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ORIGINAL PAPER

Immunochemical analysis of 3-phenoxybenzoic acid, a biomarker of forestry worker exposure to pyrethroid insecticides

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Abstract Pyrethroid insecticides widely used in forestry, agricultural, industrial, and residential applications have potential for human exposure. Short sample preparation time and sensitive, economical high-throughput assays are needed for biomonitoring studies that analyze a large number of samples. An enzyme-linked immunosorbent assay (ELISA) was used for determining 3-phenoxybenzoic acid (3-PBA), a general urinary biomarker of exposure to some pyrethroid insecticides. A mixed-mode solid-phase extraction reduced interferences from acid hydrolyzed urine and gave 110±6% recoveries from spiked samples. The method limit of quantification was 2 μg/L. Urine samples were collected from forestry workers that harvest pine cone seeds where pyrethroid insecticides were applied at ten different orchards. At least four samples for each worker were collected in a 1-week period. The 3-PBA in workers classified as high, low, or no exposure based on job analysis over all sampling days was 6.40±9.60 (n=200), 5.27±5.39 (n=52), and 3.56±2.64 ng/mL (n=34), respectively. Pair-wise comparison of the differences in least squares means of 3-PBA concentrations among groups only showed a significant difference between high and no exposure. Although this difference was not significant when 3-PBA excretion was normalized by creatinine excretion, the general trend was still apparent. No significant differences were observed among days or orchards. This ELISA method using a 96-well plate was performed as a high-throughput tool for analyzing around 300 urine samples measured in triplicate to provide data for workers exposure assessment.

Keywords Pyrethroid insecticide · Biomonitoring · Immunoassay · Forestry workers

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Esfenvalerate, permethrin, and bifenthrin are among the class of pyrethroid insecticides with extensive agricultural, forestry, horticulture, public health, and residential uses [1]. These compounds are neurotoxic, yet are very important insecticides because of their rapid control of insects, relatively low toxicity to mammals, and rapid degradation in the environment [2]. However, there is a potential for human exposure. In humans, pyrethroid insecticides are rapidly metabolized by esterases and renally eliminated [3]. The common primary metabolite of these compounds that contain the 3-phenoxybenzyl moiety is 3-phenoxybenzoic acid (3-PBA). This acid may undergo secondary metabolism to form amino acid or glucuronide conjugates. Hydroxylations of the parent compound or 3-PBA are minor routes of metabolism. Following an inhalation exposure to cyfluthrin, metabolites were eliminated in the urine with a half time of about 6 h [4]. In a study comparing dermal and oral doses of cypermethrin, greater than 90% of

the recovered metabolites was excreted within the first 72 h [5]. The mean elimination half-life for total metabolites following dermal exposure was 13 h. Since 3-PBA is a common metabolite for most pyrethroid pesticides, it has been used as a marker of pyrethroid exposure by the Centers for Disease Control and Prevention in the National Health and Nutrition Examination Survey study [6].

Few studies have been published on pesticide exposure in forestry workers. Most of those studies focused on workers that handled conifer seedlings in the nursery [7, 8] or conducted weed control on the ground [9] or aerially [10]. To our knowledge, no study has been published that examines exposure in pine cone seed harvesters to pyrethroid insecticides used in cone and seed insect control programs.

Current analytical methods for pyrethroid metabolites in urine rely upon multi-step sample clean-up procedures including hydrolysis, extraction, and derivatization. Samples are then analyzed using either liquid chromatography- mass spectrometry (LC/MS) [11] or gas chromatography with MS (GC/MS) [12]. Another method for rapid assessment of exposure to permethrin is the use of immunoassays that detect key urinary biomarkers of exposure [13]. Since the first radioimmunoassay was reported in 1960, immunoassays have found their basis in the clinical chemistry area where the analysis of large numbers of samples, requiring rapid turnaround time in a cost-effective manner, is a routine. It has been demonstrated that immunoassays can be highly sensitive and selective analytical tools to determine trace chemicals such as pesticide residues and their metabolites. Recently, a sensitive immunoassay coupled by a solid-phase extraction technology has been used to measure paraquat in the urine of Costa Rican farmworkers to assess occupational exposure [14].

The significance of the sample preparation to the total analytical performance is an important consideration. An ideal sample preparation method should involve a minimum number of working steps, be easy to perform, and economical [15]. Further, as the number of samples grows high-throughput and automated analytical techniques are desired. An immunoassay for 3-PBA, which had been demonstrated as a biomarker of exposure to pyrethroids, was developed [16]. In studies with spiked urine samples, the assay had a limit of quantitation of 5 μg/L when the urine sample was diluted 1/50 and analyzed [16]. Further work has indicated that variations among urine samples can result in interferences even when samples were diluted as much as 1/100. In addition, the increased dilution raised the limit of quantitation (LOQ). Thus, the goals in this work were to develop and validate a solid-phase extraction (SPE) method that will reduce the interferences, and reduce the LOQ to 5 μg/L or less while providing short sample preparation time and cost-effective high-throughput data generation on 96-well plates and apply it to an exposure assessment study.

