DETERMINATION OF ORGANOPHOSPHORUS PESTICIDE RESIDUES IN THE LIVER BY GAS CHROMATOGRAPHY WITH FLAME PHOTOMETRIC DETECTION

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Abstract

A method for simultaneous determination of residues of six organophosphorus pesticides in animal liver samples has been developed and validated. The method is based on the extraction of organophosphorus pesticides with a mixture of acetone-petroleum ether, clean up on a gel permeation chromatography, further clean up on a small silica gel column, and finally determination by gas chromatography with flame photometric detection. The recovery, investigated by analysing blank liver samples spiked with chlorpyrifos-methyl, chlorpyrifos, diazinon, parathion, and pirimiphos-methyl at levels of 5, 25, 50, and 75 µg/kg, and with chlorfenvinphos at levels of 5, 10, and 15 µg/kg, ranged from 75.6% to 107.5%. Performance characteristics, such as repeatability and within-laboratory reproducibility, expressed as coefficient of variation, were less than 20%, and uncertainty <21%. Linearity ranged between 5 and 1,000 ng/mL. The limits of quantification were 5 µg/kg for all compounds and allowed determination of residues below the MRLs set by the European Union. The satisfactory z-score results of international proficiency tests confirmed good accuracy, reproducibility, and reliability of the developed method.

Key words: liver, organophosphorus pesticides, gas chromatography, food analysis.

Organophosphorus pesticides (OPPs) are still widely used in agriculture as insecticides in crop protection and in veterinary practice. These compounds are typically esters, amides, or thiol derivatives of phosphoric or phosphonic acid. They are easily hydrolysed and thus do not persist in the environment for a very long time. However, their toxicity (highly toxic or moderately toxic), with the possibility of accumulation of some of them in the food chain can cause a risk for human health. The main target organs in human are the nervous system, respiratory tract, and cardiovascular system. As a result of OPPs physical-chemical properties, and their toxicological profile, organophosphorus pesticides are to be monitored in food of animal origin, in order to control food quality and thus prevent any possible risk for human health.

To protect consumers and to ensure that OPPs are not found in food at levels presenting an unacceptable risk for human consumption, maximum residue limits (MRLs) of pesticide residues in food are laid down in Regulation (EC) 396/2005 (14). For the studied pesticides, MRLs in the liver of different species of farm animals have been established at the limit of quantification: from 0.01 mg/kg (chlorfenvinphos) to 0.05 mg/kg expressed on fat bases (chlorpyrifos, chlorpyrifos-methyl, diazinon, parathion, and pirimiphos-methyl).

Several methods for determination of OPPs residues in different samples by different separation and detection methods have been published so far, but very few of them are adapted for the analysis of this type of pesticides in the liver (6, 8, 11, 12). There have been standardised protocols for determination of pesticide residues in fatty food collected in one document, which shows many different opportunities to choose (4). Most analytical methods of the analysis of OPPs in fatty products of animal origin have been based on the extraction with organic solvent (ethyl acetate, acetone, n-hexane, acetonitrile) by homogenisation, accelerate solvent extraction (ASE), Soxhlet, partitioning in an immiscible solvent, and clean up of the crude extract, followed by determination of the residues by gas chromatography (GC) coupled with selective detector such as nitrogen-phosphorus detection (NPD), and flame photometric detection (FPD) (1, 3, 9, 10, 12, 17, 19-21). The main problem in the analysis of pesticides in fatty products is the co-extraction of lipidic material, which is incompatible with GC system. Lipids cause lower sensitivity and shorter column life. In order to remove the fatty interferences, extract of OPP residues may be purified by adsorption chromatography with Florisil®, silica, octadeyl bonded silica (C18), Extrelut NT3, or primary-secondary amine (PSA), through freezing-out step or gel permeation chromatography (GPC). Among them GPC, also called size-exclusion chromatography, is an excellent technique for quantitative separation compounds from the fatty matrices on the basis of size, as it performs a selective removal of high and medium molecular weight impurities such as lipids, pigments, and proteins from extract (7, 9, 12, 15). The main
advantage of this technique is that both polar and non-polar substances can be isolated and recovered for analysis. An expanding role of GC and liquid chromatography (LC) coupled with mass spectrometry detection (MS) or tandem mass spectrometry (MS/MS) is observed in the analysis of target pesticides in fatty food samples (2, 7, 8, 13, 16).

