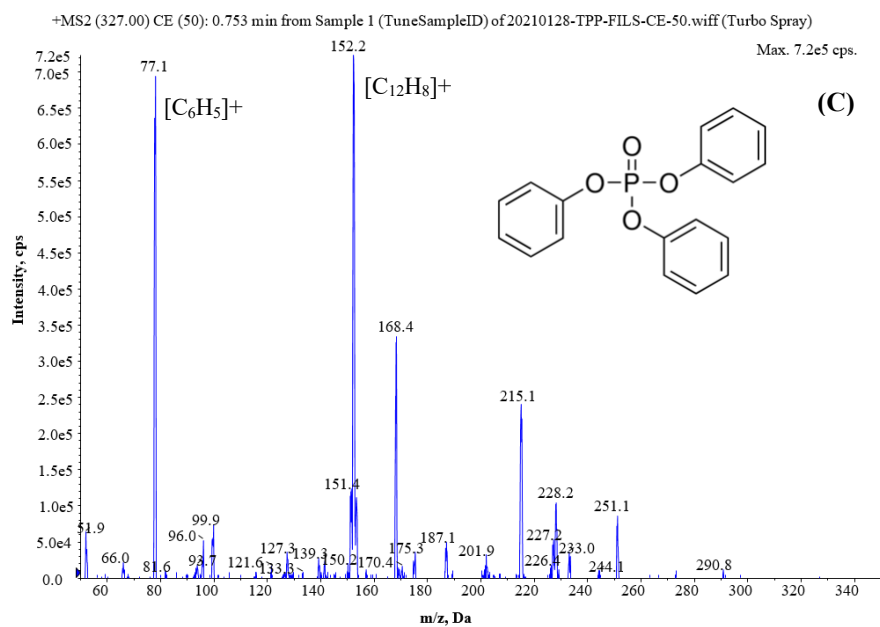
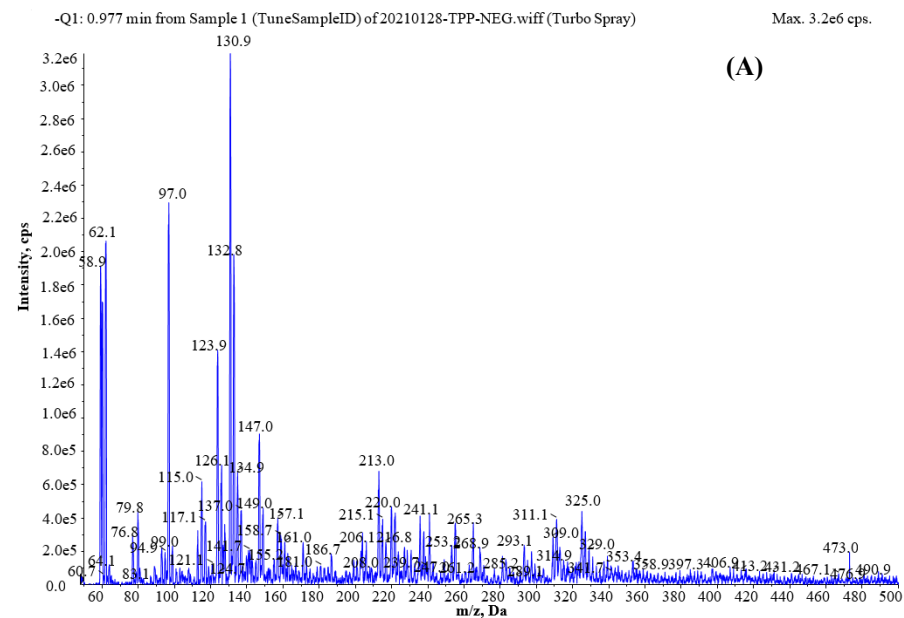
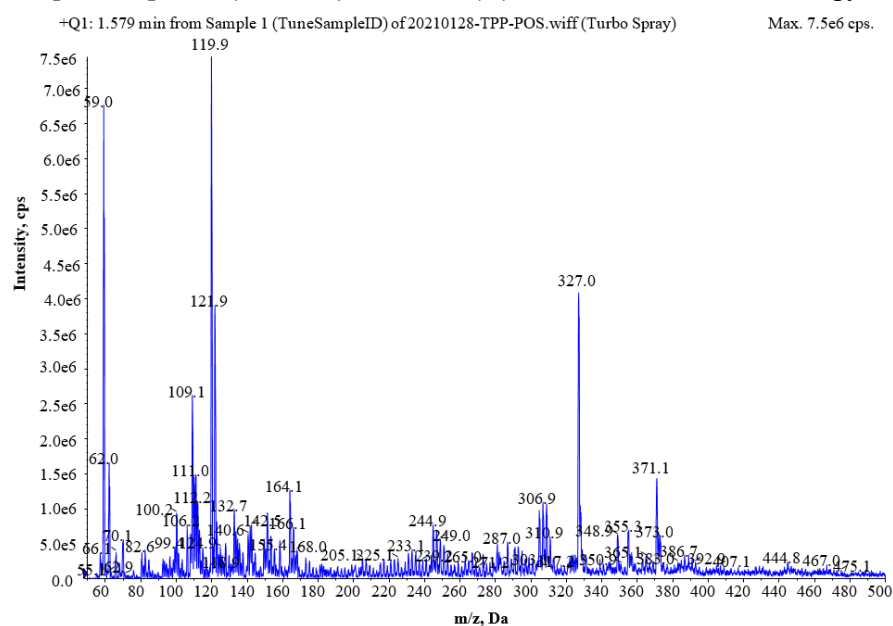
