Analysis of Organophosphorus Pesticides in whole blood by GC-MS-μECD with forensic purposes

Authors names and affiliations: Nuno I.P. Valente [a,b], Sónia Tarelho [a,*], André L. Castro [a], Armando Silvestre [b,f], Helena M. Teixeira [c,d,e]

[a] Chemistry and Forensic Sciences Service, National Institute of Legal Medicine and Forensic Sciences – North Branch, Jardim Carrilho Videira, 4050-067 Porto, Portugal.
[b] Departament of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[c] CICECO – Centre for research in ceramics & composite materials, Complexo de Laboratórios Tecnológicos, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[d] Research, Formation and Documentation Service, National Institute of Legal Medicine and Forensic Sciences, Largo da Sé Nova, 3000-213 Coimbra, Portugal.
[e] Faculty of Medicine, University of Coimbra, Rua Larga, 3004-504 Coimbra, Portugal
[f] Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal.

* Corresponding author: Chemistry and Forensic Sciences Service, North Branch of National Institute of Legal Medicine and Forensic Sciences, Jardim Carrilho Videira, 4050-067 Porto, Portugal. Fax: +351 222018069.
E-mail address: sonia.tarelho@dpinml.mj.pt

Keywords

GC-MS-ECD; Analytical method validation; Organophosphorus pesticides; Post-mortem blood samples; SPE Sep-Pak C18 cartridges

Highlights

Fully validated multiresidue methods for 10 organophosphorus pesticides in whole blood

Comparison of extraction procedures with different cartridges (Sep-Pak C18 & Oasis HLB)

GC-MS used for screening and quantitation, whilst GC-ECD for confirmation

Methods introduced in the routine analysis

Abstract

In the present work, two multi-residue methods for the determination of ten organophosphorus pesticides (OPs), namely chlorfenvinphos, chlorpyrifos, diazinon, dimethoate, fenthion, malathion, parathion, phosalone, pirimiphos-methyl and quinalphos, in post-mortem whole blood samples are presented.

The adopted procedure uses GC-MS for screening and quantitation, and GC-μECD (electron capture detector) for compound confirmation. Three different Solid Phase Extraction (SPE) procedures for OPs with Oasis® hydrophilic lipophilic balanced (HLB) and Sep-Pak® C18 cartridges were tested, and followed by GC-μECD and GC-MS analysis. The Sep-Pak® C18 cartridges extraction procedure was selected since it generated analytical signals 5 times higher than those obtained with the two different Oasis® HLB cartridges extraction procedures. The method has shown to be selective for the isolation of selected OPs as well as to the chosen internal standard (ethion) in postmortem blood samples. Calibration curves between 50 and 5000 ng/mL were prepared using weighted linear regression models ($1/x^2$). It was not possible to establish a working range for fenthion by GC-μECD due to the lower sensitivity of the detector to this compound, whereas for pirimiphos-methyl it was set between 500 and 5000 ng/mL. The limit of quantitation was established at 50 ng/mL for all analytes, except for pirimiphos-methyl by GC-μECD analysis (500 ng/mL). The average extraction efficiency ranged from 72 to 102%.

The developed methods were considered robust and fit for the purpose, and had already been adopted in the laboratory routine analysis.

1 Introduction

Organophosphorus pesticides (OPs) were developed to protect crops against damage by insects. However, their usage has been reported in suicide attempts in rural areas, despite European legislation prohibiting the use of certain pesticides, namely the most dangerous ones. This happens because, despite restrictive legislation, small farmers and Portuguese domestic users tend to keep the stock they already have when new prohibiting laws are approved.

When ingested, OPs are rapidly absorbed and distributed throughout the body, binding and therefore inhibiting acetylcholinesterase enzymes, causing accumulation of acetylcholine, which is essential for nerve impulse transmission in both vertebrate and invertebrate species. This accumulation leads to the disruption of the normal functioning of the nervous system, producing typical cholinergic symptoms (i.e. hyperactivity tremors, convulsions, paralysis and ultimately death).[1]

Since in suicide attempts people tend to ingest large amounts of pesticides, they eventually die. In order to confirm the cause of death, whole blood samples are collected and sent to toxicological analysis, requiring a sample clean-up step. Known clean-up methodologies involve Liquid-Liquid Extraction (LLE),[2, 3] Solid Phase Extraction (SPE),[4-7] and Solid Phase Micro-Extraction (SPME).[8-10]

The analytical determination of OPs in blood was first carried out by gas chromatography (GC) with flame ionization detector (FID),[5, 11] nitrogen-phosphorus detector (NPD),[9, 10, 12, 13] and flame photometric detector (FPD).[14, 15] However, in the last few decades, mass spectrometry has become the main detection technique, due to its confidence in the confirmation of peak identity and quantitation, especially if isotopically labelled internal standards are available.[2, 4, 6-9] High performance liquid chromatography coupled with mass spectrometry has also been used to detect this group of compounds.[3, 16] The use of electron capture detector (ECD) to detect OPs has been too scarce; indeed, Heinig *et al.*[17] analysed only metrifonate and one metabolite in whole blood and urine, while Pitarch *et al.*[18] developed two procedures for organochlorine and organophosphorus pesticides determination but in serum and urine samples.

