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Development of a microporous membrane liquid–liquid extractor for organophosphate esters in human blood plasma: identification of triphenyl phosphate and octyl diphenyl phosphate in donor plasma

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Abstract

An extractor has been developed for microporous membrane liquid–liquid extraction (MMLLE) of lipophilic xenobiotics at trace levels in biological fluids. This new construction allows the sample phase to be stirred, while the organic phase is pumped. The extractor was evaluated using human blood plasma with added organophosphate esters. The size exclusion properties of the membrane reduced lipid co-extraction by ~94% compared to ordinary liquid–liquid extraction. In combination with a solid-phase extraction (SPE) step, the method was shown to remove plasma lipids efficiently and thus allow gas chromatographic separation of the compounds. The clean-up method described, including the SPE step, showed a high level of reproducibility, and recoveries of between 72 and 83% were obtained for five of the organophosphate esters after a 200-min extraction period. Using this technique, triphenyl phosphate and an isomer of octyl diphenyl phosphate were detected in human plasma obtained from blood donors. The concentration of triphenyl phosphate ranged between 0.13 and 0.15 μg/g plasma. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Microporous membrane liquid–liquid extractor; Triphenyl phosphate; Octyl diphenyl phosphate

1. Introduction

1.1. Organophosphate esters — occurrence and health effects

Organophosphate esters are widely used as flame retardants or plasticiser additives in various products and materials in developed countries. For instance, electronic equipment, building materials, plastics, lubricants and varnishes may all contain large quantities of these compounds. A general structure for organophosphate esters is shown in Fig. 1.

Volatilisation of the compounds, and thus emission into their surroundings, has been reported [1]. A total of nine different organophosphate esters have so far been identified and quantified as airborne substances in a number of common indoor environments, namely school buildings, a day-care centre and an office building [2]. The compounds were shown to originate from sources in the indoor environment, and detected concentrations of tri(2-chloroethyl) phosphate were as high as 250 ng/m^3. Triphenyl phosphate has been detected at concentrations as high as

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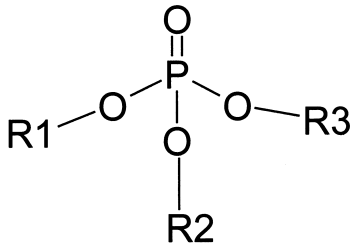

Fig. 1. General structure of organophosphate esters. R_1, R_2 and R_3 are similar or different organic substituents. The substituent may also contain halogens.

100 ng/m^3 in emissions from video display units of personal computers [3].

To date, information about the impact of organophosphates on human health is scarce in the literature. However, triphenyl phosphate is a potent inhibitor of human blood monocyte carboxylesterase [4] and has documented contact allergenic properties [5–7]. Furthermore, this substance as well as other organophosphate esters, e.g. 2-ethylhexyl diphenyl phosphate, have shown haemolytic effects [8].

Some arylated phosphates have been shown to be reproductive toxicants [9,10]. In studies on rats and mice, tri(2-chloroethyl) phosphate exhibited neurotoxicity and carcinogenic properties [11] and was shown to be distributed to all parts of the brain [12], causing lesions in the hippocampus tissue of the brain [13–15].

Fairly little has so far been described in the literature on the metabolism of organophosphates in exposed mammals. No results have so far been presented on the detection of metabolised or non-metabolised organophosphates in blood. On the other hand, three different non-metabolised organophosphate esters, i.e. tributyl phosphate, tri(butoxyethyl) phosphate and tri(1,3-dichloropropyl) phosphate, have been determined at ng/g levels in human adipose tissue [16].

1.2. Extraction of organophosphate esters

Due to the complex composition of blood plasma, several sample preparation steps are usually performed prior to final separation and detection of the target compounds. The first step traditionally consists of repeated liquid–liquid extractions (LLE) using organic solvents. This is laborious and should be performed by a skilled hand for acceptable reproducibility. Further clean-up steps are designed to reduce co-extracted matrix molecules such as plasma lipids (comprising ~0.5% of the fresh weight of plasma), which will foul the chromatographic system if not removed. If the investigated compounds are stable and acid-resistant, like highly chlorinated PCBs or polybrominated diphenyl ethers (PBDEs), lipid contents may be reduced by mixing the extract with concentrated sulphuric acid followed by separation on an open polar column, e.g. silica gel. However, for analytes such as organophosphate esters, the lipid removal has to be non-destructive. Following LLE, usually more than one technique is needed in order to achieve the required lipid reduction. Gel permeation chromatography (GPC) followed by open column chromatography on silica adsorbent is often used for lipid removal [17]. Van der Hoff et al. [18] showed that polychlorinated pesticides could be separated from lipids by high-performance liquid chromatography on a silica column.

