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A single method for detecting 11 organophosphate pesticides in human plasma and breastmilk using GC-FPD

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Abstract

Organophosphate (OP) pesticides are widely used for crop protection in many countries including Thailand. Aside from causing environmental contamination, they affect human health especially by over-stimulating of the neurotransmission system. OP pesticides, as with other non-persistent pesticides, degrade quickly in the environment as well as are metabolized quite rapidly in humans. Assessing human exposures to these compounds requires analytical methods that are sensitive, robust, and most importantly, suitable for specific laboratory settings. The aim of this study was to develop and validate an analytical method for measuring 11 OP pesticide residues in human plasma and breast milk. Analytes in both plasma and breast milk samples were extracted with acetone and methylene chloride, cleaned-up using aminopropyl solid phase extraction cartridges, and analyzed by gas chromatography with flame photometric detection. The optimized method exhibited good linearity, with the coefficients of determination of 0.996–0.999 and <7% error about the slope. Extraction recoveries from spiked plasma and breast milk samples at low and medium concentrations (0.8–5.0 and 1.6–10 ng mL⁻¹, respectively) ranged from 59.4 % (ethion) to 94.0 % (chlorpyrifos). Intra-batch and inter-batch precisions ranged from 2.3–18.9% and 5.8–19.5%, respectively. Method detection limits of plasma and breast milk ranged from 0.18–1.36 and 0.09–2.66 ng mL⁻¹, respectively. We analyzed 63 plasma and 30 breastmilk samples collected from farmworkers in Chiang Mai Province to determine the suitability of this method for occupational exposure assessment. Of the 11 pesticides measured, seven were detected in plasma

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samples and five were detected in breast milk samples. Mass spectrometry was used to confirm results. Overall, this method is rapid and reliable. It offers the laboratories with limited access to mass spectrometry a capacity to investigate levels OP pesticides in plasma and breastmilk in those occupationally exposed for health risk assessment.

Keywords

organophosphate pesticides; plasma; breast milk; GC-FPD

1. Introduction

Organophosphate (OP) pesticides are widely used for crop protection in agriculture worldwide. Exposure to OP pesticides is a matter of public concern [1,2]. Many studies reported that long-term occupational exposure to OP pesticides was associated with adverse health effects. For example, among OP pesticide applicators, their exposures were associated with signs of impaired nervous system function [3], cognitive disorders [4], changes in sex and thyroid hormone profiles [5–7], development of lung [8,9] and breast cancers [10], development of leukemia [9] and shorter relative leukocyte telomere length [11].

Some studies suggest that prenatal and postnatal exposure to OP pesticides affect the neurological development of children [12–15]. Prenatal exposure to OP pesticides was associated with impaired mental and psychomotor development, attention deficit hyperactivity disorder and pervasive development disorder [12]. Prenatal exposure to OP pesticides was also linked to adverse birth outcomes such as shorter gestation length, lower birth weight, and smaller head circumference [13–15]. In addition, early childhood exposures have been associated with similar neurodevelopment deficits such as abnormal reflex and lower motor skills [12,16].

Following exposure, OP pesticide residues have been found in human blood, plasma and serum [17], thus, the fetus can be exposed to these pesticides from maternal blood. Animal studies have documented that some OP pesticides can cross the placental barrier [18] and human studies have detected OP pesticides in umbilical cord blood and post-partum meconium [19–23]. Some OP pesticides such as chlorpyrifos, dimethoate, ethion, parathion methyl, and triazophos have low water solubility (with $\log K_{ow} < 1$). These pesticides have the potential to deposit in adipose tissues including breast milk [24,25]. Infants can be postnatally exposed to OP pesticides via breastfeeding. Timing of exposure during pre- and post-natal development is a critical factor determining the occurrence and severity of health outcomes. Thus, the ability to measure exposure levels in the maternal-fetal compartment is important and can be achieved through the development of suitable analytical methods.

In human body, OP pesticides are rapidly metabolized, and stored in adipose tissue [26]. Thion form of OP pesticides can be enzymatically converted to their oxon form, which can inhibit cholinesterase, or else, can be hydrolyzed to form metabolites which are excreted in urine [27]. For example, chlorpyrifos in blood and adipose tissue is excreted in urine within several hours to a few days [26,27]. Analysis of OP pesticide levels in human plasma provides more accurate dose in target tissue rather than metabolites in urine which is the end

of the metabolic pathway [28]. Because OP pesticides have relatively short half-lives leading to their presence in trace levels in humans, using extremely sensitive analytical methods for the quantification is recommended [29]. In most agricultural, developing countries including Thailand, native OP pesticide levels in human biological samples have never been reported while the high amount of OP use were reported [30]. Occupationally-exposed populations, especially agricultural workers, assumed to have relatively high internal dose of native OP pesticides which led to multiple health risks [31–35].

In Chiang Mai Province, Thailand, several OP pesticides have been detected in the environment including fruits and vegetables [36] regularly consumed by the locals. As a result, their metabolites including dialkylphosphates, are commonly detected in human urine with the geometric mean concentrations higher than those of the general U.S. population [37–39]. In 2011, OP pesticides were responsible for the highest number of known-pesticide poisoning cases in the upper region of northern Thailand [40]. The high use of OP pesticides, their environmental-wide contamination, and a large number of poisoning cases highlighted an urgent need for analytical methods capable of detecting OP pesticides in human biological samples to be developed.

Here we developed and validated a low-cost, an alternative method for the simultaneous determination of 11 OP pesticides in plasma and breast milk by using gas chromatography and flame photometric detection (GC-FPD). We applied this method to analyze samples collected from 63 breastfeeding farmworkers and their spouses who used pesticides regularly in Northern Thailand. This is “fit-for-purpose” method that provides reliable results for a given purpose but is the least costly is likely the best method to use. It is a more accessible method for those in low income countries without the resources to purchase mass spectrometers that still need capacity to analyze pesticides in an occupationally exposed population.