The aim of the present study was the development, optimization, and validation of a method for the determination of 10 organophosphorus pesticides (chlorfenvinphos, chlorpyrifos, diazinon, dimethoate, fenthion, malathion, parathion, phosalone, pirimiphos-methyl and quinalphos) in whole blood by SPE procedure. These pesticides were selected due to their toxicity, their presence in previously reported intoxication cases in the Chemistry and Forensic Sciences Service, and their commercialization history. Whole blood was used due to the fact that plausible binding of pesticides to erythrocytes, haemolysis and microbial degradation avoids the isolation of serum or plasma, allowing, contemporaneously, the possibility to perform screening, confirmation and quantitation procedures in the same sample.[19-21] The used apparatus consisted on single equipment, containing two independent separation systems, GC-MS

and GC-μECD, offering high sensitivity and detection based on different compound properties. Moreover, and according to good laboratory practices and internal guidelines, two different analytical procedures (granted by these two systems) are needed to issue / emit a positive result.

2 Material and methods

2.1 Reagents and materials

Pesticides standards (chlorfenvinphos 97.5%, chlorpyrifos 99.9%, diazinon 99.0%, fenthion 98.3%, malathion 97.2%, parathion 99.5%, phosalone 99.5%, pirimiphos-methyl 99.5% and quinalphos 96.2%) were purchased from Fluka. Dimethoate 96.2% and ethion 99.3% (the later was chosen as internal standard as its commercialization in Portugal is prohibited since 2003), were purchased from Supelco (Saint Louis, USA). Ammonium acetate, acetic acid, potassium chloride, sodium chloride, chloroform, diethyl ether, n-hexane, isooctane, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, isopropanol were purchased from Merck Co. (Darmstadt, Germany). Acetonitrile and methanol were purchased from Fisher Chemical and Sigma-Aldrich, respectively. Water was purified by a Milli-Q system obtained from Millipore (Molsheim, France). All solvents were of analytical or gradient grade. Extraction procedures were performed in a manual SPE equipment, using Oasis® HLB (3 cc, 60 mg) and Sep-Pak® C18 (3 cc, 500 mg) SPE cartridges, obtained from Waters™ (WATERS Corporation, USA).

2.2 Standard solutions preparation

Stock solutions of each analyte were prepared in the lab and then diluted to get appropriate pesticide standard solutions (100 and 1 μg/mL). An internal standard stock solution was also prepared and properly diluted to get a solution with a final concentration of 100 μg/mL. These solutions were stored at -20 °C. A 1 L ammonium acetate buffer solution (0.1 M) was prepared by mixing 3.3 mL acetic acid and 7700 mg ammonium acetate. The volume was adjusted with deionised water (pH 4.9). A 1 L Phosphate Buffer Saline (PBS) solution was prepared by mixing 200 mg of KCl, 8000 mg NaCl, 200 mg of KH_2PO_4 and 1150 mg of Na_2HPO_4. The volume was adjusted with deionised water.

2.3 Extraction procedures

In this work, five different SPE procedures were tested. Two of them used Oasis® HLB cartridges, were adapted from Raposo *et al.*[7] and Park *et al.*[6], and were named by "HLB_A" and "HLB_B" procedures, respectively. Other three procedures, involving the use of Sep-Pak® C18 cartridges and based on the work of Liu *et al.*[5], were tested. They differed on the volume (10 mL, 5 mL and 2 mL) of solvent or mixture used in the conditioning step, and were named "Sep-Pak_10", "Sep-Pak_5" and "Sep-Pak_2", respectively. As an example, it is presented the procedure Sep-Pak_2, which was validated during this work.

Blood samples (0.5 mL) were mixed with 5 mL deionised water and centrifuged at 4000 rpm during 30 min. Meanwhile, for cartridge conditioning, 2 mL of chloroform:isopropanol mixture (V:V 9:1), 2 mL of acetonitrile, 2 mL of acetonitrile:water mixture (V:V 1:1) and 2 mL of deionised water were passed through the cartridges. The supernatant was loaded onto the conditioned cartridge and allowed to drain at a 1–2 mL/min flow. The cartridge was then washed with 10 mL of water and vacuum dried during 15 min. After that, 3 mL of chloroform:isopropanol mixture (V:V 9:1) was passed through the cartridge to recover the analytes for a glass tube. After solvent evaporation the residues were redissolved with 100 µL isooctane, transferred to a vial with insert and injected into the GC system.

2.4 Instrumental conditions

The GC used in this work was an Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA), equipped with a Agilent 7683 series liquid autosampler, two split/splitless injectors, and two independent separation and detection systems (MS and µECD).