The aim of this study was to develop and validate an analytical method with GPC clean up for the determination of residues of the six organophosphorus pesticides by GC in animal liver samples, which fulfils the requirements of European Regulations regarding the official control of chemical residues in food of animal origin.

Material and Methods

Reagents and chemicals. Acetone, cyclohexane, ethyl acetate, petroleum ether, toluene, 2,2,4-trimethylpentane (isoctane), 95% n-hexane (Baker Ultra Arti-Resi-Analyzed) were obtained from Baker (The Netherlands). Silica gel 60 (70–230 mesh) supplied by Merck (Germany) was heated at 130°C for at least 5 h, and then deactivated with 1.5% of water before use. Anhydrous sodium sulphate supplied by POCH (Poland) was heated at 500°C for at least 5 h. Water was purified through a Milli-Q Plus system from Millipore (USA).

Standard solutions. Five certified standard solutions (10 µg/mL) were used: chlorpyrifos-methyl, 98% purity; chlorpyrifos, 98.5% purity; parathion, 99% purity; pirimiphos-methyl, 99% purity; and tributylphosphate (internal standard), 99.5% purity. All of them were purchased from Dr Ehrenstorfer (Germany). Standard substances of chlorfenvinphos and diazinon were purchased from the Institute of Industrial Organic Chemistry (Poland) and were of 98% and 99% purity, respectively. The stock (1,000 µg/mL) and intermediate (10 µg/mL) standard solutions of chlorfenvinphos or diazinon were made in cyclohexane and stored in the dark at a temperature below -18°C. The mixed working standard solutions of all pesticides were prepared by appropriate dilutions of certified standard solution and intermediate standard solutions with cyclohexane. These mixed working standard solutions with appropriate OPPs levels were used for making calibration curve, studying linearity and spiking blank liver samples, and were stable for 3 months when stored in the dark at 4°C.

Extraction. The liver samples free from pesticide residues were used as blank, to spike aliquots for recovery and precision studies. The samples were minced and deep frozen until analysis.

An accurately weighed 10 g (±0.1) sample of minced liver was placed in a centrifuge tube, thoroughly mixed with anhydrous sodium sulphate, and homogenised with 80 ml of acetone-petroleum ether mixture (2:1, v/v) for 3 min at 10,000 rpm. The sample was then centrifuged at 2,000 rpm, 2°C for 5 min. After centrifugation, the clear solvent layer was decanted through anhydrous sodium sulphate layer. To the remaining matrix, another 70 ml of acetone-petroleum ether mixture (2:1, v/v) was added and the sample was again homogenised and centrifuged under the same conditions as previously. The combined supernatants were concentrated to small volume by rotary evaporator (water bath at 35°C; reduced pressure) and evaporated to near dryness under a gentle stream of nitrogen.

Cleanup by gel permeation chromatography (GPC). The O I Analytical automated system GPC AutoPrep 2000 with UV detector was used. Chromatographic separation was achieved on a glass column packed with 60g of Bio Beads SX3, 200-400 mesh (Bio-Rad, USA). The concentrated extract with residues of organophosphorus pesticides was carefully transferred with cyclohexane-ethyl acetate mixture (1:1, v/v) to a 10 ml volumetric flask and mixed well. The sample solution was filtered through syringe filter (0.45 µm pore size, PTFE, 25 mm). The filtered extract was injected into the GPC column for clean up using the following conditions: mobile phase, cyclohexane-ethyl acetate (1:1, v/v); flow rate, 5 mL/min; detection wavelength, 254 nm; injection volume, 5 ml; dump time, 21 min; collect time, 11 min. The first fraction of the eluant (105 ml) containing lipids was discarded. The second fraction (55 ml) containing pesticides was collected and concentrated to a small volume by rotary evaporator.

Cleanup by column chromatography on a small silica-gel column. The concentrated extract from GPC in isooctane was carefully transferred onto the column containing 1 g of silica gel (deactivated with 1.5% water) with a layer of anhydrous sodium sulphate, rinsed beforehand with 10 ml of n-hexane. The pesticides were then eluted with 10 ml mixture of toluene-acetone (80:20, v/v) and the eluate was evaporated to near dryness on a rotary evaporator (water bath at 40°C; reduced pressure). The final extract was evaporated to dryness under a gentle stream of nitrogen and then dissolved in 0.5 µl of cyclohexane for GC analysis.