The objective of this study was to develop and evaluate a microporous membrane liquid–liquid extraction (MMLLE) method for non-metabolised, and thus lipophilic, organophosphates in human blood plasma. This technique utilises a hydrophobic membrane that separates an aqueous sample from the extracting organic solvent, which also fills the pores of the membrane. Thus, MMLLE is a two-phase extraction system similar to LLE. The MMLLE principle was chosen in order to develop a fast, less time- and solvent-consuming method compared to ordinary LLE. MMLLE also has the great advantage that it can be automated and coupled on-line to chromatographic methods. The use of MMLLE as a convenient method for processing plasma samples has recently been reviewed by Jönsson and Mathiasson [19,20].

2. Experimental

2.1. Chemicals

All solvents used were of analytical grade. Hexane was purchased from Fisher Chemicals (Loughborough, UK), methyl tert.-butyl ether (MTBE) from

Rathburn (Walkerburn, UK), acetone and KCl from Merck (Darmstadt, Germany), and formic acid from Riedel-de Haën (Seelze, Germany). Diphenyl methyl phosphate, triethyl, tri(*n*-propyl), tri(*n*-butyl), tri(2-chloroethyl), triphenyl, tri(2-butoxyethyl), triethyl hexyl phosphate and tritolyl phosphate were purchased from Aldrich (Milwaukee, WI, USA). Akzo Nobel (Herkenbosch, The Netherlands) kindly provided tri(2-chloropropyl) phosphate. A solution containing all nine organophosphate esters was used as an external standard for quantitative and qualitative determination. Diphenyl methyl phosphate was used as an internal volumetric standard and was added to the extract prior to GC separation. All reference substances were of analytical grade (>98%), except for diphenyl methyl phosphate. The latter compound was purified from a technical quality mixture (80%), containing triphenyl phosphate as an impurity. By using semi-preparative reversed-phase HPLC, methyl diphenyl phosphate could be obtained with a purity exceeding 99%.

2.2. Cleaning procedures

In previous investigations we have found that organophosphate esters are ubiquitous indoor air pollutants and consequently may contaminate the laboratory utensils. Therefore all glassware was thoroughly cleaned by extensive rinsing with acetone prior to use. Before MMLLE, a new membrane was washed with the extracting solvent, i.e. MTBE-hexane (1:1, v/v). After extraction, the extractor was cleaned using detergent and water and subsequently rinsed with deionised water.

2.3. Equipment

2.3.1. The extractor

The extraction device, which was constructed from polytetrafluoro ethylene (PTFE), is shown in Fig. 2. The lower part is a 12-ml chamber (diameter 40 mm, height 10 mm) for the plasma sample, also containing the stirring bar. The upper part has a serpentine like channel (2-mm width and 0.5-mm depth), with a volume of ~0.5 ml, through which the organic phase is pumped. The sample phase and the organic phase are separated by a PTFE membrane (TEU 20 (TE 35 without the stabilising polyester support), 0.2-μm pore size, 50-mm diameter; Schleicher & Schuell, Dassel, Germany). A tray, with ribs matching the serpentine channel, mechanically supports the membrane. All fittings are of low-pressure fingertight type. The construction allows the sample phase to be stirred and the extracting organic phase to be pumped (either continuously or in a stopped-flow mode). A syringe equipped with a six-port valve was used to introduce the sample and other solutions into the extractor. A Varian 9001 HPLC pump was used to pump the organic phase that after MMLLE was led to a glass tube and continuously evaporated.

2.3.2. GC–NPD

Quantitative GC analyses of the organophosphate esters were performed using a CE Instruments 8000 Top gas chromatograph equipped with a TS-2 nitrogen-phosphorus detector (CE Instruments, Italy) and a DB-5 column (30 m×0.32 mm I.D.; film thickness, 0.1 μm; J&W Scientific, CA, USA). Nitrogen was used as carrier gas at a constant flow of 2.0 ml/min. The column (oven) temperature was programmed as follows: 50°C isothermally for 2 min during injection, followed by a linear temperature increase of 40°C/min up to 200°C, then 10°C/min up to 250°C and finally 40°C/min up to 300°C, held for 2 min. Samples were injected in splitless mode with the split closed for 2 min during injection. The injector and detector temperatures were set to 300°C. A personal computer based laboratory data system (ELDS Win Pro, Chromatography Data System, Sweden) was used for recording and processing the data signal.