2. Materials and methods

2.1 Analytical standards, reagents, and materials

Chlorpyrifos, diazinon, dicrotophos, dimethoate, ethion, malathion, parathion-methyl, pirimiphos-ethyl, profenofos, prothiophos triazophos and triphenylphosphate [TPP]; used as an internal standard (ISTD)] were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Isotopically labeled internal standards for analysis using gas chromatography-tandem mass spectrometry (GC-MS/MS) were purchased from Cambridge Isotope Laboratories (MA, USA). Acetone, dichloromethane, ethyl acetate, methanol and toluene were HPLC Grade and purchased from JT Baker (PA, USA). Hexane was also HPLC Grade and purchased from Labscan (Bangkok, Thailand). Sodium sulfate was purchased from Merck (Damstadt, Germany). Ultrapure water was generated in house using an ELGA UHQ PSII system (Bucks, UK).

The 3-mL aminopropyl, an anion exchanger, solid phase extraction cartridges (NH₂-SPE) were obtained from Vertical (Nonthaburi, Thailand). The 6-mL C₁₈ SPE cartridges were from Phenomenex (CA, USA).

2.2 Biological materials

Samples used for validating the method were fetal bovine serum (Gibco, USA) and commercial cow's pasteurized milk with 3% fat (Dutchmill, Thailand). Plasma samples from the studied subjects were pooled together (2 ml from each individual sample) to create a pooled plasma sample that was later used for storage stability testing. Breast milk samples were also pooled together (5 ml from each individual sample) for the same purpose. Information regarding subject recruitment, biological sample collection, and preparation is provided in section 2.7.5 and 2.8.

2.3 Preparation of stock solutions, standard mixtures, and quality control solutions

Each individual stock solution of 11 OP pesticides was prepared separately by dissolving approximately about 10 mg of each standard into 10 mL acetone using a 10-mL volumetric flask to create a solution with $\sim 1 \text{ mg mL}^{-1}$ concentration. After that, they were used to prepare intermediate solutions by serial dilution with ethyl acetate to yield a final concentration of $50 \text{ } \mu\text{g mL}^{-1}$ for all standard solutions. Then, the intermediate solutions were used to prepare a mixture solution ($1 \text{ } \mu\text{g mL}^{-1}$) in ethyl acetate. This mixture, including the TPP ISTD, was injected into the GC-FPD in order to determine retention times of the target analytes and to adjust for optimum chromatographic condition.

Prior to preparing the calibration solutions, individual intermediate solutions ($50 \text{ } \mu\text{g mL}^{-1}$) were diluted with ethyl acetate into a set of solutions covering a concentration range of 120–1,000 ng mL^{-1} . With varying amounts injected, the sensitivity of each analyte across different levels was carefully assessed. The calibration range was then selected for each analyte. The calibration mixture was subsequently prepared so that spiking different volumes into the working biological matrices would result in calibrants having the appropriate calibration range (ethion: 0.3–3.0 ng mL^{-1} , diazinon and primiphos ethyl: 0.4–4.0 ng mL^{-1} , prothiophos: 0.5–5.0 ng mL^{-1} , chlorpyrifos and parathion methyl: 0.8–8.0 ng mL^{-1} , malathion 1.5–15 ng mL^{-1} , dimethoate and profenophos 2.0–20 ng mL^{-1} and dicrothphos and triazophos 2.5–25 ng mL^{-1}).

Quality control (QC) solutions, containing low and medium levels of the target analytes were prepared in the same manner as the calibration solutions. The specific concentrations of the QC samples can be seen in Table 3. All standard solutions were kept at $-20 \text{ }^{\circ}\text{C}$ until use.

2.4 Preparation of calibration standards and quality control samples

This method employed matrix-matched calibration. The 7-point calibration curves were derived from spiking 5, 10, 15, 20, 25, 50, 100 μL of the calibration mixture and 20 μL of $1 \text{ } \mu\text{g mL}^{-1}$ TPP ISTD into 2 mL of working matrices (commercial bovine serum or cow milk). The calibrants were then extracted and cleaned up using the same procedure as the samples. Following evaporation to dryness, they were reconstituted with 100 μL of ethyl acetate before injection. One microliter of these calibrants was injected into GC-FPD.

QC samples at two concentration levels (low and medium) were prepared by spiking the quality control solutions into 2 mL of either commercial bovine serum or cow milk. They were processed concurrently with the samples using the same procedure.

2.5 Sample preparation

Two milliliters of plasma or milk were spiked with 20 μL of 1 $\mu\text{g mL}^{-1}$ ISTD then 5 mL of acetone was added and the mixture was vortex mixed for 5 min. According to previous reports, freezing-lipid precipitation has been an effective lipid-removal technique in analysis of biological samples (with approximately 70–90% removal efficiency) [41–44]. Therefore, to precipitate out proteins and lipid, the samples were put into a $-20\text{ }^{\circ}\text{C}$ freezer for 30 min. After that, the cold, cloudy samples were immediately centrifuged at 1200 $\times g$ for 3 min and quickly filtered through Whatman No.1 paper filter. For this step, refrigerated centrifugation should be used to process a large number of samples. In addition, the samples should be kept in the ice bath while undergoing filtration.

After that the filtrates were collected and vortex mixed with 5 mL dichloromethane (DCM) briefly (~ 10 seconds). The mixtures were centrifuged again at 800 $\times g$ for 3 min in order to aid the removal of the upper aqueous layer and lipid-precipitated residue at the interface. Then, for each sample, the bottom organic layer (acetone-DCM layer) was loaded onto an NH_2 -SPE cartridge that had been conditioned with 3 mL of deionized water and 3 mL of DCM. The eluate was collected immediately in the glass tube containing 1 g of anhydrous sodium sulfate. The cartridge was eluted again with 1 mL of DCM. The eluates were dried under a gentle nitrogen gas stream and the extracts were re-dissolved in 100 μL of ethyl acetate. One microliter was injected into GC-FPD. The remainder of the extract was kept in a $-20\text{ }^{\circ}\text{C}$ freezer until GC-MS/MS analysis.