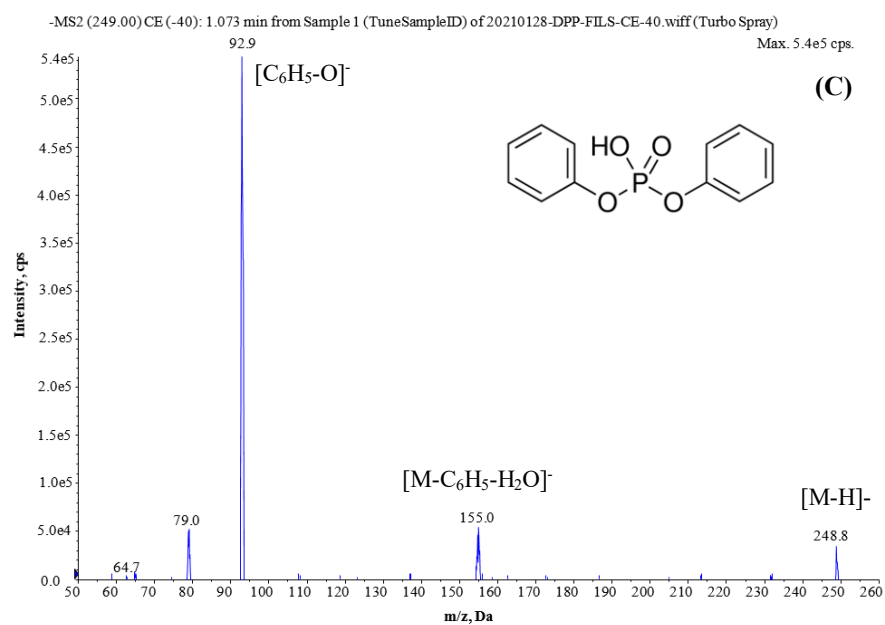
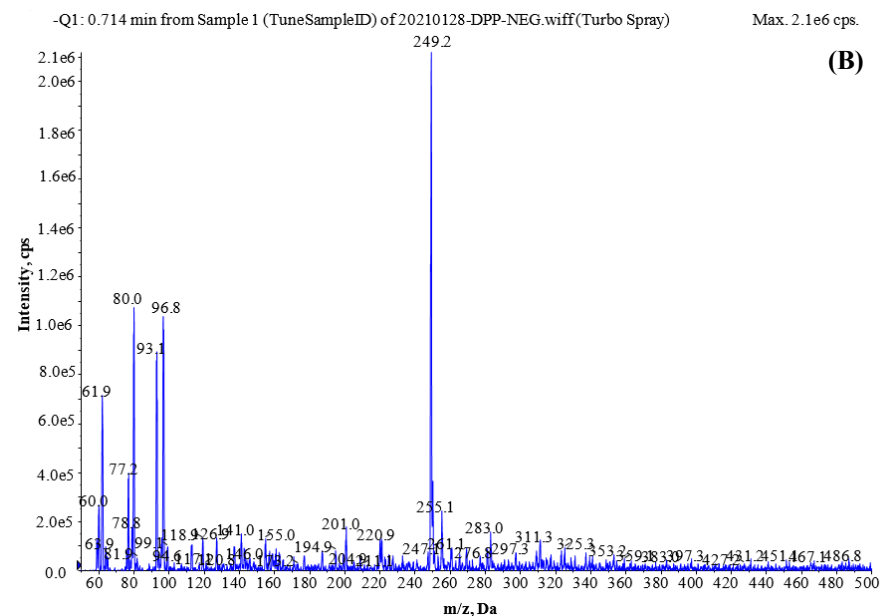
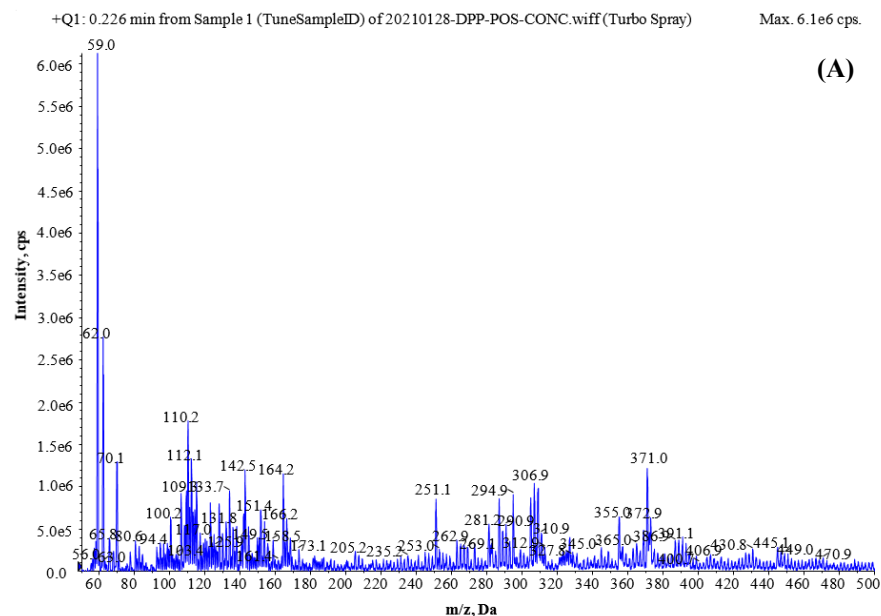