2.3.3. GC–MS-EI

For identifying organophosphate esters in non-spiked human blood plasma, GC–MS analyses were performed using an Automass Multi system (ThermoQuest, France), with the same type of GC column (DB5) and temperature program as used in the GC–NPD, and a split-splitless injector kept at 300°C. Helium was used as carrier gas with a constant flow of 1.0 ml/min. The temperature of the transfer line between the GC and the mass spectrometer was held at 280°C. Analyses were made in electron ionisation (EI) mode, with an ionisation energy of 70 eV and an ion source temperature of 150°C. Scanning was performed in full-scan mode, using a scan cycle of 50–500 Th in 0.5 s.

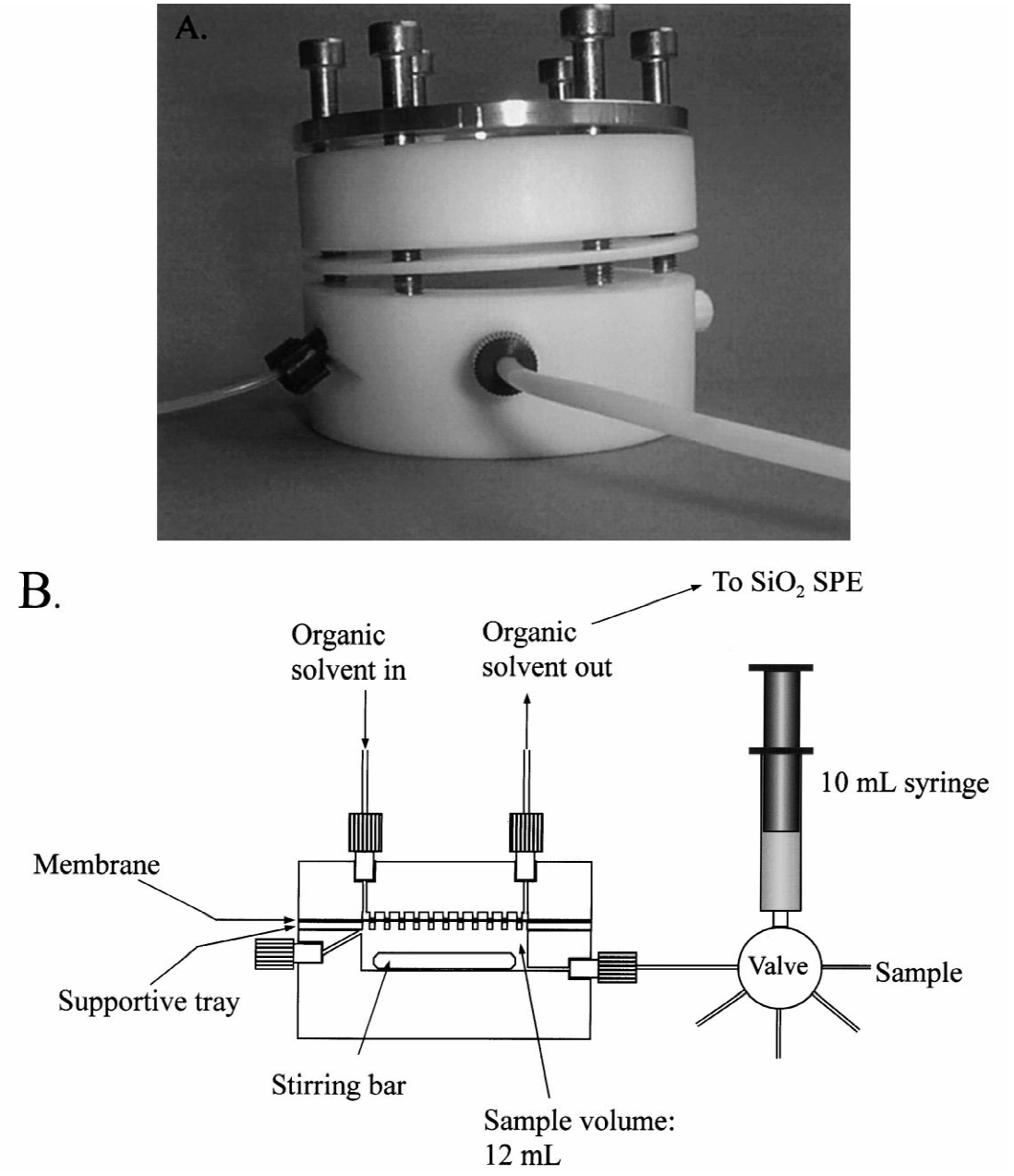

Fig. 2. (A) Photograph of the extraction device. (B) Schematic diagram of the extraction device in cross-section. All parts including the membrane are made of PTFE. The syringe is used for handling samples, and as a communicative vessel by removing the plunger.

2.4. MMLLE and SPE procedures

2.4.1. Sample preparation

Human plasma in blood storage containers was obtained from the Karolinska Hospital, Stockholm, Sweden. The plasma was transferred to glass tubes in 5.0-g portions and kept frozen at −20°C until analysis. Spiked samples, used for evaluation of the extraction method, were produced by adding between 500 and 800 ng of each organophosphate

ester, in 10 μl of acetone-cyclohexane, 1:1, to 5.0 g plasma. The sample was then mixed on a vortex mixer for 15 s and placed on a mixing table for 20 min. Non-spiked samples, used to investigate the occurrence of organophosphate esters in human blood plasma, and spiked samples were treated in an identical manner using the procedures described below. To denature the plasma proteins, 5 ml of concentrated formic acid was added to the sample before it was transferred into the extractor.

2.4.2. MMLLE

The performance of the MMLLE device was evaluated using spiked plasma samples. For each analysis a new cleaned membrane was mounted in the extraction device. Samples were introduced into the extractor via a six-port valve using a 10-ml glass syringe with Teflon® sealed plunger. In order to fill the sample volume of the extractor completely, all air was evacuated through a hole that was sealed with a fingertight screw after filling. To maintain an appropriate