Gas chromatography. An Agilent Technologies gas chromatograph model 6890 Plus, equipped with 7683B series autosampler, split/splitless injector, and flame photometric detector was used. Chromatographic separation was achieved on a DB 1701 capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness, J&W Scientific, USA). The following oven temperature programme was used: initial temperature of 90°C hold for 0.75 min, increase to 180°C at 40°C/min and hold for 9 min, increase to 220°C at 35°C/min and hold 5 min, increase to 235°C at 90°C/min and hold for 3.9 min, and then increase to 275°C at 120°C/min and hold for 20.4 min. The injector and detector were operated at 260°C and 250°C, respectively. The carrier gas was helium with flow rate of 0.7 mL/min. The injection volume was 3 µl in the pulsed splitless mode. The GC was controlled by ChemStation software.

Validation. Blank liver samples spiked with OPPs at the levels corresponding to 0.5, 1 and 1.5 MRLs were used for evaluation of recovery and precision (repeatability and within-laboratory reproducibility). Spiked samples at each concentration level were analysed in three series, each on the different day, and
each in six replicates. The accuracy determined by average recovery was calculated by comparing the determined concentrations of spiked samples to their target level. The precision was determined by calculating the coefficient of variation (CV). The linearity was checked using mixed standard solution and analysing each of them in duplicate at seven concentrations between 5 and 1,000 µg/L. The limit of quantification (LOQ) of OPPs was tested as the minimum concentration of the analyte that can be quantified with acceptable accuracy and precision. The limit of detection (LOD) was established as the half of the LOQ. The uncertainty was estimated in accordance with the recommendations of Eurachem/CITAC Guide (5).

Results

Typical chromatograms obtained from the standard solution of OPPs, extract of blank porcine liver, and spiked liver extract are shown in Fig. 1. For the applied analytical procedure and chromatographic conditions of the separation, no interference peaks of other groups of pesticides, like organochlorine pesticides and pyrethroids, or matrix components were observed in the retention times of the analytes.

To confirm that the developed method is suitable for its intended use, and to fulfil the aim of this study, the validation process was carried out. All validation results obtained for OPPs determinations in spiked liver samples are listed in Table 1. The quantification of OPPs was obtained using calibration curve and tributylphosphate as an internal standard. Estimated validation parameters of the method were satisfactory. The accuracy of the method was expressed as mean recoveries, and they were higher than 70% for all spiked levels and all OPPs. Repeatability of measurements and within-laboratory reproducibility, expressed as coefficients of variation (CV) were lower than 9.6% and 18.4%, respectively. Regression curve for all pesticides showed good linearity with regression coefficients ($R^2$) values of at least 0.999.

Fig. 1. GC-FPD chromatograms: A – standard solution of OPPs at 100 µg/L (except chlorfenvinphos at 30 µg/L), B – blank liver extract, C – liver spiked with OPPs at the level of 5 µg/kg.
Table 1
Validation parameters obtained for liver matrix