Experimental section

Chemicals and instruments Bovine serum albumin (BSA), goat anti-rabbit IgG-horseradish peroxidase conjugate, 3,3′,5,5′-tetramethylbenzidine sodium salt, and Tween 20 were from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade or better from Fisher Scientific (Pittsburgh, PA). An ELISA was performed on 96-well microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Sunnyvale, CA) in dual wavelength mode (450–650 nm). The plates were washed with an automated strip washer (Bio-tek Instruments, Inc., Winooski, VT). Phosphate-buffered saline (PBS) was 10 mmol/L phosphate buffer (pH 7.5) containing 0.8% NaCl. For sample preparation, a mixed-mode SPE column (100 mg, Strata Screen-A, 50 μm, 70 Å, Phenomenex, Torrance, CA), a SPE vacuum manifold processing station (Supelco, Bellefonte, PA), a dry heat block (Thermolyne Dri-Bath), and a centrifugal vacuum concentrator (ATR, Laurel, MD) were used. Analytical grade solvents were from Fisher Scientific Inc. (Pittsburgh, PA).

Urine sample collection A total of 73 forestry workers that harvest pine cone seeds where the pyrethroid pesticide was applied were enrolled at ten forest sites in the Southern states. Supervisors attended an orientation meeting in Pensacola, FL, where study goals and objectives were discussed. Guidelines for the conduct of human research were reviewed and supervisors completed an online tutorial administered by the Office of Research Integrity, University of California Riverside. As part of the training, supervisors were provided notebooks that included instructions for enrolling volunteers, forms for obtaining informed consent, and formats for recording the nature of work tasks and related field data. All study records were maintained by supervisors at the respective orchards during the study period except three sites where supervisors were assisted by UC Riverside study staff. Urine was collected at least four times within seven successive harvesting days and volumes were measured. On average, as many as 43 individual urine samples per day were collected. Although there was no specific control of the work activities of the harvesters, urine samples of the workers previously not occupationally exposed to the pyrethroid insecticide were collected. The urine samples were frozen without any treatment until analysis.

In order to compare non-occupational human exposure to pyrethroid insecticides, additional urine spot samples

were collected once during the day from ten individuals in the laboratory who were not involved in this project.

Urine sample preparation The field samples were shipped to the laboratory where they were kept frozen until the analysis. After thawing for around 16 h at room temperature, the urine was shaken for 30 s and then left to stand for about 2 h. The supernatant was used for 3-PBA and creatinine analyses.

Hydrolysis Urine was subjected to acid hydrolysis to free 3-PBA that might be conjugated [3]. A 0.5-mL aliquot of the clear supernatant was added to a 3.5-mL tube. In the fume hood, a 0.1-mL aliquot of 6 N HCl was added and the tube capped. The capped tube was placed in a heating block set at 100 °C for 1 h in the fume hood. After cooling, the acid mixture was neutralized with about 95 μL of 6 N NaOH (Caution: add base slowly to avoid "bumping" or foaming). Sodium acetate buffer (0.1 M, pH 4.5, 1 mL) was added to the tube and mixed thoroughly.

SPE The neutralized hydrolysate was subjected to mixed-mode SPE (C8+anion exchange). The method was slightly modified from a method published previously [17]. A set of 24 SPE tubes was placed into the vacuum manifold. A 16×100-mm test tube was placed below each tube and the manifold cover sealed. For each addition of liquid to the tube, the liquid was allowed to flow through under gentle vacuum at a flow rate of about 2–3 mL/min. When the liquid reached the top of the resin bed, the next solution was added. To precondition the mixed-mode tube, 1 mL of methanol was added to the column followed by 1 mL of water, then 1 mL of sodium acetate buffer (pH 4.5). The hydrolyzed urine mixture (about 1.5 mL) was added to the tube, followed by 1 mL water and 1 mL of methanol to remove interferences. The tube was then dried under high vacuum (10 in. Hg) for 5 min. The manifold cover was removed, and the glass tube was removed and replaced with a fresh 12×75-mm tube. The manifold cover was then replaced. The 3-PBA was eluted by the addition 1.5 mL of 1% acetic acid in a mixture of hexane and ethyl acetate (70:30 *v*/*v*) to the tube. The tubes containing the eluate were removed from the manifold and placed in a centrifugal vacuum evaporator where they were evaporated to dryness at least for 3 h. The residue was dissolved in methanol (0.25 mL) followed by the addition of 2.25 mL of PBS to make a 5-fold dilution based on the original urine amount. Each urine sample was processed in triplicate.