2.6 GC-FPD and GC-MS/MS conditions

GC-FPD—A Hewlett Packard 6890 GC-FPD (Agilent Technologies, CA, USA) was used for analysis of 11 OP pesticides. The analytical column was an Agilent HP-5 (30 m $\times$ 0.25 mm. id, 0.25 μm film thickness). The oven temperature program was: 100 $^{\circ}\text{C}$ starting temperature, then raised at a rate of 25 $^{\circ}\text{C min}^{-1}$ to 180 $^{\circ}\text{C}$, then raised at a rate of 4 $^{\circ}\text{C min}^{-1}$ to 200 $^{\circ}\text{C}$, and finally raised again at a rate of 10 $^{\circ}\text{C min}^{-1}$ to 250 $^{\circ}\text{C}$. The post-run temperature was set at 290 $^{\circ}\text{C}$ for 2 min. Detector temperature was set at 250 $^{\circ}\text{C}$. Helium (99.99% purity) was used as a carrier gas. The inlet flow rate was set at 1.0 mL min^{-1} . The injection was set in a splitless mode and the injected volume was 1 μL . The total run time was 16.20 min.

GC-MS/MS monitored in multiple-reaction monitoring (MRM) (for confirmation)—An Agilent 7890A GC (Agilent Technologies, CA, USA), equipped with a 5975C quadrupole MS (GC-MS/MS; Agilent Technologies, CA, USA) was used to confirmed the results obtained by GC-FPD. The system was equipped with a split/splitless injector and a 7693 autosampler (Agilent Technologies, CA, USA). The analytical column was the same as for GC-FPD. A multi-stepwise-gradient temperature program was applied to achieve an optimum resolution, using the following setup: 90 $^{\circ}\text{C}$ held for 0.1 min, then raised at rate of 25 $^{\circ}\text{C min}^{-1}$ to 200 $^{\circ}\text{C}$ and held for 1 min, then raised at a rate of 40 $^{\circ}\text{C min}^{-1}$ to 250 $^{\circ}\text{C}$ and held for 1 min.

min⁻¹ to 220 °C and held for 8 min, then raised at a rate of 50 °C min⁻¹ to 310 °C and held for 5 min. The post-run temperature was set at 100 °C for 2 min. The front inlet temperature was set at 250 °C; the inlet pressure was set at 20 psi. Helium (99.99% purity) was used as a carrier gas. The splitless injection volume was 1 µL. The total run time was 22.8 min. Mass spectrometric parameters were set as follows: 300 °C auxiliary temperature, 70 eV electron impact energy, 1447 V electron multiplier, 230 °C MS source temperature, and 150 °C MS quadrupole temperature. Quantification and confirmation ions were monitored using a multiple reaction monitoring (MRM) mode for each native pesticide, while only quantification ions were monitored for the isotopically labeled internal standards. Masses for each ion monitored for analysis are shown in Table 1. Noteworthy, the GC-MS/MS method was set to confirm only 9 OP pesticides. Dicrotophos and dimethoate were not included due to lack of neat standard materials.

2.7 Method validation

2.7.1 Limits of detection (LODs)—For each compound, the LOD was estimated based on the method published by Taylor [45] using the following equation $LOD = 3SD/\text{slope}$; where SD was the estimated uncertainty (standard deviation) of the responses (slopes of regression lines) and the slope was from calibration curve [46].

2.7.2 Extraction recovery—Commercially available bovine serum and milk samples were used to evaluate extraction recoveries. Prior to extraction, the first group of samples (consisting of 3 replicates of 2 mL working matrix) was spiked with target analytes at the concentrations equivalent to low levels of QC samples; while the second group was not spiked. Both groups were extracted as previously described. The samples of the second group were spiked after extraction to yield the same concentrations as in the first group. All samples were analyzed using GC-FPD. The absolute and relative recoveries were determined using the peak ratios of pre-spiked samples to post-spiked samples. The absolute and relative recoveries were calculated by comparing peak areas and concentrations, respectively, obtained from samples spiked with standards before extraction to samples spiked with standards after extraction which represented 100% recovery. This procedure was repeated again using concentrations equivalent to medium levels of QC samples.

2.7.3 Accuracy—Accuracy was determined from the results of samples spiked with all analytes at concentrations equivalent to low and medium levels (level 2 and 4) of QC samples. Relative recoveries were calculated by comparing the concentrations of the extracted samples with those from the calibration curves. The acceptable recovery for the concentration in ng mL⁻¹ is in the range of 70–125% [47].

2.7.4 Precision—Precision of the method was evaluated using replicates of QC samples (low and medium levels) prepared and analyzed in different analytical batches. For intra-batch precision, 20 replicates of QC samples were analyzed for each level. The inter-batch precision was evaluated using a total of 10 replicates of quality control samples derived from 5 analytical batches (5 duplicates from 5 analytical runs). Precision of the method was expressed as relative standard deviations (%RSD).

2.7.5 Storage stability—The storage stability was determined by using spiked samples. Pooled plasma and breast milk samples (as defined in section 2.2) were spiked with all analytes to yield two different levels (equivalent to low and medium levels of QC samples) and, then, mixed well by stirring on an ice bath for 1 hour to ensure homogeneous distribution of the target pesticides in the samples. The samples were analyzed immediately and the results represented a day-0 storage period. The rest of the samples were aliquot (2 mL each) and stored at -20°C for 6 months. During which time, each set of these samples (consisting of 3 replicates per one spiked level) was taken out of the freezer, prepared, and analyzed every day for 7 days. Then, they were taken out, prepared, and analyzed again at day 14th, 30th, 60th, 90th, 120th, and 180th, respectively. The recoveries were used to evaluate the storage stability of target analytes.