Fig. 2 : Full scan mass spectra in the m/z 50-500 range of DPhP in positive (A) and negative (B) ionization mode, MS/MS mass spectrum of the [M - H]<sup>-</sup> ion (m/z 249) of DPhP (C) with -40 V collision energy.





## **2.2. Sample collection and mice exposures**

Five-week-old FVB mice from Charles River Laboratories (L'Arbresle, France) were used for the method development and exposures. The exposures experiments were carried out in the accredited ANICAN experimental animal facility. Groups of 5 individuals were exposed to various doses of TPhP or DPhP, firstly solubilized in a water/corn oil mixture, either by feeding or by venous injection. Additional groups fed or treated with the same material without TPhP nor DPhP was used as a control. Blood samples were collected one hour after administration.

For the development, a batch from a mixture of blood from several mice, not intentionally exposed to TPhP or DPhP, was used. It was considered blank, but due to the ubiquitous presence of flame retardants throughout the laboratory environment, traces of DPhP were detected. As the development of the method involved comparisons between the different parameters tested, this did not hinder the conclusions.

## **2.3. Sample preparation**

A volume of 250  $\mu\text{L}$  of whole blood and 50  $\mu\text{L}$  of internal standard solutions (50 ng/mL of DPhP-d10 and TPhP-d15 in distilled ACN) were introduced into 2 mL polypropylene tube containing six zirconium beads with a diameter of 3 mm (Dutcher, Brumath, France). The mixture was swirled on a vortex mixer for 5 s at 1,400 rpm. The extraction was performed with 950  $\mu\text{L}$  of ACN introduced in the tube followed by cell lysis in a Beadbug<sup>TM</sup> microtube homogenizer (Benchmark Scientific, Sayreville, USA) for 2 min at 4000 rpm. After centrifugation at 10,000 rpm for 10 min at 5 °C (Sigma Laboratory Centrifuges 3K30H, Fisher Bioblock Scientific), 500  $\mu\text{L}$  of supernatant were transferred into a 10 mL pyrex centrifuge tube. This extraction was repeated twice but with recovery volumes of 1 mL supernatant. In total, 2.5 mL were recovered. The combined extract was evaporated to dryness under a stream of nitrogen at 40°C and reconstituted at the time of UHPLC-MS/MS analysis with 500  $\mu\text{L}$  of distilled MeOH.

## **2.4. LC-MS/MS analysis**

The liquid chromatographic separation was performed on a 1200 HPLC system from Agilent Technologies (Massy, France). The separation was achieved with a Kinetex XB C18 column (50 x 2,1 mm ID, 1,7  $\mu\text{m}$  porosity, 100 Å) from Phenomenex (Le Pecq, France). The mobile phase was composed of 0.01% FA and 0.1 mM AmAc in distilled pure water (A) and 1 mM

AmAc in distilled MeOH (B). The following gradient was used: from 5% to 100% (B) in 2 min and 100% (B) for 5 min. The equilibration time between each analysis was set to 5 min. The column oven was set at 60 ° C; the injection volume was 5 µL and the analytical flow rate was 700 µL/min.

The chromatographic system was coupled to a triple-stage 5500 QTrap from ABSciex (Les Ulis, France) with a Turbo V<sup>TM</sup> source (ABSciex) equipped with a TurboIonSpray® probe for electrospray ionization. A negative/positive polarity switching at 3.1 min allowed the acquisition of multiple reaction monitoring (MRM) transitions in both ionization modes from a single injection. The MS/MS settings and the parameters of the electrospray ionization (ESI) source were optimized by manual infusion of standards prepared at 100 ng/L in MeOH/H<sub>2</sub>O (50/50; v/v). They are presented in Table 1. The Turbo V<sup>TM</sup> Ion source parameters were as followed: the ion spray voltage was 5500V in positive and -4500 in negative; the operating pressures of the nitrogen flow for the nebulizer gas (GS1) and for the turbo gas (GS2) were 50 and 55 Psi, respectively, and the source temperature was 700°C.

The Analyst software (1.6.2) was used for instrument control, data acquisition and data processing.

The quantification was performed with a matrix-matched calibration with correction by ISs.

## **2.5. Method validation**

The validation strategy followed the International Conference on Harmonisation (ICH) guidelines [37]. The specificity of the method was assessed by both chromatographic and mass spectrometry characteristics of the analytes. DPhP and TPhP were unequivocally identified by their retention times ( $\pm 0.1$  min), two of their characteristic ion transitions and the specific ratios of the intensities of their product ions (deviation < 20%).

The validation was insured by two operators who worked independently (i.e. preparing different mobile phases, with different sets of pipettes and materials). It was performed on the basis of the extractions then analysis of spiked blank samples with a concentration range from C0 to C8: C0 represented a sample without addition of standards (blank sample) whereas C1-C8 was 1-2-4-8-16-24-32-48 ng/mL. A minimum of 5 concentrations is recommended by ICH [37] for the establishment of linearity. Linearity was verified over 4 calibration curves, built from C0, C1, C2, C4, C5, C6 and C8 for TPhP and from C0, C1, C3, C4, C5, C7 and C8 for DPhP. It was examined by the square correlation coefficient ( $r^2$ ) of the matrix-matched calibration curve corrected by ISs. The accuracy, that should be established across the specified range of the analytical procedure [37] was evaluated for these 6 concentration levels

(n=4), as well as for C3 and C7 (for TPhP) or C2 and C6 (for DPhP). The repeatability was studied at 3 concentration levels (C1-C2-C4) and was expressed as the relative standard deviation (RSD -%).

The limit of quantification (LOQ) was determined as the lower concentration for which the RSD value of the accuracy and intraday precision were < 20%. The limit of detection (LOD) was evaluated on the basis of signal-to-noise ratio of 3 in matrix-spiked samples.