The front system was equipped with a HP-5MS Agilent column (30 m x 0.25 mm x 0.25 µm) and an Agilent 5973N series mass selective detector (MS), containing a single quadrupole. Injector and MS transfer line temperatures were both set at 280 °C. The oven temperature was initially held at 80 °C for 1.0 min, and then increased to 227 °C (35 °C/min), held for 6.0 min, and then increased to 275 °C (10 °C/min) and held for 2.0 min (total run time: 18.0 min). Gas chromatograph was operated in split mode (ratio 38.5:1) and a Helium BIP gas flow of 1.3 mL/min (constant flow mode) was used. The MS was used in SIM mode both for qualitative and for quantitative analysis, with a

solvent delay of 5.00 min, and at least three ions were monitored for each analyte, except for the internal standard (Table 1). Chlorfenvinphos standard showed two chromatographic peaks due to its *E* and *Z* isomers.

The back system was equipped with a Factor Four VF-5MS column from Varian (60 m x 0.32 mm x 0.25 μm) and a G2397A micro-cell Electron Capture Detector (μECD) from Agilent. Injector and μECD temperatures were set at 280 and 320 °C, respectively. The column temperature was initially held at 80 °C for 1.0 min, increased to 200 °C (20 °C/min), held for 9.0 min, and then increased to 275 °C (10 °C/min) and held for 8.0 min (total run time: 31.5 min.). This system was operated in splitless mode, and Helium BIP (1.2 mL/min) and Nitrogen BIP (60.0 mL/min) were used as carrier and make-up gas, respectively, with constant flows. Retention times are shown in Table 1.

2.5 Analytical validation

For the validation of the methodologies, the following criteria were used (based in international guidelines:[22, 23] confirmation of identity and selectivity, work range and linearity, limit of detection (LOD), limit of quantitation (LOQ), carryover, extraction efficiency, accuracy, and robustness. Through the study of confirmation of identity and selectivity, a pool of six blood samples was prepared and used: cardiac and peripheral, collected post-mortem, without OPs present (confirmed by a previous analysis using another analytical procedure). Blank blood samples used in the remaining parameters of this work were obtained from the Portuguese Blood Institute. The discussion of these parameters is presented below.

3 Results and discussion

3.1 Extraction procedures

The choice of the extraction procedure started with the comparison among HLB_A, HLB_B and Sep-Pak_10 procedures. Blank and spiked samples were prepared simultaneously, according to these three procedures in two different days and averages of the obtained peak areas were compared for all the pesticides (Figure 1).

The average of all peak areas of samples prepared with HLB cartridges were five times smaller than those obtained with C18 cartridges. The overall average peak areas

obtained in GC-μECD corresponded to 19.8% for HLB_B and 18.0% for HLB_A when compared with Sep-Pak_10. However, the results for dimethoate stood out. The difference of peak areas of dimethoate was smaller (59.6 and 43.3%, for HLB_A and HLB_B, respectively). The same samples were analysed by GC-MS to assess the presence of interfering peaks. Interfering peaks were only observed near the retention time of phosalone when HLB cartridges were used. Thus, the procedure with Sep-Pak® C18 was chosen to proceed with the study.

In order to optimize the extraction procedure with Sep-Pak® C18, the conditioning step was tested with three different volumes of each solvent or mixture, namely 10 mL, 5 mL and 2 mL, and named Sep-Pak_10, Sep-Pak_5 and Sep-Pak_2, respectively. The average of the results obtained in two different days showed no significant differences among Sep-Pak_10, Sep-Pak_5 and Sep-Pak_2. Using the peak areas obtained with Sep-Pak_10 procedure as a reference, those obtained with Sep-Pak_5 corresponded to 95.7%, while those obtained with Sep-Pak_2 corresponded to 107.6%. The biggest differences were registered for dimethoate, phosalone and pirimiphos-methyl (Figure 1).

Sep-Pak_2 procedure was selected to continue this study since it allows some solvent savings, a faster preparation step and high extraction efficiency.

3.2 Methods validation

3.2.1 Confirmation of identity and Selectivity

Selectivity and specificity are often used indistinctly, being given the same meaning. Selectivity can be referred as the capacity of a system to separate physically and determinate an analyte (or a group of analytes) present in a complex mixture without significant interference from other components of the mixture, whereas specificity is seen as the ultimate degree of selectivity, in an absolute sense, such as "specific or not". Following this line, IUPAC recommends that the term 'specificity' should not be used to avoid confusion.[22, 24, 25]

Selectivity was studied by analysing ten blank and ten spiked samples. All the analytes were successfully identified[26] in all spiked samples by GC-μECD and GC-MS when compared to a control sample analysed contemporaneously. Additionally, all spiked and blank samples were free of co-eluting peaks at the retention times of the studied OPs and of the internal standard. Figure 2 shows no interferences from sample matrix (absence of any interfering peak) and, thus, demonstrates that the method

involving GC-μECD was selective to chlorfenvinphos, chlorpyrifos, diazinon, dimethoate, malathion, parathion, phosalone, pirimiphos-methyl and quinalphos. However, due to lack of sensitivity of the detector to fenthion, which might be explained by a hypothetic lower electronegativity of the compound, this method was not considered fit to perform an analysis involving this pesticide. The GC-MS method also showed no interfering peaks for all selected ions and was also considered selective for all of ten studied pesticides.