pressure in the sample volume, the syringe was used as a communicative vessel by first filling it to a certain level (over the membrane) with 0.9% $KCl_{(aq)}$ and then removing the plunger. The organic acceptor flow, hexane-MTBE, 1:1, was started first, and then the stirring bar, rotating at a speed of ~360 rpm. The stirring bar used in this study was 35 mm long and 7 mm thick.

A stopped-flow technique was used, with a 2-min flow of 0.5 ml/min every 20 min for a total time of 202 min. The organic phase was led to a pre-weighed, 1.75-ml glass tube where it was continuously evaporated using a gentle stream of nitrogen. The extract was evaporated to dryness in order to determine the amount of co-extracted plasma lipids.

2.4.3. SPE

Following MMLLE, further removal of plasma lipids is required before GC analysis is performed. Therefore, after the co-extracted lipids were weighed, the extract was dissolved in ~200 μl of acetone and MTBE in equal proportions and then transferred to a silica SPE column (1 g, 6 ml; IST, UK) for further clean-up. The SPE column was conditioned using 6 ml acetone-MTBE (1:1, v/v). The organophosphate esters were eluted using 4 ml of acetone-MTBE (1:1, v/v). Prior to GC separation, the volume of the SPE extract was reduced by a gentle stream of nitrogen to ~200 μl and 526 ng of methyl diphenyl phosphate was added as an internal volumetric standard. After GC analysis, the extract was evaporated to dryness to determine the final amount of co-extracted lipids after both MMLLE and SPE.

2.5. Recovery calculations

Recoveries of the nine organophosphates from spiked plasma samples were calculated by subtracting the average background values of the corresponding non-spiked plasma samples. Among the nine compounds, only triphenyl phosphate was present in the non-spiked plasma.

Blank samples, consisting of aqueous solutions of 0.9% KCl, were subjected to the same treatment and analyses as the plasma samples. No detector signals corresponding to organophosphate esters were observed.

2.6. Determination of the amount of co-extracted lipids

The amount of lipids in the samples, defined as the mass of co-extracted plasma compounds, was determined gravimetrically after both the MMLLE and SPE steps. The results were compared to those obtained using a previously reported LLE method [17].