The extraction efficiency (or extraction recovery) was evaluated by the ratio of the peak area of the compound in the blank matrix spiked before extraction ( $A_{\text{before extraction}}$ ) on the peak area of the compound in the blank matrix spiked after extraction ( $A_{\text{after extraction}}$ ). Matrix effect represented the ratio of the compound in the blank matrix spiked after extraction on the peak area of the compound in solvent at the same concentration. A value greater than 100 indicated an enhancement of the signal, while a value less than 100 indicated ion suppression. Correction of the response of the compounds by ISs was used to ensure the overall robustness of each analysis.

### **3. Results and discussion**

In the proposed method, we first optimized the detection of TPhP and DPhP by tandem mass spectrometry, then the chromatographic conditions for their liquid chromatography separation. Next, we optimized the sample preparation based on liquid-liquid extraction, to extract, purify and preconcentrate DPhP and TPhP from a low volume of mice whole blood (250  $\mu\text{L}$ ). Thus, we selected the liquid-liquid extraction solvent, the cell lysis conditions and the purification conditions. Performances of the different tests were evaluated by determination of recoveries and matrix effects from two replicates. After optimization of the extraction and LC-MS/MS conditions, the method was validated and tested during a short exposure of mice.

#### **3.1. Use of distilled solvents**

As organophosphate flame retardants are ubiquitously present throughout the analytical material in particular in the chromatographic system, one of the challenges was to limit or stabilize the release of the target compounds. For this, we tested the influence of the quality of solvents by distillation. To highlight the impact of the distillation on the mobile phase solvents, the instrumental limits of quantification were evaluated with distilled or undistilled solvent. A decrease of a factor of 4 for DPhP and 9 for TPhP was observed on the instrumental limit of quantification by distilling the eluents. To limit the sources of pollution,

the solvents used for the extraction step were also distilled. Distilled solvents could be kept at 4°C during three days.

### 3.2. LC-ESI-MS/MS optimization

First, the manual infusion into the electrospray source of the compounds of interest made it possible to select the ionization mode of DPhP and TPhP as well as the precursor ion and product ions. The majority precursor ion of TPhP corresponded to its proton adduct  $[M+H]^+$  (Fig. 1A and 1B). The fragmentation of this  $m/z$  327 ion formed two intense product ions: one at  $m/z$  152 probably resulting from the reaction between  $C_6H_4$  and  $C_6H_5^+$  ( $C_6H_4$  and  $C_6H_5^+ \rightarrow C_{12}H_8^+ + H$ ) and the phenyl cation ( $m/z$  77,  $C_6H_5^+$ ) (Fig. 1C). DPhP has a deprotonation site due to its high acidity, therefore the deprotonated  $[M-H]^-$  ion was chosen as a precursor (Fig. 2A and 2B). The most intense product ions (Fig. 2C) corresponded to the loss of the phenolic part of the molecule ( $m/z = 155$  and 93). As a consequence, a switch of polarity of the ionization source during the run was required to analyse both TPhP and DPhP. Two MRM transitions were monitored: the most abundant fragment ion that resulted in the lowest limit of quantification was used for quantitation and the second for confirmation. The selected declustering potentials (DP) and collision energies (CE) are summarized in Table 1.

Through injection of blood sample into the chromatographic system in flow injection analysis, the second step was the optimization of source parameters and mainly the source temperature (also called desolvation temperature corresponding to the temperature of the heating resistors). Three temperatures were tested, 600, 650 and 700°C. DPhP ionisation was not sensitive to the desolvation temperature because the responses were the same whatever the temperature. On the other hand, the signal of TPhP increased by a factor 1.8 between 650°C and 700°C. Therefore, the source temperature was set to 700 °C. As 750°C was the maximum temperature of the Turbo V source, it was decided not to test and work at this limit value for ensure the spray stability and avoid premature degradation of the heating resistors. The optimization of the ESI parameters lead to an optimal spray for both analytes.

After optimizing the ESI-MS/MS ionization and detection, the liquid chromatography conditions were optimized by searching a separation of the two target analytes suitable with a switch of ionization polarity during the analytical run. A Kinetex XB C18 column with a weak porosity was used in order to work at a flow rate of 700  $\mu$ L/min (due to Core-Shell technology), flow compatible with the source geometry to get an optimal ionization. A separation of 0.4 min between the two compounds was obtained.

Table 1 : MS/MS acquisition method parameters: transitions, declustering potential (DP), collision energy (CE), collision cell exit potential (CXP)

Compound	Ionisation Mode	RT (min)	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Transition ratio
DPhP	ESI -	2.9	249	93	-135	-40	-11	11
			249	155	-135	-30	-13	
DPhP-d10	ESI -	2.9	259	98	-40	-54	-13	
TPhP	ESI+	3.3	327	77	126	65	10	1.1
			327	152	126	55	10	
TPhP-d15	ESI+	3.3	342	82	176	73	10	

### 3.3. Selection of liquid-liquid microextraction conditions

The choice of 250  $\mu$ L as initial amount of whole blood for the extraction was dictated by the quantity of blood that can be easily recovered from one mouse. To work at the level of one individual, a miniaturization of the extraction protocol had to be adjusted. To facilitate the release of the compounds of interest and to extract them from the matrix with good recoveries, cell lysis was used. Several techniques were commonly used to ensure the destruction of cell membranes such as the use of an acid, a solvent, a detergent, ultrasound or grinding. For our development, this last technique was applied to blood samples.