3.2.2 Working range and linearity

Weighted calibration curves were established by analysis of blank blood samples spiked with concentrations ranging from 50 (500 for pirimiphos-methyl in GC-μECD) to 5000 g/mL to compensate for heterocedasticity. After testing several weighting factors (1, $1/x$, $1/x^2$, $1/x^{1/2}$, $1/y$, $1/y^2$, $1/y^{1/2}$), $1/x^2$ was chosen because it presented the smallest sum of squares of the deviations between data[27] for almost all pesticides (Table 2). The only exception was $1/y^2$ for pirimiphos-methyl in GC-μECD.

3.2.3 Limits

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated from extracted samples spiked with the studied OPs. The LOD and LOQ were established using the residual standard deviation ($S_{y/x}$) and the slope of the weighted linear regression (b), as LOD = 3.3 $S_{y/x}/b$ and LOQ = 10 $S_{y/x}/b$ [25] of calibration curves prepared between 44 and 66 ng/mL (somewhere near to a reasonable defined value, based on the laboratory experience and literature review[6, 7]). Regardless of the detection technique, LOD values obtained were very similar for almost all pesticides. With the exception pirimiphos-methyl in GC-μECD, LOD varied from 2.46 to 5.19 ng/mL (Table 2). Noticeably, pirimiphos-methyl and fenthion are the only two thiophosphates with two methoxy groups bonded to the phosphorus and a third group linked via an oxygen atom. These features might be responsible for the lower electronegativity of the pesticides and, therefore, lower sensitivity of the detector to the compounds. The same behaviour was observed for LOQ, due to the relation between these two limits (LOD = 3.3/10 LOQ): values ranged from 7.46 to 15.73 ng/mL. These values of LOD and LOQ are similar to or lower than other published involving GC analysis.[3, 4, 6] Although some published results using HPLC analysis of whole blood[16] and serum[28] showed lower limits, the lower costs and larger availability of GC based procedures are still attractive.

However, since the lowest pesticide concentration determined in fatal cases and published was, to our knowledge, 50 ng/mL for quinalphos,[7] the limit of quantitation was established at a higher level than those obtained from parameters of calibration curves, namely 50 ng/mL (the lower limit of working range). The exception was, again, pirimiphos-methyl in samples analysed by GC-μECD, 500 ng/mL. The coefficient of variation was lower than 20% and bias within ±20%, respecting the criteria established by U.S. Food and Drug Administration's guideline.[19]

3.2.4 Carryover

The analysis of three blank samples, interspersed with spiked samples at the upper limit of the working range (5000 ng/mL), showed no evidence of carryover phenomena in both GC-μECD and GC-MS procedures. This means that there was no analyte transfer between samples and, thus, sample contamination was avoided. If this had happened, it could generate false positive or inflate blood concentration results.

3.2.5 Extraction efficiency

The efficiency of the extraction procedure was assessed by running three replicates at the relative low and high concentrations, within the 1st and 4th quarter of working range, respectively. Efficiency is often denominated as recovery, but this term will not be used to avoid confusion with recovery related to accuracy of the method. Efficiency was calculated as a direct comparison between the A_A/A_{IS} ratio (quotient between the peak area of analyte and the peak area of IS) of samples spiked before extraction and the A_A/A_{IS} ratio of samples spiked after extraction (IS was added before the extraction in both situations). The results (n=3) obtained in GC-MS are shown in Table 3.

In general, average efficiencies higher than 70% were obtained, and for some of the compounds, as dimethoate and pirimiphos-methyl, efficiencies close to 100% were obtained. These results (including lower ones) were higher than or similar to other values presented elsewhere,[4, 7] and were considered fit for the purpose.

3.2.6 Accuracy

Accuracy, in analytical chemistry, comprises some doubts and disagreements. According to the English version of International vocabulary of metrology,[29] measurement accuracy is the 'closeness of agreement between a measured quantity value and a true quantity value of a measurand', and it 'is not a quantity and is not given

a numerical quantity value'. It is related to Precision ('closeness of agreement between measured quantity values') and Trueness ('closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value'). The better the performance of the assay in these two parameters, the better is the accuracy and, consequently, the better is the measurement uncertainty, a non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used.[29, 30]

The study of the accuracy began with the determination of precision based on the analysis of variance (ANOVA) of the results generated by 3 replicates at each of 8 days at two concentration levels. It was studied the repeatability standard deviation (s_r), between-run standard deviation (s_{run}), and intermediate standard deviation (s_i), as proposed by Maroto and colleagues,[31] and the repeatability limit (r) based on ISO 5725 – part 6.[32] However, aiming an application in laboratory routine and an optimization of costs and lower blood sample consumption, a possible use of only two aliquots was evaluated. Two results of each day were used on a new ANOVA, and the repeatability limits obtained where compared to those obtained with three aliquots, for each pesticide and each method. A t-student test, with a confidence level of 95%, showed that limits weren't significantly different, and thus the methods could be applied using only two aliquots. The results of r are shown in Table 4.

The estimation of measurement uncertainty was based on the same validation results as predicted by ISO 5725. This approach consisted on the sum of standard uncertainty associated to precision with standard uncertainty associated to trueness.