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Linearity</th>
<th>Spike level (µg/kg)</th>
<th>Recovery % (n=18)</th>
<th>Repeatability y CV, % (n=6)</th>
<th>Within-laboratory reproducibility CV, % (n=18)</th>
<th>Uncertainty y (%)</th>
</tr>
</thead>
</table>
| Chlorpyrifos-methyl | $y = 3.95x + 5.16$  
$R^2 = 1.000$ | 25                  | 83.7              | 9.3                         | 8.8                                           | 20.1             |
|                    |            | 50                  | 95.0              | 9.1                         | 7.2                                           |                  |
|                    |            | 75                  | 105.0             | 3.0                         | 4.6                                           |                  |
| Chlorpyrifos        | $y = 3.75x + 18.88$  
$R^2 = 0.999$ | 25                  | 75.6              | 9.1                         | 9.5                                           | 19.5             |
|                    |            | 50                  | 90.2              | 9.0                         | 7.2                                           |                  |
|                    |            | 75                  | 99.0              | 5.1                         | 3.9                                           |                  |
| Diazinon            | $y = 3.59x + 4.32$  
$R^2 = 0.999$ | 25                  | 82.0              | 9.6                         | 6.3                                           | 20.1             |
|                    |            | 50                  | 90.7              | 8.8                         | 7.3                                           |                  |
|                    |            | 75                  | 92.6              | 6.2                         | 9.6                                           |                  |
| Parathion           | $y = 4.47x + 20.19$  
$R^2 = 0.999$ | 25                  | 99.5              | 6.2                         | 10.3                                          | 16.5             |
|                    |            | 50                  | 94.9              | 6.2                         | 6.2                                           |                  |
|                    |            | 75                  | 92.5              | 7.2                         | 4.8                                           |                  |
| Pirimiphos-methyl   | $y = 3.91x + 14.29$  
$R^2 = 0.999$ | 25                  | 75.9              | 8.9                         | 7.7                                           | 20.8             |
|                    |            | 50                  | 90.2              | 8.9                         | 7.7                                           |                  |
|                    |            | 75                  | 95.3              | 6.5                         | 7.4                                           |                  |
| Chlorfenvinphos     | $y = 5.38x - 33.78$  
$R^2 = 0.999$ | 5                   | 81.9              | 8.3                         | 18.4                                          |                  |
|                    |            | 10                  | 105.4             | 8.4                         | 7.1                                           | 17.6             |
|                    |            | 15                  | 100.7             | 4.5                         | 7.0                                           |                  |

Table 2
Validation parameters obtained for liver matrix spiked at the LOQ level

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>MRL (µg/kg)</th>
<th>LOQ (µg/kg)</th>
<th>Recovery, % (n=20)</th>
<th>Repeatability, CV, % (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos-methyl</td>
<td>50</td>
<td>5</td>
<td>102.0</td>
<td>17.5</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>50</td>
<td>5</td>
<td>90.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Diazinon</td>
<td>50</td>
<td>5</td>
<td>81.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Parathion</td>
<td>50</td>
<td>5</td>
<td>107.5</td>
<td>10.1</td>
</tr>
<tr>
<td>Pirimiphos-methyl</td>
<td>50</td>
<td>5</td>
<td>85.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Chlorfenvinphos</td>
<td>10</td>
<td>5</td>
<td>78.2</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Fig. 2. GC-FPD overlaid chromatograms of the liver spiked with OPPs at 50 µg/kg (chlorfenvinphos at 10 µg/kg): A – two step cleanup (GPC and silica gel column), B – one step cleanup (GPC only).
The parameters expressed accuracy and precision obtained for liver samples at the LOQ level of 5 µg/kg are listed in Table 2. The LOD was established as 2.5 µg/kg. The LOD and LOQ values are considerably lower than the MRLs.

Discussion

The presented method allowed for simultaneous determination of six organophosphorus pesticides in animal liver by GC-FPD. Matched chromatographic conditions allowed the separation of all pesticides determined by this method. Using a capillary column with a low/midpolarity (14%-cyanopropyl-phenyl)-methylpolysiloxane stationary phase was appropriate. Some interferences from the sample matrix on the typical chromatogram of liver extract were noted, but they did not cover with any of the analyte. The only maintenance performed on the system was to change the injection liner and septum after a large number of injections. When performance was poor, the peak shape changed noticeably. The analytes, which eluted first from the column, diminished their peak height. This phenomenon is called “matrix-induced chromatographic response enhancement” and is caused by interactions between matrix components and active sites in the injector. The presence of coextracted sample impurities may block the active sites inside the injection inlet of the GC leading to an increase in the amount of the analyte being transferred to the column (20). Matrix enhancement is a phenomenon commonly encountered in GC analysis of pesticides in food.