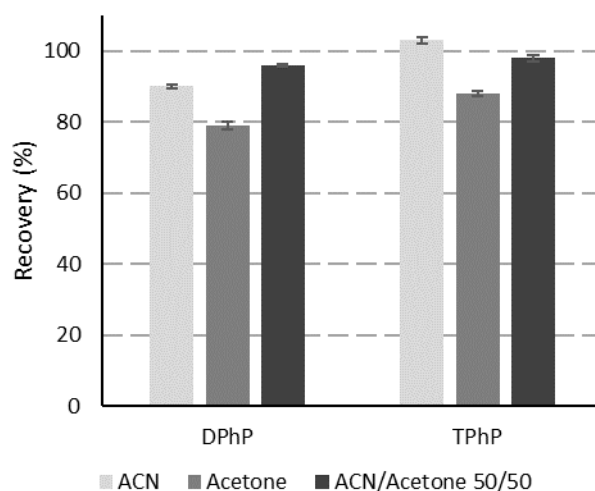
Mechanical grinding step of the biological cells from blood samples enhanced the extraction efficiency of the method. The adding of beads in the extraction tube facilitated cell lysis and disruption of analyte-matrix interactions. For example, a gain of + 46% on the recovery of TPP was observed when we used beads compared to an extraction without beads for ACN extraction solvent.

The extraction of 250  $\mu$ L of whole blood was first performed by 1000  $\mu$ L of ACN, acetone or a mix ACN/acetone (50/50) introduced in the tube of 2 mL polypropylene tube containing six zirconium beads. Solvents were chosen in accordance with the log  $K_{ow}$  of the compounds of interest. After agitation with a vortex and a centrifugation step at 10,000 rpm for 10 min at 5  $^{\circ}$ C, 500  $\mu$ L of supernatant were transferred into a 10 mL pyrex centrifuge tube. This extraction was repeated twice.

As shown in Fig. 3, the extraction efficiency at 40 ng/mL from whole blood was superior to 80% for all 3 solvents. It was however a little weaker with acetone comparatively with ACN or ACN/acetone (50/50) for both DPhP and TPhP. Indeed, recoveries were superior to 90%

whether for ACN or acetone but did not exceed 85% with acetone. On the other hand, the extraction method has to be efficient to extract a maximum of the compound of interest but in the same time has to be selective so as to limit the presence of interfering compounds leading to matrix effects. For DPhP, the matrix enhanced its ionization (Fig. 4) which is not commonly observed in literature [38]. For TPhP, as the matrix effects was negative, namely components of whole blood caused suppression of signal during ionization, the extraction solvent was chosen to minimize them. So, ACN was finally selected: DPhP and TPhP were extracted with recoveries superior to 90% and limited matrix effects (31% and -28%, respectively) before even considering a purification step.

Fig. 3: Recoveries of DPhP and TPhP at 40 ng/mL, after  $\mu$ LLE (with grinding, no dSPE) using different extraction solvents (n=2)



#### 3.4. Addition of a purification step

The effect of an additional purification of the extract with the dispersive phase PSA was tested after the liquid-liquid microextraction. PSA was chosen because of its universal purification properties to trap the matrix interferences, while not trapping the molecules of interest [39]. In addition, it is a very fast technique, much faster than the more traditional cartridge-based purification [35, 36], and does not require the additional use of solvent. After three successive LLE by ACN with 6 zirconium beads, 2.5 mL of the extract were placed in a 10 mL pyrex centrifuge tube containing 350 mg of PSA. After shaking vigorously by hand and with vortex during 5s at 1400 rpm, the tube was centrifuged during 10 min 2500 rpm at 5 °C. A volume of 2 mL of the purified extract were transferred into another tube to be

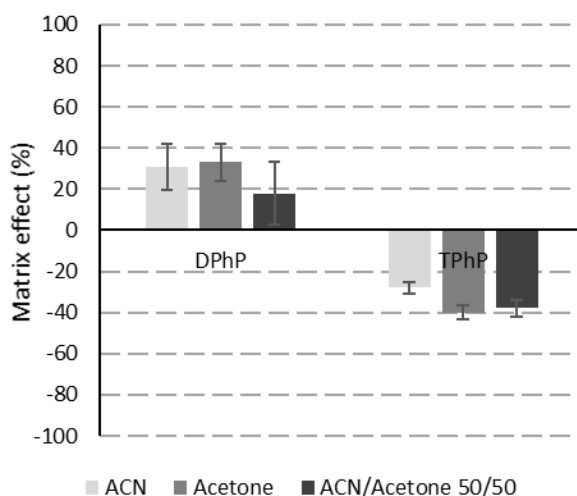
evaporated under nitrogen to dryness at ambient temperature and reconstituted in 400  $\mu$ L before analysis.

For DPhP, the matrix effects were reduced by a factor 2 with the dSPE purification (Fig. 4). On the contrary, for TPhP, the dSPE purification increased the matrix effects: 53% of signal inhibition with dSPE and 34% without dSPE.

On the other hand, the extraction recovery values indicated a loss of the compounds of interest. DPhP and TPhP seemed to be adsorbed on the dispersive phase used. The recoveries were reduced of 9% for the DPhP and 42% for the TPhP, compared to the values obtained without a purification step.

The final extract obtained after the liquid-liquid microextraction was sufficiently limpid, without insoluble particles in suspension which would quickly contaminate the injector and decrease the life of the chromatographic column. In addition, successive injections of spiked samples did not show an increase in the signal, indicating the absence of carry over or interfering substances with the analysis. In order to maintain an optimal extraction in terms of efficiency and speed of implementation, a method without purification step was chosen. The measured matrix effects (less than 40%) remain below what is reported in the literature for these compounds. Indeed, Hou et al [36] measured effects of 69% and 92% for DPhP and TPhP, respectively, despite a purification step on an ENVI-18 SPE cartridge. ACN is often used for the precipitation of proteins. Combined with a strong grinding of the sample, the use of ACN was therefore sufficient to obtain a sample with little interferences to TPhP and DPhP.