The standard uncertainty associated to precision ($u_{precision}$) corresponded to standard deviation ($s_{precision}$) of the previously mentioned results.[32, 33]

Once this parameter was estimated, relative standard uncertainty associated to average recovery, $u(R_m)$, was also determined for all pesticides in both methods. The standard uncertainty associated to trueness can be resumed to $u(R_m)$ admitting careful choice of analytical standards and gravimetric and/or volumetric operations.[32] The average recovery, R_m, served as an estimate of trueness error and whenever a 95% bilateral t-student test showed that average recovery was significantly different of 1 (essentially for samples spiked at 600 ng/mL) results were corrected. The average recovery ranged between 85 and 110%, and the lowest values were obtained with samples spiked at 600 ng/mL.

Lastly, expanded uncertainty was determined taking into account standard uncertainty associated to precision and standard uncertainty associated to average recovery[32]:

$$U(y) = 2 \times \sqrt{\left(u_{precision}\right)^2 + (u(R_m))^2} \quad \text{(equation 1)}$$

Table 4 shows that expanded uncertainty values ranged from 11.70 to 28.31%. The expanded uncertainty was higher at smaller concentrations. The method of GC-MS exhibited a smaller overall expanded uncertainty when compared to GC-μECD (17.00 vs. 18.43 %).

3.2.7 Robustness

During the validation process, several slightly changes were introduced. Different stock solutions, pipettes, manual extractors, centrifuges, evaporation equipment, among others, were used and no differences were detected. Thus, the methods were considered robust.