Immunochemical analysis The specific antibody and competing heterologous hapten for the target analyte (3-PBA) were previously described [16]. Briefly, the polyclonal antibody (Antiserum 294) was produced against a conjugate of hapten (3-((2-oxoethoxy)ethoxy)phenoxybenzoic acid) and thyroglobulin. The preparation of the buffers and the procedure for the indirect competitive ELISA were previously described [16]. The IC_{50} value, an expression of the sensitivity of immunoassay, and the limit of detection (LOD) defined as the IC_{20} value were obtained from a four-parameter logistic equation. Borosilicate glass tubes were used to prepare standard and sample solutions. Briefly, 96-well plates were coated overnight at 4 °C with the coating antigen 3-PBA-BSA (1/5,200 dilution of a stock solution with a concentration of 2.6 mg protein/mL) in a 0.05 M sodium carbonate–bicarbonate buffer (pH 9.6). The following day, the coating antigen was washed off the plate with a PBS solution containing 0.05% Tween 20 (PBST), pH 7.5, and the wells were blocked for 30 min with 200 μL 0.5% of BSA in PBS. In a separate microtiter plate, 70 μL of sample or standard in 10% methanol-PBS and 70 μL of 3-PBA antibody diluted 1/5,000 in PBST were added to each well and mixed on a plate shaker for 15 min. After washing the coated and blocked plate, the mixture was pipetted into each well (100 μL/well) in triplicate and incubated at room temperature for 1 h. The sample matrix and excess reagents were washed away leaving only the antibodies bound to the coating antigen. Then, 100 μL of the goat anti-rabbit IgG-horseradish peroxidase conjugate diluted 1/3,000 in PBST was added into each well, and the plate incubated for 1 h. After a wash, procedures were performed according to the published method [16]. The concentration of 3-PBA in the unknown samples was calculated based on the standard curve run on each plate. The software package Softmax Pro (Molecular Devices, Sunnyvale, CA) was used for fitting the 11-point sigmoidal standard curve based on a four-parameter logistic method of Rodbard [18].

Quality control and assurance Random field urine samples, spiked with 10 μg/L 3-PBA, were extracted with each batch of samples to verify that there were no matrix effects from the sample extract. The results of the spiked samples were determined by the ELISA and compared with those of unspiked 3-PBA samples after the SPE. A method blank was prepared along with all field samples each time the vacuum manifold was reloaded for extraction. The 3-PBA calibration curve was run in the presence of the same volume of method blank that corresponds to the volume of a sample eluate. The triplicate sample analyses were performed to verify the precision of analyte preparation procedures. When the coefficient of variation (CV %) for a sample measured in triplicate was >30%, or the concentration was outside the linear range, the sample was measured again. Urine samples containing over 55 μg/L 3-PBA (i.e., out of the

linear range) were reanalyzed after 10- and 20-fold dilution. Only 2.3% or seven out of 300 samples were reanalyzed. Three samples had concentrations that were too high and four were near the limit of detection.

In order to compare the degree of free and conjugated 3-PBA in urine, the concentration of 3-PBA was determined in a subset of samples before and after hydrolysis. For the recovery study, 3-PBA dissolved in 50% methanol-PBS was spiked at a range of 1–50 μg/L into 0.5 mL of urine samples in which the concentration of 3-PBA was less than LOQ. The samples were hydrolyzed and extracted as describe above. Each sample was analyzed in quadruplicates. Recovery values for this standard reference material of 60–135% were deemed acceptable, and no correction to the data were made. For samples with recoveries less than 60% or greater than 135%, the sample was reanalyzed.

Creatinine analysis The supernatant urine samples were diluted 1:10, 1:20, and 1:40 in distilled water. Ten microliters of diluted sample, standard or control, was pipetted into each well of a 96-well microtiter plate (Dynatech, Chantilly, VA), in triplicate, followed by 100 μL of picrate reagent. The picrate reagent consisted of a 1:1 mixture of 0.3 N NaOH and 1% picric acid in water (Ricca Chemical Co., Arlington, TX). The plates were incubated at room temperature for 15 min, and then read in a microplate reader at a wavelength of 480 nm. Concentrations were calculated by comparing the absorbance of the sample to a linear regressed fit of the absorbances of the standards (0.01, 0.03, and 0.1 mg creatinine/mL distilled water). If the absorbances of the samples fell outside the calibration range, the samples were reanalyzed at either greater or lesser dilution as appropriate. The creatinine value selected for reporting among the three dilutions was the one with the absorbance value nearest the absorbance of the 0.03 mg/mL standard.