Fig. 4: Matrix effects of DPhP and TPhP at 40 ng/mL after  $\mu$ LLE (with grinding, no dSPE) using different extraction solvents (n=2)



### 3.5. Method validation

The performance characteristics are compiled in Table 2.

Good linearity was observed for the two compounds on the range 1– 48 ng/mL: regression coefficient  $R^2$  was higher than 0.997 (n=4). For DPhP and TPhP, accuracy was between 98.5% and 101.4% and between 92.5% and 106.7% respectively at all the concentration levels.

For DPhP, LOQ was validated at 1 ng/mL with an accuracy RSD < 20%. Regarding the TPhP, the intraday precision RSD was 74.2% at 1 ng/mL. The LOQ for TPhP was therefore validated at 2 ng/mL. The LOD were evaluated at 0.3 and 0.4 for DPhP and TPhP, respectively. This is higher than Zhao et al. (0.02 ng/mL for TPhP) and Hou et al. (0.006 ng/mL for DPhP) but of the same order as Hou et al. for DPhP (0.31 ng/mL) for an initial volume of blood twice as large (500  $\mu$ L). Due to the use of organophosphorus flame retardants in a lot of equipment used in a laboratory or animal facility, including within the analytical instruments themselves, a residual background noise of DPhP was observed in samples used as blank. In this case, the standard deviation of procedural blank (n=10) was calculated and the LOQ was calculated by the initial concentration subtracting the blank values, as already mentioned for this kind of compounds [36]. DPhP levels in unintentionally exposed mice were found to vary between batches of mice from LOD to 4 ng/mL.



Table 2: Performances and validation parameters for the quantification of DPhP and TPhP in whole blood by  $\mu$ LLE-LC-MS/MS

<b>DPhP</b>						<b>TPhP</b>							
	ng/mL	Accuracy (n=4)	RSD (%) accuracy	Extraction efficiency (%)	Repeat. % RSD	Matrix effect (%)		ng/mL	Accuracy (n=4)	RSD (%) accuracy	Extraction efficiency (%)	Repeat. % RSD	Matrix effect (%)
C1	1	103	16.4		4.0 (n= 6)		C1	1	83.9	74.2		32.1 (n=6)	
C2	2	98.5	6.9		3.2 (n= 6)	107.9	C2	2	92.5	7.9		4.6 (n= 6)	92.1
C3	4	100.8	4.5	85.0			C3	4	98.5 (n=3)	2.7	82.0		
C4	8	101.4	4.1		1.3 (n= 10)	101.9	C4	8	99.6	5.8		4.7 (n= 10)	91.0
C5	16	100.8	2.6				C5	16	104.8	3.8			
C6	24	101.4	2.7	86.8			C6	24	104.9	3.8	103.4		
C7	32	101.4	0.6				C7	32	106.2	5.0			
C8	48	99.1 (n=3)	0.1		0.9 (n=3)		C8	48	97.7 (n=3)	1.1 (n=3)			
R <sup>2</sup> (n=4)	0.9995						R <sup>2</sup> (n=4)	0.9972					
LOD	0.3						LOD	0.4					
LOQ	1						LOQ	2					

### 3.6. Application of the method to whole blood of mice exposed to DPhP or TPhP

The analytical method was evaluated in two short term TPhP or DPhP intoxication experiments in mice. The mice were exposed either by feeding or by intravenous injection. Blood samples were taken 1 hour after administration of the compounds. The results are summarized in Table 3. The first experiment was conducted by feeding, with a high dose of TPhP and slightly lower doses of TPhP and DPhP. It should be noted that the method allowed for the determination of TPhP in blood only during acute TPhP intoxication (1 mg). For a feeding of 0.01 mg TPhP, it was found in only one blood sample. On the other hand, DPhP was quantified in all mice fed with TPhP or DPhP, which confirms a rapid metabolism of TPhP to DPhP. Finally, DPhP was found in the blood of mice not exposed to flame retardants, indicating chronic environmental exposure. The second experiment was carried out at lower doses, according to two routes of administration, i.e. by feeding or intravenous injection. These tests confirm the fact that TPhP is rapidly metabolised to DPhP, which should therefore be considered as a good marker of TPhP exposure. It can also be noted that the amount of DPhP in blood is quantified in a dose-dependent manner, which shows the relevance of the method developed to monitor intoxication or chronic exposure to TPhP or DPhP.

Table 3: DPhP and TPhP values measured in whole blood of mice after feeding or intravenous injection with DPhP or TPhP

	DPhP (ng/mL)		TPhP (ng/mL)	
	mean	% RSD	mean	% RSD
<i>1<sup>st</sup> exp. Feeding (n=5)</i>				
Control (ng/mL)	5.2	42.1	< LD	-
1 mg TPhP - feed	23.7	26.7	0.7	54
0.01 mg TPhP - feed	5.9	20.2	2.0 (n=1)	-
0.01 mg DPhP - feed	56.5	29.7	< LD	-
<i>2<sup>nd</sup> exp. Feeding or intravenous (n=5)</i>				
Control (ng/mL)	< LD	-	< LD	-
0.1 µg DPhP - IV	< LQ	-	< LD	-
0.1 µg DPhP - feed	1.3	0.3	< LD	-
1 µg DPhP - IV	4.7	0.4	< LD	-
1 µg DPhP - feed	12.2	3.1	< LD	-
0.01 mg TPhP - IV	< LQ	-	2.3 (n=1)	-
0.01 mg TPhP - feed	1.9	1.5	2.5	4.4
0.1 mg TPhP - IV	1.3	0.7	< LD	-
0.1 mg TPhP - feed	4.0	5.4	< LD	-