2.8 Method application to human samples

This method was tested for its suitability by analyzing the 11 OP pesticides in 63 plasma and 30 breast milk samples that were provided by 33 breastfeeding farmworkers and 30 spouses. The participants lived and worked in an agricultural community in Fang District, Chiang Mai Province, Thailand, where OP pesticides have been used intensively. In this area, several OP pesticides have been detected in the environment including in fruits and vegetables [36] that were locally consumed. Several reports documented high exposure levels of OP pesticides among different populations of this region [37–39]. Mean (min-max) age of the subjects was 26.8 (18–50) years. Based on the questionnaire data, 30 (48%) subjects reported that their farms were recently applied pesticide in less than 1 week. Subjects living on the farm, within 1 km from the farm, and more than 1 km from the farm were 32%, 22% and 46%, respectively. Participant recruitment and biological sample collection were done during September 2013. An interview questionnaire was administered to collect demographics and work-associated information. This study protocol was approved by the Office of Research Ethics, Research Institute for Health Sciences, Chiang Mai University (Ref.no. 54/2012).

2.9 Mass spectrometric confirmation

Extracted plasma and breast milk samples were re-analyzed using GC-MS/MS at the Laboratory of Exposure Assessment and Development in Environmental Research (LEADER) within the Rollins School of Public Health and the HERCULES Exposome Research Center's Analytic Core of Emory University. A subset of plasma samples (n=15) was randomly selected and analyzed using GC-MS/MS as described above.

3. Results and discussions

3.1 Optimization of the extraction procedure and GC-FPD

Several organic solvents were used during the extraction of OP pesticides from human plasma and breast milk. Acetone was used to precipitate protein in plasma and breast milk. From previous analytical methods dealing with lipid-rich matrices [43,48–50], samples should be frozen at either -20°C or -70°C for various time ranges for additional precipitation of macromolecules including lipids. Therefore, after initial treatment with acetone, the samples were stored at -20°C for 30 minutes. The cold samples were

immediately centrifuged to separate the extract from the precipitate. Previously, freezing lipid precipitation (FLP) has been used in many methods using high lipid matrices [41–44]. These methods eliminated lipids by freezing at about -20°C . The lipid content was found to be able to reduce more than 70 % of the total lipid content. These methods also provided good recovery of both persistent compounds (e.g. organochlorines) and semi-persistent compounds (e.g. organophosphates). Hence, FLP was applied for removing lipids in human plasma and breast milk in our method.

In our method, after freezing the samples for 30 min, centrifugation was immediately done for only 3 min. After which time, they were still cold. We added more references to the text and provided more precise detail on this FLP procedure in section 3.1 Optimization of the extraction procedure. We also recommend that the samples should be centrifuged using a refrigerated centrifuge and placed in an ice bath while undergoing paper filtration.

After the removal of most proteins and lipids, several solvents were tested for further extraction. For instance, dichloromethane, ethyl acetate, and a mixture of these two solvents (1:1 v/v) were used. Dichloromethane was chosen mainly because of its low boiling point which resulted in a shorter evaporation time compared to other organic solvents.

After liquid-liquid extraction, the extractants were further cleaned up using an NH_2 -SPE cartridge. The initial results showed that the extraction recoveries of 11 OP pesticides from both serum and milk using C_{18} was lower than those using NH_2 -cartridges (Fig. 2). Therefore, the final protocol consisted of a cleanup process using the NH_2 -SPE cartridge. The total time spent for this extraction procedure was 2 hours for a maximum of 24 samples.

It must be noted here that use of TPP as an ISTD should be cautioned. TPP is one of the most prominent organophosphate flame retardant and was detected in human biological samples [51]. Screening of pooled sample(s) collected from a target population is recommended in order to investigate the presence of TPP. Our preliminary results indicated no detection of TPP in either pooled plasma or breast milk samples. Therefore, using TPP as an ISTD standard was acceptable for this method.

3.2 Linearity and LODs

Calibration curves of 11 OP pesticides in plasma and milk exhibited good linearity with regression coefficient (R^2) values between 0.998–0.999 and 0.995–0.999, respectively (shown in Table 3). The quantification ranges of 11 OP pesticides were different depending on their individual response to the detector. LODs in serum and milk samples were from 0.18 (chlorpyrifos) to 1.36 (dicotophos) ng mL^{-1} and 0.09 (parathion methyl) to 2.66 (dicotophos) ng mL^{-1} , respectively.

In this work, our primary focus was chlorpyrifos because it was the most frequently detected OP pesticide in agricultural areas in Chiang Mai Province [39] and it currently ranks first among the imported OP pesticides (comprising 85% of total imported OP pesticides) in Thailand [52]. Thus, the analytic parameters were optimized to achieve the lowest possible LOD for this compound in each biological matrix. From the results of this study, LODs of chlorpyrifos in serum and milk were 0.18 and 0.22 ng mL^{-1} , respectively.

3.3 Extraction efficiency

For serum, the extraction recoveries were in the range of 61–94 % and 61–95% at low and medium QC levels, respectively. For milk, the extraction recoveries were in the range of 60–90 % and 61–95% at low and medium QC levels, respectively. Chlorpyrifos had extraction recoveries above 75% in both matrices.

3.4 Precision and accuracy

Precision and accuracy results were shown in Table 4. Replicates of QC samples, fortified at two concentrations (low and medium) were analyzed to determine intra-batch precision (20 replicates) and inter-batch precision (10 replicates; 5 pairs from 5 analytical runs). The intra-batch precisions of 11 OP pesticides in serum and breast milk (%RSD) were between 2.3–12% and 3.9–17%, respectively. Their inter-batch precisions for serum and breast milk were 7.8–19% and 5.8–19 %, respectively. These precisions were all below 20%.