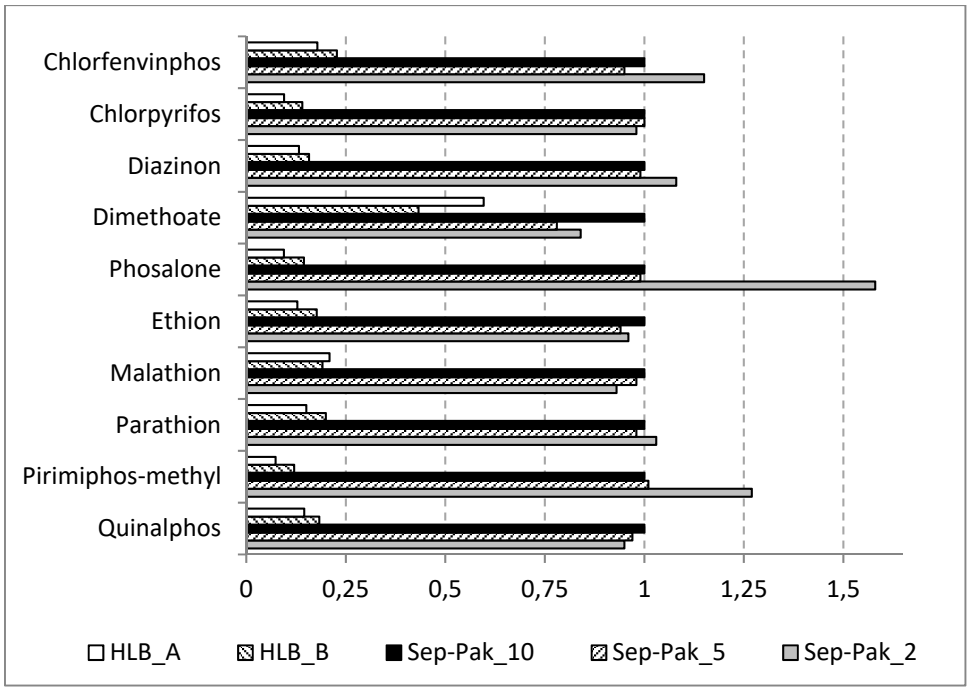
4 Conclusion

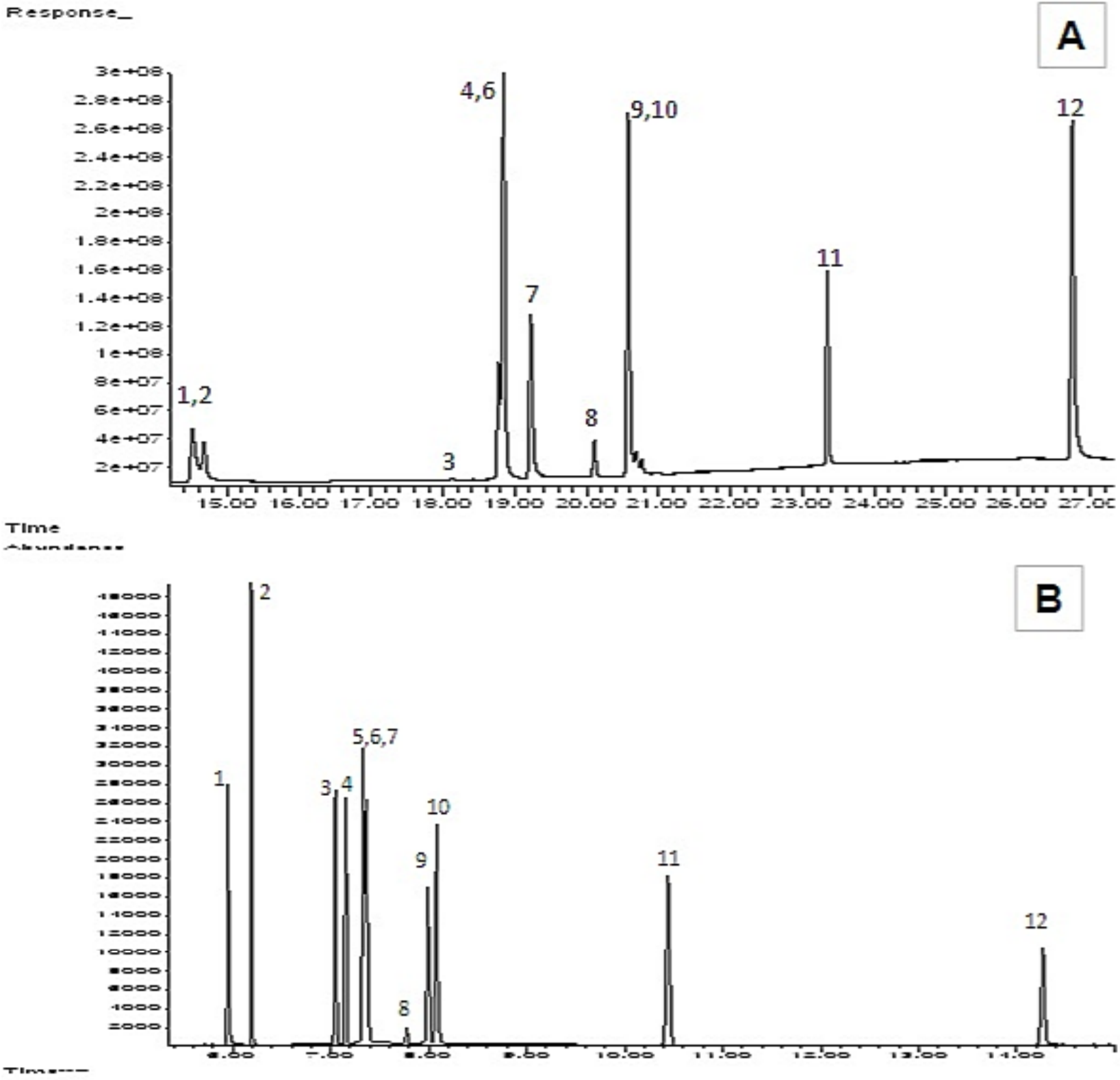
The first two multi-residue methods for analysis of organophosphorus pesticides in whole blood samples have been presented in this paper. Methodologies involved an SPE procedure followed by GC-MS and GC-μECD analyses. In a preliminary phase, three extraction procedures (using C18 or HLB cartridges) were evaluated. The one with C18 cartridges was chosen, followed by further tests in order to reduce the solvents consumption and the time of conditioning step. The developed and validated methods were rapid, robust and selective. Average extraction efficiencies were higher than 70%, while the defined LODs and LOQs were considered fit for the purpose (< 50 ng/mL). The lack of sensitivity of μECD prevents its use in the analysis of fenthion and limits its usage in pirimiphos-methyl analysis. For this reason, the methodology with GC-MS was chosen for an initial screening step, GC-μECD for confirmation (excepting positive results for fenthion) and GC-MS for quantitation.