#### **4. Conclusion**

In this study, a simple, rapid and effective sample preparation for the quantification of the organophosphate flame retardants TPhP and DPhP, in mouse whole blood is presented, using a liquid-liquid microextraction followed by a LC-MS/MS analysis. The method requires a small initial volume of blood (250  $\mu$ L) and consumes only a few mL of solvent for preparation. Without the need for an additional purification step, the method is very fast compared to previously developed protocols. The analytical performances in terms of accuracy and intraday precision allowed the quantification in the range of ng/mL of the compounds in mouse whole blood and highlighted the applicability of the  $\mu$ LLE-LC-MS/MS method for small volumes of biological samples available during chronic or acute exposure-based studies.

#### **Acknowledgments**

This project receives financial support from ITMO Cancer under the Cancer Plan 2014-2019.

#### **Compliance with ethical standards**

Animal experiments were performed in compliance with French and European regulations on protection of animals used for scientific purposes (EC Directive 2010/63/EU and French Decree 2013–118). They were approved by Ethics Committee and authorized by the French Ministry of Research (reference 01017.01).

#### **Conflict of interest**

The authors declare no conflict of interest.

## References

1. V. Preston E, D. MC Clean M, Claus Henn B, M. Stapleton H, E. Braverman L, N. Pearce E, M. Makey C, F. Webster T. Associations between urinary diphenyl phosphate and thyroid function. *Environment International*. 2017; 101: 158-164
2. Cairns RA, Harris IS, and Mak TW, Regulation of cancer cell metabolism. *Nat Rev Cancer*, 11 (2011) 85-95
3. Van der Veen I, de Boer J. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. *Chemosphere*. 2012; 88: 1119-1153
4. Cui K, Wen J, Zeng F, Zhou X, Li S, Zeng Z. Determination of organophosphate ester flame retardants and plasticizers in soil samples by microwave-assisted extraction coupled with silica gel/alumina multilayer solid-phase extraction and gas chromatography-mass spectrometry. *Analytical Methods*. 2017; 9(6): 986–993
5. Rosca I.D, Vergnaud B. Approach for a testing system to evaluate food safety with polymer packages. *Polymer Testing*. 2006; 25:532-543
6. André C, Castanheira I, Cruz J.M, Paseiro P, Sanches-Silva A. Analytical strategies to evaluate antioxidants in food : a review. *Trends in Food Science Technology* 21. 2010; 229-246
7. Mendelsohn E, Hagopian A, Hoffman K, M. Butt C, Lorenzo A, Congleton J, F. Webster T, M. Stapleton H. Nail polish as a source of exposure to triphenyl phosphate. *Environment International*. 2016; 86: 45-51
8. D. Meeker J, M. Cooper E, M. Stapleton H, Hauser R. Urinary Metabolites of Organophosphate Flame Retardants: Temporal Variability and Correlations with House Dust Concentrations. *Environ Health Perspect*. 2013; 121(5): 580-585
9. Wang D, Zhu W, Chen L, Yan J, Teng M, Zhou Z. Neonatal triphenyl phosphate and its metabolite diphenyl phosphate exposure induce sex- and dose-dependent metabolic disruptions in adult mice. *Environmental Pollution*. 2018; 237: 10-17
10. K. Jayatilaka N, Restrepo P, Williams L, Ospina M, Valentin-Blasini L, M. Calafat A. Quantification of three chlorinated dialkyl phosphates; diphenyl phosphate, 2,3,4,5,-tetrabromobenzoic acid, and four other organophosphates in human urine by solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2017; 409: 1323-1332

11. Pouech C, Kiss A, Lafay F, Léonard D, Wiest L, Cren-Olivé C, Vulliet E. Human exposure assessment to a large set of polymer additives through the analysis of urine by solid phase extraction followed by ultra high performance liquid chromatography coupled to tandem mass spectrometry. *Journal of Chromatography A*. 2015; 1423:111-123
12. E. Petropoulou S-S, Petreas M, Park J-S. Analytical methodology using ion-pair liquid chromatography-tandem mass spectrometry for the determination of four di-ester metabolites of organophosphate flame retardants in California human urine. *Journal of Chromatography A*. 2016; 1434: 70-80
13. Kosarac I, Kubwabo C, G. Foster W. Quantitative determination of nine urinary metabolites of organophosphate flame retardants using solid phase extraction and ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). *Journal of Chromatography B*. 2016; 1014: 24-30
14. Cequier E, Marcé R.M, Becher G, Thomsen C. A high-throughput method for the determination of metabolites of organophosphate flame retardants in urine by ultra performance liquid chromatography-high resolution mass spectrometry. *Analytica Chimica Acta*. 2014; 845: 98-104
15. Van den Eede N, Neels H, G. Jorens P, Covaci A. Analysis of organophosphate flame retardant diester metabolites in human urine by liquid chromatography electrospray ionisation tandem mass spectrometry. *Journal of chromatography A*. 2013; 1303: 48-53
16. Cooper E.M, Covaci A, van Nuijs A. L. N, Webster T. F, Stapleton H.M. Analysis of the flame retardant metabolites bis(1,3-dichloro-2-propyl) phosphate (BDCPP) and diphenyl phosphate (DPP) in urine using liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2011; 401: 2123-2132
17. Schindler B.K, Förster K, Angerer J. Determination of human urinary organophosphate flame retardants metabolites by solid-phase extraction and gas chromatography-tandem mass spectrometry. *Journal of chromatography B*. 2009; 877: 375-381
18. Castorina R, Butt C, M. Stapleton H.M, Avery D, G. Harley K, Holland N, Eskenazi B, Bradman A. Flame retardants and their metabolites in the homes and urine of pregnant women residing in California (the CHAMACOS cohort). *Chemosphere*. 2017; 179: 159-166
19. Rappaport, S.M. Implications of the exposome for exposure science. *J. Expo. Sci. Environ. Epidemiol*. 2011; 21, 5–9