The method accuracies (relative recoveries) for the majority of analytes in serum and milk samples were between 95.2–125% which is considered acceptable according to AOAC guidelines (acceptable range 70–125%) [47]. Analytes with relative recoveries outside the acceptable range were dimethoate, parathion-methyl and profenophos. These compounds were more difficult to extract compared to the others. Thus, they should be deemed semi-quantitative.

3.5 Storage stability

The results of the stability tests from spiked, pooled human plasma and breastmilk stored at –20°C for 180 days are shown in Fig. 4. For any compounds measured, the acceptable stability should be within 70–125% of the originally measured value (pre-storage concentration). In plasma samples, 9 pesticides that included dicotophos, dimethoate, parathion methyl, malathion, chlorpyrifos, pirimiphos ethyl, prothiophos, profenophos and triazophos were found to be relatively stable under these storage conditions. The recoveries of diazinon and ethion, however, were below 70% at day 1 and day 3, respectively. In breast milk samples, the recovery of ethion was below 70% at day 3 and the recovery of profenophos was out of range at day 60. The others were considered stable for 180 days. In previous studies, OP pesticides were tested for their stability and the results varied by matrices and storage conditions [53–56]. These results point to the importance of having biological samples analyzed as quickly as possible upon collection.

3.6 Application to human samples

To evaluate the method performance, 63 plasma and 30 breast milk samples from breastfeeding female farmworkers (n=33) and their spouses (n=30) in an agricultural community of Fang District, Chiang Mai Province, Thailand were analyzed. The analysis results are shown in Table 5.

Detection of target analyte in the samples was determined using the following criteria: 1) the retention time of the detected peak is matched with the one detected in the calibrants or QC samples (within ± 0.2 min range); 2) the signal-to-noise ratio of the detected peak is ≥ 3 ; 3) the detected peak should be of good analytical quality. If a peak of a target analyte was

detected and met the above criteria but its calculated concentration was below the LOD; it was reported as “detected but not quantified” (refers to as DNQ). Only quantitative result was reported when the calculated concentration of the detected peak was greater than the LOD (refers to as DAQ).

Results indicated that malathion, chlorpyrifos, pirimiphos ethyl, profenofos, prothiophos, ethion and triazophos, each was detected in at least one plasma sample. Meanwhile, dicrotophos, dimethoate malathion, chlorpyrifos and ethion, each was detected in at least one breastmilk sample. Chlorpyrifos was the most frequently detected OP pesticides in both plasma (46%) and breast milk (73%) samples. In some plasma samples, chlorpyrifos, malathion, profenophos and triazophos were detected with the concentrations above their LODs. In some breast milk samples, chlorpyrifos and malathion were found to have their concentrations exceeding their LODs.

All samples detected with OP pesticides above the LODs were from the subjects who worked on the farm that was recently applied pesticides (less than one week) or who lived on or within 1km proximity to the farm. The highest concentrations of malathion and chlorpyrifos were detected in plasma and breast milk samples of a couple who applied pesticides in less than 24 hours prior to sample collection. The couple was reported to live on their farm as well. Although biological half-lives of these OP pesticides in human are in the range of hours to days [27,57,58]; chlorpyrifos and malathion including profenophos have high log K_{ow} (4.7, 3.5 and 4.4, respectively) and tend to partition themselves in lipid-rich matrices [58–60].

Chlorpyrifos, malathion, and profenophos were among the list of Thailand’ highly imported pesticides [37]. Detection of these OP pesticides in biological samples of Thai farmworkers had long been foreseen but finally revealed for the first time in this study. Low level detection of these compounds in plasma and breast milk samples is due to: 1) fast rate metabolism as mentioned before, 2) an inability to collect samples within hours of application; 3) a portion of these compounds had been converted into their oxon form and was precipitated out with their covalently-bound macromolecules during the sample preparation process. It is noteworthy that parathion-methyl which was banned for use in Thailand in 2007 [30] was not detected in any samples in the present study.

OP pesticides in plasma and breastmilk from this study were detected in low ng mL^{-1} range while previous studies in non-farmworkers found the background concentrations of OP pesticides in the pg mL^{-1} range [61–66]. Therefore, the present method might not be sensitive enough to analyze background levels that were found in the general population. However, this method showed some degrees of suitability towards the determination of OP pesticides in occupational exposure setting such as farmworkers.

3.7 Mass spectrometric confirmation of the GC-FPD results

Although GC-FPD is a selective detector for phosphorus- and sulphur-containing compounds, identification of target analyte is according to its retention time. Co-elution of unknown compounds might have occurred and could bring about false positive identification. In this study, 15 plasma samples were selected for mass spectrometric

confirmation of the results obtained from the GC-FPD method, mainly for the presence of chlorpyrifos and malathion, using the GC-MS/MS method, under the conditions described above. The examples of selected ion monitoring chromatograms were shown in Fig. 5.

The 15 samples consisted of the plasma samples initially reported by the GC-FPD method as non-detected, DNQ, and DAQ samples. For chlorpyrifos, all DNQ and DAQ samples of the GC-FPD method were confirmed for its presence. For the samples that were not detected with chlorpyrifos before using the GC-FPD method, they were found to have chlorpyrifos in the range of 0.04–0.10 ng ml⁻¹. These concentrations were below the LOD of the GC-FPD method which was 0.18 ng ml⁻¹. The LOD of chlorpyrifos in the GC-MS/MS method was 0.01 ng ml⁻¹.

For malathion, it was the same with chlorpyrifos in the sense that all DNQ and DAQ samples initially reported by the GC-FPD method were confirmed for its presence in plasma samples using the GC-MS/MS method. Four out of the 13 samples that were initially reported as non-detected samples by the GC-FPD method were detected with malathion at the concentration around 0.01 ng ml⁻¹. This concentration was below the LOD of the GC-FPD method which was 0.36 ng ml⁻¹. The LOD of malathion in the GC-MS/MS method was 0.06 ng ml⁻¹.