3. Results and discussion

3.1. MMLLE and SPE

The overall recoveries for all investigated organophosphate esters, in spiked blood plasma, using the combined MMLLE–SPE method with an MMLLE period of 202 min are presented in Table 1. Data is based on spiking of two different plasma samples, both in triplicate. The two groups were not statistically separated with respect to precision (F-test, $P=0.05$) or averages (t-test, $P=0.05$) and therefore the results were pooled, giving $n=6$. For most of the compounds, fully acceptable recoveries were obtained. Furthermore, the reproducibility for

Table 1
Recoveries of nine different organophosphate esters using the described MMLLE–SPE method ($n=6$)

Organophosphate ester	Recovery (%)	RSD	LOD (ng/g)[a]
Triethyl phosphate	4.7	34	0.9
Tri(*n*-propyl) phosphate	50	3.4	0.07
Tri(*n*-butyl) phosphate	75	2.7	0.06
Tri(2-chloroethyl) phosphate	25	12	0.4
Tri(2-chloropropyl) phosphate	70	3.9	0.2
Triphenyl phosphate[b]	75	2.7	0.2
Tri(2-butoxyethyl) phosphate	81	5.9	0.3
Tri(2-ethylhexyl) phosphate	4.3	16	4
Tritolyl phosphate	76	6.7	0.5

[a] The method LOD values are based on the extraction of 5 g plasma.
[b] The recovery is calculated by subtracting the average background values of the corresponding non-spiked plasma samples.

these compounds was high, with relative standard deviations (RSD) of 3–7%. Except for tri(2-ethylhexyl) phosphate, the three compounds with the shortest alkyl chains exhibit the lowest recoveries of all the analytes. This is most likely due to the more polar properties of these compounds and their correspondingly weaker tendency to be extracted in MMLLE by the organic phase. The low recovery of tri(2-ethylhexyl) phosphate, on the other hand, may be due either to the compound binding to the plasma matrix, or to its bulky, lipophilic structure leading to a slow mass transfer over the membrane. However, this requires further study.

In order to maximise the extraction efficiency in MMLLE, the sample and the organic phase are both most often pumped at very low flow-rates, either in parallel or in opposite directions, through channels with high area to volume ratios. Since the analytes may bind to plasma proteins, some form of denaturation is required. This may lead to protein aggregation and clogging and thus unwanted pressure changes. If the pressure difference between the two sides of the membrane gets too high it may lead to leakage of organic solvent into the aqueous phase [21]. Therefore, to avoid clogging, the whole sample is stirred instead of being pumped in narrow channels, in the MMLLE method presented here. The choice of organic phase is also critical, since the polar properties of the phosphate esters require a relatively polar composition, i.e. a large amount of polar modifier like MTBE, to give good recoveries. On the other hand, MTBE has a tendency to diffuse into the plasma phase. In order to prevent the plasma phase from a too extensive alteration of its composition, the concentration of MTBE in the organic phase should be held within a certain limit, i.e. not more than 50%.

In the SPE step, all organophosphate esters could be eluted from the column with recoveries higher than 90%. The relatively polar properties of most of the organophosphate esters investigated require the use of a polar composition of the eluent. If a non-polar solvent such as hexane is used, both phosphate esters and lipids will be trapped on the silica phase. By adding a polar modifier, e.g. MTBE, to hexane to a final composition of 1:1, some of the less polar phosphate esters will elute, but unfortunately, so will also most of the lipids. The even more polar composition used in this study (MTBE-acetone, 1:1) has the advantage of eluting all the investigated phosphate esters while most of the lipids are still retained.

For the investigated phosphate esters using the presented MMLLE–SPE method, the method limits of detection (LOD) are presented in Table 1. The method LODs were calculated from the detector signal at three times the noise level, corrected for the clean-up recoveries and based on extraction of 5 g plasma. For triphenyl phosphate, the method LOD was found to be less than 0.2 ng/g plasma.

3.2. Lipid removal

The MMLLE step alone was shown to reduce co-extracted lipids by ~94% compared to ordinary LLE [17]. This is due to the size exclusion properties of the membrane, the extent of mass transfer being

dependent on the molecular size of the compounds. This high efficiency reduced the need for subsequent time-consuming separations on GPC or other chromatographic systems. Instead, the SPE method presented was shown to quickly remove more than 50% of the remaining lipids. The system gave a total lipid reduction of over 97% compared to ordinary LLE.