References

1. Synthetic insecticides II: organophophorus and carbamate compounds. In: Cremlyn RJ, editor. *Agrochemicals: preparation and mode of action*. Chichester: John Wiley & Sons; 1991, p. 105-56.
2. Gottzein AK, Musshoff F, Doberentz E, Madea B. Combined suicide by oxydemeton-methyl (Metasystox (R)) ingestion and hanging. *Forensic Sci Int* 2009;**189**:e21-e5.
3. Inoue S, Saito T, Mase H, Suzuki Y, Takazawa K, Yamamoto I, et al. Rapid simultaneous determination for organophosphorus pesticides in human serum by LC-MS. *J Pharm Biomed* 2007;**44**:258-64.
4. Lacassie E, Dreyfuss MF, Gaulier JM, Marquet P, Daguet JL, Lachatre G. Multiresidue determination method for organophosphorus pesticides in serum and whole blood by gas chromatography-mass-selective detection. *J Chromatogr B* 2001;**759**:109-16.
5. Liu J, Suzuki O, Kumazawa T, Seno H. Rapid isolation with Sep-Pak C18 cartridges and wide-bore capillary gas chromatography of organophosphate pesticides. *Forensic Sci Int* 1989;**41**:67-72.
6. Park M-J, In S-W, Lee S-K, Choi W-K, Park Y-S, Chung H-S. Postmortem blood concentrations of organophosphorus pesticides. *Forensic Sci Int* 2009;**184**:28-31.
7. Raposo R, Barroso M, Fonseca S, Costa S, Queiroz JA, Gallardo E, et al. Determination of eight selected organophosphorus insecticides in postmortem blood samples using solid-phase extraction and gas chromatography/mass spectrometry. *Rapid Commun Mass Sp* 2010;**24**:3187-94.
8. Kasiotis KM, Souki H, Tsakirakis AN, Carageorgiou H, Theotokatos SA, Haroutounian SA, et al. Headspace solid phase micro extraction gas chromatographic determination of fenthion in human serum. *Int J Mol Sci* 2008;**9**:906-13.
9. Tsoukali H, Raikos N, Theodoridis G, Psaroulis D. Headspace solid phase microextraction for the gas chromatographic analysis of methyl-parathion in post-mortem human samples. *Forensic Sci Int* 2004;**143**:127-32.
10. Tsoukali H, Theodoridis G, Raikos N, Grigoratou I. Solid phase microextraction gas chromatographic analysis of organophosphorus pesticides in biological samples. *J Chromatogr B* 2005;**822**:194-200.
11. Liu JT, Suzuki O. Conditions of solid-phase extraction for the mixture of organophosphates and synthetic pyrethroids in human body fluids. *Forensic Sci Int* 1999;**99**:159-61.
12. Lee XP, Kumazawa T, Sato K, Suzuki O. Detection of organophosphate pesticides in human body fluids by headspace solid-phase microextraction (SPME) and capillary gas chromatography with nitrogen-phosphorus detection. *Chromatographia* 1996;**42**:135-40.
13. Pavlic M, Haidekker A, Grubwieser P, Rabl W. Fatal intoxication with omethoate. *Int J Legal Med* 2002;**116**:238-41.
14. Kojima T, Yashiki M, Ohtani M, Chikasue F, Miyazaki T. Determination of dimethoate in blood and hemoperfusion cartridge following ingestion of formothion - a case-study. *Forensic Sci Int* 1990;**48**:79-88.
15. Nolan RJ, Rick DL, Freshour NL, Saunders JH. Chlorpyrifos - pharmacokinetics in human volunteers. *Toxicol Appl Pharm* 1984;**73**:8-15.
16. Salm P, Taylor PJ, Roberts D, de Silva J. Liquid chromatography-tandem mass spectrometry method for the simultaneous quantitative determination of the

organophosphorus pesticides dimethoate, fenthion, diazinon and chlorpyrifos in human blood. *J Chromatogr B* 2009;**877**:568-74.
17. Heinig R, Zimmer D, Yeh S, Krol GJ. Development, validation and application of assays to quantify metrifonate and 2,2-dichlorovinyl dimethylphosphate in human body fluids. *J Chromatogr B* 2000;**741**:257-69.
18. Pitarch E, Lopez FJ, Serrano R, Hernandez F. Multiresidue determination of organophosphorus and organochlorine pesticides in human biological fluids by capillary gas chromatography. *Fresen J Anal Chem* 2001;**369**:502-9.
19. *FDA guidance for industry: bioanalytical method validation*: U.S. Food and Drug Administration - Center for Drug Evaluation and Research; 2001.
20. Drummer OH. Requirements for bioanalytical procedures in postmortem toxicology. *Anal Bioanal Chem* 2007;**388**:1495-503.
21. Skopp G. Preanalytic aspects in postmortem toxicology. *Forensic Sci Int* 2004;**142**:75-100.
22. *The fitness for purpose of analytical methods: a laboratory guide to method validation and related topics*: EURACHEM; 1998.
23. *Guia RELACRE 13 - validação de métodos internos de ensaio em análise química*: RELACRE; 2000.
24. Vessman J, Stefan RI, Van Staden JF, Danzer K, Lindner W, Burns DT, et al. Selectivity in analytical chemistry - (IUPAC Recommendations 2001). *Pure Appl Chem* 2001;**73**:1381-6.
25. Performance parameters, calculations and tests. In: Ermer J, Miller JHM, editors. *Method validation in pharmaceutical analysis - a guide to best practice*. Weinheim: Wiley-VCH; 2005, p. 21-194.
26. *TD2010IDCR - Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry*: WADA Laboratory Committee; 2010.
27. Almeida AM, Castel-Branco MM, Falcao AC. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J Chromatogr B* 2002;**774**:215-22.
28. Sancho JV, Pozo OJ, Hernández F. Direct determination of chlorpyrifos and its main metabolite 3,5,6-trichloro-2-pyridinol in human serum and urine by coupled-column liquid chromatography/electrospray-tandem mass spectrometry. *Rapid Commun Mass Sp* 2000;**14**:1485-90.
29. *International vocabulary of metrology - basic and general concepts and associated terms (VIM)*. 3ª ed: Joint Committee for Guides in Metrology (JCGM); 2012.
30. Peters FT, Drummer OH, Musshoff F. Validation of new methods. *Forensic Sci Int* 2007;**165**:216-224.
31. Maroto A, Boque R, Riu J, Rius FX. Estimation of measurement uncertainty by using regression techniques and spiked samples. *Anal Chim Acta* 2001;**446**:133-45.
31. *ISO 5725: Accuracy (trueness and precision) of measurement methods and results*: International Organization for Standardization (ISO); 1994.
32. Guide for the Uncertainty Quantification in Chemical Assays [*OGC007 - Guia para quantificação de incerteza em ensaios químicos*]: Instituto Português de Acreditação (IPAC); 2007.