3.8 Comparison of the present and other published methods

This method was compared to other published methods which were developed and validated for detecting OP pesticides in serum, blood, and breastmilk samples (Table 6). LODs of this method were in the same range with the GC-MS and GC-NPD methods. Generally, higher LODs were noted from both techniques comparing to GC-MS/MS.

As an approach, the GC-FPD might be considered low-performance compared to mass spectrometry, but it is not lower in robustness. When it comes to the analysis of pesticides in human biological samples, the more highly advanced instruments such as mass spectrometers may actually be less robust because of their extreme sensitivity to matrix effects as well as existing isobaric interferences [67]. In LC/MS, matrix effects are well known factors that reduce the robustness of an analytical method. Thus several measures are required for effective management of these effects in quantification. On the other hand, GC/MS is not free from matrix effects either. Matrix-induced ion enhancement is a typical phenomenon associated with this technique. The GC-FPD is however free from matrix effects. By design, it responds well and very consistently with high degree of specificity to sulphur or phosphorous containing compounds even in very dirty matrices like biological samples.

It must be noted here that the current method is not suitable to analyze biological samples of environmentally exposed populations, unless a series of method modifications is implemented to improve the overall sensitivity (as described in section 3.9). However, it offers an unprecedented capacity for monitoring of OP pesticides in farmworkers in Thailand and other agro-centric areas in South East Asia and elsewhere.

3.9 Suggestion for sensitivity improvement

The LODs reported in this present method could be improved by several method modifications. First, more volume of plasma and breast milk sample could be used to increase the final amount of target analytes reaching the FPD. Injection volume could be increased for the same purpose. In this present method, only 1 µL was used. Reducing the final volume of dried residue reconstitution, from 100 µL to lower volume, is another way that will lead to an increased response of the target analytes. Last, the newer version of GC-FPD with a signal enhancement capability could be used to gain better sensitivity of the method.

4. Conclusion

In conclusion, we developed an analytical method that is rapid and reliable for monitoring of OP pesticides in both human plasma and breastmilk of farmworkers in Thailand. This method was tested for its suitability by analyzing 63 plasma and 30 breast milk samples from breastfeeding farmworkers. Although the present method could detect OP pesticides in those samples, its quantitative capacity should be improved through a series of methodologic modifications.

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Highlights

- A single extraction method for detecting in plasma and breastmilk was developed
- Both matrices were extracted with acetone and dichloromethane and cleaned-up by SPE
- Matrix-matched calibration curves were used for reducing matrix effect
- 46% and 73% of plasma and breastmilk samples were detected OP pesticides
- This method was successfully applied in occupationally exposed population

Fig. 1.

Chemical structures of 11 OP pesticides measured in this study. * represents the carbon-positions of isotope labeled in parathion-methyl-D6, malathion-D10, chlorpyrifos-D10, ethion-D20.

Fig. 2.
Extraction recoveries of 11 OP pesticides using C18- and NH₂- cartridges

Fig. 3.

Typical GC-FPD chromatograms of blank solvent (A), level 3 standard solution (B), level 3-spiked serum (C), level 3-spiked milk (D), unknown plasma sample (E) and unknown breastmilk sample (F).

Fig. 4.

The extraction recoveries of 11 OP pesticides in stored plasma (—●—) and breast milk (—◆—) for 180 days at -20°C . The X and Y axis represent number of storage days and % deviation of originally measured value, respectively. The dashed lines bracket the acceptable measurement range of 70–125%.

Fig. 5. Extracted chromatograms of GC-MS/MS analysis of chlorpyrifos (ion transitions: 314→258) and malathion (ion transitions: 173→99) in solvent (a), serum matrix (b), serum matrix spiked with standard solution (c), positive plasma sample (d), milk matrix (e), milk matrix spiked with standard solution (f) and positive breast milk sample (g).

Quantification and confirmation ions of 9 OP pesticides in plasma and milk from multiple reaction monitoring mode of GC-MS/MS

Table 1

Pesticides	Molecular weight	Retention time (min)	Mass-specific information					
			Quantification Ions			Confirmation Ions		
			Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Native pesticides								
Diazinon	304	6.75	304	179	10	179	137	20
Parathion-methyl	263	7.52	263	109	15	263	125	15
Malathion	330	8.11	173	99	15	173	117	20
Chlorpyrifos	350	8.38	314	258	15	316	260	15
Pyrimiphos-ethyl	333	8.80	333	318	10	333	304	10
Prothiophos	345	10.67	267	221	20	269	223	20
Profenofos	373	10.75	339	269	15	337	267	15
Ethion	384	12.77	231	128	30	231	174	15
Triazophos	313	13.29	161	134	10	161	106	15
Isotopically labeled internal standard								
Parathion-methyl-D ₆	269	7.45	131	50	10	–	–	–
Malathion-D ₁₀	340	8.05	183	100	20	–	–	–
Chlorpyrifos-D ₁₀	360	8.30	324	260	15	–	–	–
Ethion-D ₂₀	404	12.41	241	209	5	–	–	–

Table 2

Calibration data and LODs of 11 OP pesticides in serum and milk samples.