3.3. Identification of organophosphate esters in human blood plasma

Triphenyl phosphate and an isomer of octyl diphenyl phosphate could be identified in all non-spiked plasma samples investigated, after clean-up using the combined MMLLE–SPE method. The structures were confirmed by GC–MS-EI, exhibiting molecular ions with m/z values of 326 and 362. Fig. 3 shows the GC–MS and GC–NPD chromatograms for one of the non-spiked samples. The high selectivity of the NPD is clearly demonstrated, with LOD for triphenyl phosphate being less than 1 pg.

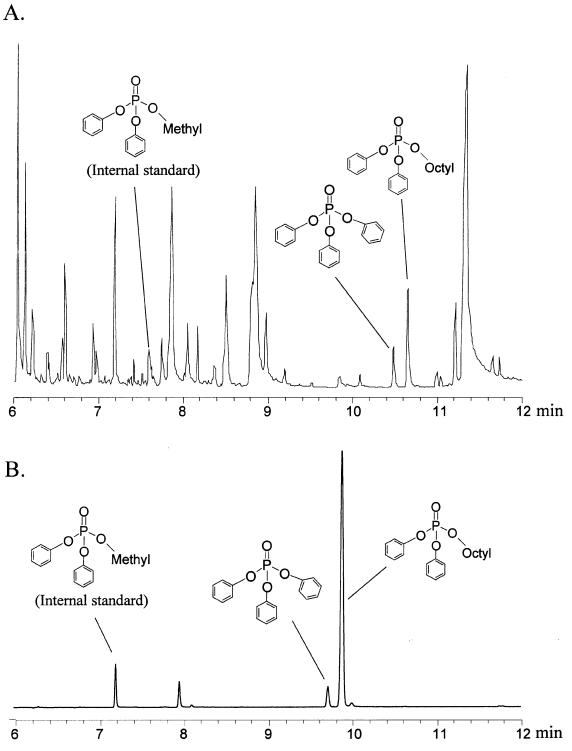

Fig. 3. GC–MS-EI in full-scan mode (A) and GC–NPD (B) chromatograms of a non-spiked plasma sample. For chromatographic conditions, see Experimental section. The peak eluting at ~7.9 min in the GC–NPD chromatogram was not identified.

Table 2
Concentrations of triphenyl phosphate and an isomer of octyl diphenyl phosphate in non-spiked plasma samples obtained from three different individuals[a]

	Triphenyl phosphate[b], conc. (μg/g)	Octyl diphenyl phosphate[c], conc. (μg/g)
Individual 1	0.14/0.15	0.61/0.74
Individual 2	0.13/0.15	1.4/1.8
Individual 3	0.12/0.13	1.3/1.5

[a] Duplicate samples were analysed for each individual.
[b] The concentrations of triphenyl phosphate are corrected for 80% recovery.
[c] Only tentatively quantified, since no standard was available (and thereby not corrected for recovery), assuming identical NPD response as for triphenyl phosphate.

The group of organophosphate esters is quite heterogeneous. Thus, the use of deuterium or ^{13}C-labelled surrogate standards corresponding to each individual analyte would provide an optimal quantification method. However, that would preclude the use of the nitrogen-phosphor detector that is very selective as well as sensitive with respect to organophosphate esters. Since no other suitable surrogate standard has been found so far, quantification was performed by determination of analyte recovery followed by comparison of the detector response with that of an external standard. An internal volumetric standard was added to the extract prior to GC analysis.

The concentrations of triphenyl phosphate in plasma from three different individuals are presented in Table 2. The other compound, an isomer of octyl diphenyl phosphate, was only tentatively quantified, since no reference substance was available. The compounds were assumed to give identical NPD responses.

4. Conclusions

The presented method was shown to enable efficient removal of lipids from plasma samples. The

amount of co-extracted matrix compounds using MMLLE was reduced by 94% compared to an ordinary LLE method.

The individual extraction steps were both shown to give high recoveries and reproducibility and, furthermore, to require fairly small volumes of extracting solvents. Therefore, they should be ideal for on-line coupling. The method presented here required much less laborious handling than ordinary LLE. Another advantage is the dynamic properties of the extraction. When using ordinary static LLE methods, the extractions usually have to be repeated twice or more. Furthermore, the LLE methods most often have to be combined with time- or solvent-consuming lipid removal steps, such as GPC.

The identification of organophosphate esters in human blood plasma is of interest because of the reported contact allergenic effects of triphenyl phosphate and, even more importantly, because of the impact of these compounds on human blood monocytes and their haemolytic effects. It is, however, important to point out that the origin of the two compounds has not been investigated in this study.

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