Chlorfenvinphos
Chlorpyrifos
Diazinon
Dimethoate
Phosalone
Ethion
Malathion
Parathion
Pirimiphos-methyl
Quinalphos
0
0,25
0,5
0,75
1
1,25
1,5
HLB_A
HLB_B
Sep-Pak_10
Sep-Pak_5
Sep-Pak_2


A
Response_
3e+08
2.8e+08
2.6e+08
2.4e+08
2.2e+08
2e+08
1.8e+08
1.6e+08
1.4e+08
1.2e+08
1e+08
8e+07
6e+07
4e+07
2e+07
1,2
3
4,6
7
8
9,10
11
12
15.00 16.00 17.00 18.00 19.00 20.00 21.00 22.00 23.00 24.00 25.00 26.00
Time
B
1
2
3
4
5,6,7
8
9
10
11
12

Insecticide	GC-MS		GC-ECD
	RT (min)	Selected ions (*m/z*)	RT (min)
Dimethoate (1)	5,95	87[a], 93, 125, 229	14,51
Diazinon (2)	6,20	137, 153, 179, 304	14,68
Pirimiphos-methyl (3)	7,06	276, 290, 305	18,13
Malathion (4)	7,16	127, 158, 173	18,77
Fenthion (5)	7,34	79, 153, 278	-
Chlorpyrifos (6)	7,37	197, 286, 314	19,12
Parathion (7)	7,38	139, 186, 235, 291	19,22
Chlorfenvinphos (8)	7.78 // 8.01[b]	267, 295, 323	20.10 // 20.57[c]
Quinalphos (9)	8,1	118, 146, 157, 298	20,69
Ethion (PI) (10)	10,47	231, 153	23,35
Phosalone (11)	14,29	121, 154, 182, 367	26,75

Table 1 - GC parameters used for the selected insecticides
RT = retention time
(a) quantitation ions used in GC-MS are underlined; (b) and (c) peaks with retention time of 8.01 and 20.57 min corresponded to the Z isomer, the most abundant isomer.

Pesticide	GC-MS				GC-ECD			
	Range (ng/mL)	w	LOD (ng/mL)	LOQ (ng/mL)	Range (ng/mL)	w	LOD (ng/mL)	LOQ (ng/mL)
Chlorfenvinphos	50 - 5000	$1/x^2$	2,98	9,05	50 - 5000	$1/x^2$	4,53	13,72
Chlorpyrifos	50 - 5000	$1/x^2$	3,66	11,10	50 - 5000	$1/x^2$	4,50	13,64
Diazinon	50 - 5000	$1/x^2$	3,17	9,61	50 - 5000	$1/x^2$	4,52	13,69
Dimethoate	50 - 5000	$1/x^2$	3,24	9,83	50 - 5000	$1/x^2$	3,54	10,74
Fenthion	50 - 5000	$1/x^2$	4,50	13,63	-	-	-	-
Malathion	50 - 5000	$1/x^2$	4,34	13,14	50 - 5000	$1/x^2$	4,81	14,56
Parathion	50 - 5000	$1/x^2$	3,54	10,72	50 - 5000	$1/x^2$	3,52	10,66
Phosalone	50 - 5000	$1/x^2$	3,12	9,45	50 - 5000	$1/x^2$	3,39	10,26
Pirimiphos-methyl	50 - 5000	$1/x^2$	3,20	9,70	500 - 5000	$1/y^2$	35,30	106,98
Quinalphos	50 - 5000	$1/x^2$	2,46	7,46	50 - 5000	$1/x^2$	5,19	15,73

Table 2 - Data obtained in the study of the linearity
w - weighting factor.

Pesticide	100 ng/mL	4000 ng/mL
Chlorfenvinphos	80,93	90,23
Chlorpyrifos	79,08	69,26
Diazinon	86,3	92,95
Dimethoate	93,28	101,31
Fenthion	71,76	72,89
Malathion	78,29	85,96
Parathion	82,47	79,25
Phosalone	88,14	65,38
Pirimiphos-methyl	114,31	90,24
Quinalphos	79,58	82,34

Table 3 - Extraction efficiency (%) for the 10 pesticides at two different concentrations

Pesticide	r (%)		U (y) (%)	
	GC-MS	GC-ECD	GC-MS	GC-ECD
Chlorfenvinphos	21,44	23,7	16,42	17,78
Chlorpyrifos	18,31	29,28	14,72	22,64
Diazinon	27,03	25,25	21,05	19,75
Dimethoate	22,32	38,43	17,27	28,31
Fenthion	30,73	-	23,71	-
Malathion	17,63	24,83	14,22	18,71
Parathion	14,64	19,35	11,88	15,52
Phosalone	16,84	14,29	13,37	11,7
Pirimiphos-methyl	31,78	25,56	24,25	19,18
Quinalphos	16,88	13,96	13,07	12,29

Table 4 - Study of repeatibility limits and expanded uncertainty of the methods