20. Wild CP. Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. *Cancer Epidemiol. Biomark. Prev.* 2005; 14: 1847-1850
21. Rezg R, El-Fazaa S, Gharbi N, Mornagui B. Bisphenol A and huma, chronic diseases: current evidences, possible mechanisms and future perspectives. *Environ. Intern.* 2014; 64: 83-90
22. V. Krivoshiev B, Dardenne F, Covaci A, Blust R, J. Husson S. Assessing in-vitro estrogenic effects of currently-used flame retardants. *Toxicology in vitro.*2016; 33: 153-162
23. Chen G, Jin Y, Wu Y, Liu L, Fu Z. Exposure of male mice to two kinds of organophosphate flame retardants (OPFRs) induced oxidative stress and endocrine disruption. *Environmental Toxicology and Pharmacology.* 2015; 40: 310-318
24. Schang G, Robaire B, F. Hales B. Organophosphate flame retardants act as endocrine-disrupting chemicals in MA-10 mouse tumor leydig cells. *Toxicological Sciences.* 2016; 150(2): 499-509
25. M. Plassmann M, Schmidt M, Brack W, Krauss M. Detecting a wide range of environmental contaminants in human blood samples-combining QuEChERS with LC-MS and GC-MS methods. *Anal Bioanal Chem.*2015; 407: 7047-7054
26. S. Iqbal, M. Muntazir Iqbal, M. Javed, A. Bahadur, S. Yasien, Najam-ud-din, A. Hurr, N. Ahmad, M. Raheel, G. Liu, Modified QuEChERS extraction method followed by simultaneous quantitation of nine multi-class pesticides in human blood and urine by using GC-MS, *Journal of Chromatography B* 1152 (2020) 122227
27. Medvedev Y.V, Ramenskaya G.V, Shokhin I.E, Yarushok T.A. HPLC and UPLC for Determining Drugs in Blood (A Review). *Pharmaceutical Chemistry Journal* 47. 2013; 225-230
28. Rezaee M, Yamini Y, Hojjati M, Faraji M. Novel extraction method based on the dispersion of the extraction solvent for extraction of letrozole from biological fluids. *Analytical Methods* 2. 2010; 1341-1345
29. Borges K.B, Freire E.F, Martins I, de Siqueira M. Simultaneous determination of multibenzodiazepines by HPLC/UV: Investigation of liquid-liquid and solid-phase extractions in human plasma. *Talanta.* 2009; 78: 233-241

30. Chang H.C, Li J.Y, Li J, Guan X.D, Sun F.L, Qian Z.Z, Bi K.S, Fan G.R. Simultaneous determination of amlodipine and bisoprolol in rat plasma by a liquid chromatography/tandem mass spectrometry method and its application in pharmacokinetic study. *Journal of Pharmaceutical and Biomedical Analysis*. 2012; 71: 104-110
31. Li P, Jin J, Wang Y, Hu J, Xu M, Sun Y, Ma Y. Concentrations of organophosphorus, polybromobenzene, and polybrominated diphenyl ether flame retardants in human serum, and relationships between concentrations and donor ages. *Chemosphere*. 2017; 171: 654-660
32. Tarazona I, Chisvert A, Salvador A. Determination of benzophenone-3 and its main metabolites in human serum by dispersive liquid-liquid microextraction followed by liquid chromatography tandem mass spectrometry. *Talanta*. 2013; 116: 388-395
33. Roche S, Sewell L, Meiller J, Pedersen K, Rajpal R, O'Gorman P, Clynes M, O'Connor R. Development, validation and application of a sensitive LC-MS/MS method for the quantification of thalidomide in human serum, cells and cell culture medium. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. 2012; 902: 16-26
34. Andrews R, Paterson S. A validated method for the analysis of cannabinoids in post-mortem blood using liquid-liquid extraction and two-dimensional gas chromatography-mass spectrometry. *Forensic Science International*. 2012; 222: 111-117
35. F. Zhao, Y. Wan, H. Zhao, W. Hu, D. Mu, T.F. Webster, J. Hu, Levels of blood organophosphorous flame retardants and association with changes in human sphingolipid homeostasis. *Environmental Science & technology* 50 (2016) 8896-8903.
36. M. Hou, Y. Shi, Q. Jin, Y. Cai, organophosphate esters and their metabolites in paired human whole blood, serum, and urine as biomarkers of exposure. *Environment International* 139 (2020) 105698
37. International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use. *Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, ICH Q2(R1)*
38. Marchi I, Viette V, Badoud F, Fathi M, Saugy M, Rudaz S, Veuthey J.L. Characterization and classification of matrix effects in biological samples analyses. *Journal of Chromatography A*. 2010.1217: 4071-4078

39. C. Pouech, M. Tournier, N. Quignot, A. Kiss, L. Wiest, F. Lafay, M.M. Flament-Waton, E. Lemazurier, and C. Cren-Olive, Multi-residue analysis of free and conjugated hormones and endocrine disruptors in rat testis by QuEChERS-based extraction and LC-MS/MS. *Analytical and Bioanalytical Chemistry* 402 (2012) 2777-2788.