During the initial stage we tested three different mixtures of different polarity organic solvents: A – cyclohexane-ethyl acetate (1:1), which was chosen by Pang et al. (13) for their multiresidue study, B – ethyl acetate-acetone (3:2), which is the combination of solvents typically used in pesticide residue analysis, and C – acetone-petroleum ether (2:1), which was a modification of n-hexane-acetone mixture (2:1) proposed for the extraction of pesticides from meat samples by official document (4). For that, 10 g of blank liver samples were spiked with chlorfenvinphos at the level of 10 µg/kg and with the rest investigated OPPs at 50 µg/kg. Extracts with appropriate dilutions were analysed on GC. At this step, the extraction with the mixture A gave the lowest level of matrix interferences. Further, GPC clean up was done, and after this one step clean up, extraction with mixture C gave visually the most purified extract. In consequence, acetone-petroleum ether mixture (2:1) was chosen as the most appropriate for the analysis of OPPs in lipophilic tissue. In our laboratory we have much experience with the use of this mixture for extraction of pesticides from fatty matrices. In the latest proficiency tests organised in 2010 and 2011 by EURL for Pesticides in Food of Animal Origin, CVUA, Freiburg, Germany, the proper extraction of fat from pork and poultry meat samples were also evaluated, and the extraction with acetone-petroleum ether mixture (2:1) gave very good efficiency.

Navas Diaz et al. (11) used GPC column packed with 10 g of Bio-Beads SX3 with mobile phase hexane-chloroform-acetone (75:20:5, v/v) supplied by gravity to clean up crude extracts in determination of two OPPs in liver sample, but GPC was not automatic. Sannino et al. (19) also used GPC for clean up of OPPs in fatty processed foods, but methylene dichloro-cyclohexane mixture (15:85, v/v) was used as eluant in semiautomatic purification process. Garrido Frenich et al. (8) compared two methods for analysing OPPs in liver tissue: homogenisation of liver samples followed by purification at two on-line connected Envirogel GC columns, and matrix solid phase dispersion (MSPD) extraction with C18 sorbent combined with Florisil clean up and ethyl acetate elution. Both tested methods used gas chromatography with electron impact ionisation tandem mass spectrometry GC-(EI) MS/MS for OPPs determination. Because of poor recoveries for more polar pesticides due to non-polar nature of the C18 sorbent used for extraction, five among 13 studied OPPs gave recoveries below 30%, the GPC method was finally selected.

The GPC clean up gave an excellent efficiency for the lipid removal, even though some small amounts of coextracted impurities still remained in the fraction with pesticides. Therefore, further clean up steps are often necessary because these small amounts of macromolecules would interfere with the subsequent GC analysis. The complex samples require a two-step clean up, which combines different chromatographic techniques in the series. In the second step, extracts are separated on silica-gel by polarity. The combination of these two techniques results in a powerful two-dimensional clean up by molecular size and polarity (15). Chromatograms of liver extracts obtained with one or two-step clean up procedures are shown in Fig. 2. Due to the nature of animal origin samples, especially the presence of fat, selection of the appropriate technique of preconcentration and purification of the analytes, and its optimisation is the most laborious part of sample preparation, but constitutes a very important aspect of the analysis.

The validation was performed with the recommendations of the document SANCO (18). This guidance document describes the method of validation and analytical quality control requirements to support the validity of data used for checking compliance with maximum residue limits, enforcement actions, or assessment of consumer exposure to pesticides in the EU. This document is complementary and integral to the requirements in ISO/IEC 17025. Validation parameters obtained for liver matrix demonstrate that the developed analytical method meet the method performance acceptability criteria (mean recoveries in the range 70%–120%, precision with CV ≤ 20%, LOQ ≤ MRL).

For quality assurance and quality control, our laboratory participates regularly in proficiency tests. The proficiency test is one of the most important elements of laboratory quality assurance. Participation in these tests allows comparison of the analytical results with the results from other laboratories. Our laboratory (accredited according to ISO 17025) successfully
participated in proficiency tests organised by FAPAS (Food Analysis Performance Assessment Scheme), The Food and Environmental Research Agency, UK (2010, test 0573) and by The European Union Reference Laboratory for Pesticides in Food of Animal Origin, CVUA, Freiburg, Germany (2006 – 2011, EUPT AO 01 – 06, pesticides in fatty foods). These satisfactory z-score results confirm good accuracy, reproducibility, and reliability of the developed method.

The method for the determination of residues of six organophosphorus pesticides in animal liver samples proved to be reliable, accurate, and precise, and the results of validation and verification of the method show its usefulness. The efficiency of the automated GPC clean up combined with selective detection by GC-FPD made this method very sensitive and suitable for use.

References

4. EN 1528 Fatty food - Determination of pesticides and polychlorinated biphenyls (PCBs) – part 1-4, 1996.