	Retenti on time (min)	Serum samples					Milk samples				
		LOD (ng mL ⁻¹)	Range (ng mL ⁻¹)	Slope ^a	% Error of slope ^b	Regressi on coefficie nt ^a (R ²)	LOD (ng mL ⁻¹)	Range (ng mL ⁻¹)	Slope ^a	% Error of slope ^b	Regressi on coefficie nt ^a (R ²)
Dicrotophos	6.89	1.36	0.80–8.0	0.07	6.4	0.999	2.66	0.80–8.0	0.06	4.5	0.998
Dimethoate	7.62	0.39	0.40–4.0	0.04	6.8	0.999	0.84	0.40–4.0	0.04	6.2	0.999
Diazinon	8.19	0.38	2.5–25	0.09	4.1	0.999	0.66	2.5–25	0.09	4.9	0.998
Parathion-methyl	10.25	0.26	2.0–20	0.04	6.3	0.999	0.09	2.0–20	0.04	4.3	0.998
Malathion	10.42	0.36	0.30–3.0	0.04	5.0	0.999	0.12	0.30–3.0	0.05	5.1	0.998
Chlorpyrifos	10.97	0.18	1.5–15	0.06	6.4	0.999	0.22	1.5–15	0.06	5.5	0.998
Pirimiphos-ethyl	12.85	0.24	0.80–8.0	0.06	7.4	0.999	0.13	0.80–8.0	0.05	4.5	0.995
Prothiophos	12.98	0.47	0.40–4.0	0.12	6.7	0.999	0.57	0.40–4.0	0.11	3.2	0.999
Profenofos	14.28	0.51	2.0–20	0.03	7.1	0.999	1.23	2.0–20	0.03	5.1	0.999
Ethion	14.70	0.27	0.50–5.0	0.09	6.1	0.998	0.57	0.50–5.0	0.09	4.4	0.996
Triazophos	15.83	0.33	2.5–25	0.05	4.7	0.999	1.22	2.5–25	0.04	4.9	0.999

Note: LOD= limit of detection;

^aFrom a regression analysis of the concentration and the ratio of analyte's area and internal standard's area;

^bFrom a calculation of slope error by LINEST function (Excel software)

Table 3

Extraction recoveries of 11 OP pesticides at low and medium levels in serum and milk samples.

Pesticides	QC concentration ; low, medium) (ng mL ⁻¹)	Absolute recovery ; mean ± SD				Relative recovery ; mean ± SD			
		Serum samples		Milk samples		Serum samples		Milk samples	
		Low level (n=3)	Medium level (n=3)	Low level (n=3)	Medium level (n=3)	Low level (n=3)	Medium level (n=3)	Low level (n=3)	Medium level (n=3)
Dicrotophos	1.6, 3.2	39±8.9	50±10.6	45±8.1	65±9.3	69±2.4	68±3.5	75±13	71±0.2
Dimethoate	0.8, 1.6	37± 172	52±4.2	42±9.0	69±8.9	62±2.9	72±4.1	65±7.9	79±3.7
Diazinon	5.0, 10	49±5.6	57±2.3	48±6.5	65±4.3	61±1.2	90±3.6	72±20	81±8.6
Parathion-methyl	4.0, 8.0	49±2.1	50±5.7	54±8.9	71±9.5	66±1.8	78±3.9	90±11	82±1.8
Malathion	0.6, 1.2	50±4.8	51±7.7	53±3.8	67±4.1	76±1.7	93±2.8	86±12	78±4.9
Chlorpyrifos	3.0, 6.0	55±5.2	53±2.5	58±8.4	74±5.6	84±0.6	95±2.8	84±14	78±8.6
Pirimiphos-ethyl	1.6, 3.2	58±5.6	54±9.2	51±5.4	68±2.8	94±1.1	86±3.6	60±14	79±2.1
Prothiophos	0.8,1.6	38±3.5	51±6.9	58±1.4	66±2.4	68±1.4	61±2.8	72±16	71±5.7
Profenofos	4.0,8.0	36±6.3	46±2.3	54±4.1	69±7.9	62±2.1	95±2.7	68±7.1	81±2.1
Ethion	1.0,2.0	38±5.4	51±5.9	45±2.3	61±4.7	63±1.8	59±1.7	68±7.1	84±3.4
Triazophos	5.0,10	39±2.5	43±6.0	51±3.6	71±4.9	61±2.0	76±1.9	64±2.3	85±1.6

S.D. = standard deviation

Table 4

Precision and accuracy of 11 OP pesticides in serum and milk samples

Pesticides	Serum samples				Milk samples					
	Precision (%RSD)		Accuracy (%recovery)		Precision (%RSD)		Accuracy (%recovery)			
	Intrabatch		Interbatch		Intrabatch		Interbatch			
	low (n=20)	medium (n=20)	low (n=5)	medium (n=20)	low/ (n=20)	medium (n=20)	low (n=5)	medium (n=20)		
Dicrotophos	7.8	2.5	10	98	100	8.7	9.2	14	123	105
Dimethoate	8.6	5.1	9.8	97	99	6.9	10	8.9	160	143
Diazinon	8.7	2.6	8.7	96	100	7.2	16	8.5	115	104
Parathion-methyl	8.6	4.9	11	98	102	6.9	9.4	9.9	162	128
Malathion	7.2	2.8	15	102	95	6.7	10	17	94	74
Chlorpyrifos	5.3	3.5	9.4	100	101	8.7	11	9.0	125	108
Primiphos-ethyl	11	2.6	7.8	100	96	10	12	5.8	119	102
Prothiophos	4.5	2.3	8.2	100	101	16	7.4	7.9	77	92
Profenofos	7.3	5.2	20	100	101	10	5.9	19	90	34
Ethion	10	6.5	12	101	121	17	6.9	11	105	96
Triazophos	7.1	2.3	11	103	100	5.2	3.9	10	105	102

Table 5

Application of the developed method for determining OP pesticides in plasma and breastmilk samples from Thai farmworkers

Samples	Pesticides	N	Concentration (ng mL ⁻¹)								
			% detected	%DNQ	% DAQ	LOD	Range	Percentile			
								25	50	75	90
Plasma	Malathion	6 3	11	9.4	1.6	0.36	<LOD-0.50	<LOD	<LOD	<LOD	0.13
	Chlorpyrifos	6 3	46	35	11	0.18	<LOD-0.66	<LOD	<LOD	0.13	0.19
	Pirimiphos ethyl	6 3	1.6	1.6	0.0	0.24	<LOD-0.08	<LOD	<LOD	<LOD	<LOD
	Prothiophos	6 3	7.9	4.7	3.2	0.47	<LOD-1.68	<LOD	<LOD	<LOD	<LOD
	Profenofos	6 3	3.2	3.2	0.0	0.51	<LOD-0.07	<LOD	<LOD	<LOD	<LOD
	Ethion	6 3	4.8	4.8	0.0	0.27	<LOD-0.09	<LOD	<LOD	<LOD	<LOD
Breast milk	Triazophos	6 3	19	17.4	1.6	0.33	<LOD-0.74	<LOD	<LOD	<LOD	0.14
	Dicrotophos	3 0	3.3	3.3	0.0	2.66	<LOD-0.14	<LOD	<LOD	<LOD	<LOD
	Dimethoate	3 0	3.3	3.3	0.0	0.84	<LOD-0.12	<LOD	<LOD	<LOD	<LOD
	Malathion	3 0	23	3	20	0.12	<LOD-0.35	<LOD	<LOD	0.02	0.30
	Chlorpyrifos	3 0	73	63	10	0.22	<LOD-0.46	<LOD	0.1	0.15	0.22
	Ethion	3 0	20	20	0.0	0.57	<LOD-0.14	<LOD	<LOD	<LOD	0.11

Note % detected was from the number of all positive sample; % DNQ was from detected but not quantified sample; %DAQ was from the number of positive sample detected above LOD. All % were calculated divided by number of total sample and then, multiplied with 100.

Table 6
Comparison of the present and other published methods of detecting OP pesticides in human serum, blood, and breastmilk samples using gas chromatography

Studies	Matrix	Preparation method	Analytical instrument	LOD	LOQ	% Extraction recovery	Subjects	Detected concentration
This study	Serum	LLE (acetone and dichloromethane) and $\text{NH}_2\text{-SPE}$	GC-PPD	0.10–0.80 ng mL^{-1}	0.40–1.5 ng mL^{-1}	59–95	Farmworkers	ND–0.66 ng mL^{-1}
Barr et al., 2002 [61]	Serum	Denatured protein with $\text{NH}_4\text{SO}_4\text{-SPE}$ (OASIS)	GC-HRMS	0.5–12 pg g^{-1}	NA	15–27	Female	2 (Diazinon), 9 (Chlorpyrifos) pg g^{-1}
Pitarch et al., 2003 [62]	Serum	C18 SPE	GC-MS/MS	0.1–3.0 ng mL^{-1}	0.4–9.0 ng mL^{-1}	71–102	Exposed and non-exposed agricultural workers and general population	0.8 ng mL^{-1} (Chlorpyrifos in exposed subject)
Inoue et al., 2007 [55]	Serum	LLE (acetonitrile)	LC-APCI-MS	0.125–1 ug mL^{-1}	0.25–1.25 ug mL^{-1}	82–107	Male attempted suicide by ingesting approximately 100 mL of both 5% fenitrothion and acephate emulsion	7.2 (Acephate) and 4.5 (fenitrothion) ug mL^{-1}
Singh and Dogra, 2009 [64]	Serum	LLE (mixture of acetone and diethyl ether), acidifying, LLE (diethyl ether)	GC-NPD	NA	0.25 ug mL^{-1} (lowest level)	76–93	Employees working in a pesticide manufacturing unit	0.5–0.8 ug mL^{-1} (malathion)
Perez et al., 2010 [68]	Plasma	Mixed-phase SPE	GC-HRMS	16–21 pg mL^{-1}	NA	100–144	Cohort of African American and Dominican women	ND
Tarbah et al., 2001 [56]	Blood	LLE (toluene)	GC-MS, GC-PND	NA	NA	56–133	Non-fatal clinical cases of acute E 605 intoxication	0.31, 0.75 (parathion ethyl) and 0.04 (paraoxon) ug mL^{-1}
Corrion et al., 2005 [21]	Blood	Sonication, methanol/phosphate buffer adding, LLE (hexane)	GC-MS	<0.1–0.2 ng mL^{-1}	NA	118–140	Pregnant women	ND
Park et al., 2009 [69]	Blood	PBS adding, sonication, OASIS HLB SPE	GC/MS	0.04–0.09 ug mL^{-1}	0.13–0.17 ug mL^{-1}	75–94	Person suspected to have ingested OPs.	0.21–19.64 ug mL^{-1}

Studies	Matrix	Preparation method	Analytical instrument	LOD	LOQ	% Extraction recovery	Subjects	Detected concentration
This study	Breastmilk	LLE and NH ₂ -SPE	GC-FPD	0.1–1.4 ng mL ⁻¹	0.4–4.8 ng mL ⁻¹	71–85	Farmworkers	ND-0.46 ng mL ⁻¹
Sanghi et al., 2003 [70]	Breastmilk	Mixing with sodium chloride and dichloromethane, LLE (n-hexane)	GC-ECD	0.01 mg kg ⁻¹	NA	89	Women belonging to the lower socioeconomic class	0.01–0.043 ug mL ⁻¹
González Rodríguez et al., 2005 [71]	Breast milk	Acid adding, LLE (pentachlorobenzene in acetone), SPME	GC-MS	0.01–0.15 ug mL ⁻¹	0.02–0.50 ug mL ⁻¹	87–103	NA	ND
Srivastava et al., 2011 [72]	Breastmilk	NA	GC-ECD	NA	NA	NA	Rural mothers	4–744 ng g ⁻¹ fat
Chen et al., 2014 [42]	Breastmilk	LLE and GCB/PSA SPE	GC-MS/MS	7.6 pg mL ⁻¹	NA	80–90	Human milk	0.022–0.711 ng mL ⁻¹ (chlorpyrifos)

Note: LOD= limit of detection; LOQ=limit of quantitation; NA=not available; ND=Not detected; LLE =liquid-liquid extraction; SPE=solid phase extraction