LC/MS/MS analysis of 3-PBA For validation of the ELISA method, a set of urine samples after SPE were analyzed by an LC/MS/MS method [19]. Chromatographic separation was performed using a Shimadzu AP-10 separation module (Shimadzu, Japan) equipped with a C18 Atlantis column (150×2.1 mm, 3 μm; Waters, Milford, MA) held at 25 °C. A solvent system consisting of 0.1% (*v*/*v*) formic acid in water (solvent A) and 0.1% (*v*/*v*) formic acid in acetonitrile (solvent B) was used. Before injection, 100 μL of 50 μg/L ^{13}C-3-PBA in acetonitrile was added to a glass tube containing a dried SPE extract and mixed using a Vortex mixer for 30 s. Water (100 μL) was further added to the glass tubes and mixed for 30 s. Finally, a 150-μL aliquot of the sample was transferred into glass inserts in HPLC autosampler vials. The samples were kept at 10 °C in the autosampler. The injection volume was 25 μL. The target analytes were loaded on the column at 5% B and a 0.5 mL/min flow rate. The flow was diverted into waste for 2 min to wash polar impurities from the column (0 min). After 2 min of wash, the flow was diverted back into detector and solvent B was ramped to 30% with a reduction of flow rate to 0.3 mL/min in 10 s (2.1 min). The isocratic flow of 30% B at 0.3 mL/min was kept for 2.9 min (5 min). After 5 min, solvent B was ramped to 100% in 2 min (7 min). Non-polar impurities were washed from the column at 0.3 mL/min for 4.5 min (11.5 min), returned to initial conditions of 5% B at 0.5 mL/min (11 min), and allowed to equilibrate for 1 min (12 min). The target analytes were detected by tandem quadrupole mass spectrometry in multiple reaction monitoring mode (MRM) using a Quattro Ultima tandem quadrupole mass spectrometer (Micromass, UK). Nitrogen gas flow rates were fixed with a cone gas flow of 50 L/h and a desolvation gas flow of 650 L/h. Electrospray ionization was performed in negative mode with a capillary voltage fixed at 3.00 kV using a source temperature of 125 °C and a desolvation temperature of 400 °C. Argon was used as collision gas (2.3×10^{-3} mbar). An optimal transition of *m*/*z* 213>*m*/*z* 93 for 3-PBA, and *m*/*z* 219>*m*/*z* 99 for ^{13}C-3-PBA was monitored using a cone voltage of 40 V, and a collision voltage of 25 V. A LOD (S/N >3) was estimated at 0.2 μg/L (5 pg on column) and a LOQ (S/N >10) of 0.6 μg/L (15 pg on column) in urine was estimated using root mean square signal-to-noise measurements of the analytical signal from serial dilutions of 3-PBA.

Statistical analysis Log-transformed PBA values were analyzed using mixed model analysis of variance methods. An initial model was run that accounted for the effect of group (exposure level), orchard, and day, where subject effects were treated as random effects. In this analysis, none of the effects were statistically significant, and so the analysis was rerun as a one-way mixed model for the group effect, again treating the subject as a random effect. Analysis of the residual errors from these analyses was done using a Wilk–Shapiro test of normality and a Levene test of homoscedasticity. No problems were detected with either of these assumptions.

Results and discussion

Immunoassay The developed 3-PBA immunoassay was used as a high-throughput tool to analyze about 300 urine samples in triplicate to provide data for workers exposure assessment. Immunoassays are subject to specific interferences due to structurally similar compounds. Among the compounds tested for cross-reactivity, 4-fluoro-3-phenox-

ybenzoic acid (FPBA), 4-hydroxy-3-PBA, and 3-phenoxybenzaldehyde have significant responses at values of 72%, 103%, and 75%, respectively. FPBA is a metabolite derived from cyfluthrin; 4-hydroxy-3-PBA is a minor metabolite; 3-phenoxybenzyl aldehyde is an intermediate that oxidizes to 3-PBA. These metabolites can be considered as a minor factor in this human exposure study of pyrethroid insecticides as they are unlikely present at levels that would make an impact on the results. Other metabolites such as the glycine conjugates of 3-PBA and FPBA, 3-phenoxybenzyl alcohol, and 4-hydroxybenzoic acid as well as parent compounds such as permethrin, cypermethrin, esfenvalerate, deltamethrin, and cyfluthrin do not cause any interference in the assay up to concentrations tested (10,000 μg/L) [16]. A representative standard curve is shown in Fig. 1. The average 4-parameter curve fit parameters for 12 assays during the project period were 0.96 (A_{max}), 0.03 (A_{min}), 0.77 (IC_{50}), and 1.23 (slope). The A_{max} is the maximum absorbance at zero analyte. The IC_{50} is the concentration at which the absorbance was inhibited by 50%. This value is used to compare relative sensitivity among assays. The linear range is defined as the concentration range corresponding to 20% and 80% inhibition. The LOD was set as the value of IC_{20}.

Sample preparation As seen in Table 1, 3-PBA concentrations in urine samples of forestry workers were higher in acid hydrolyzed samples than those in non-hydrolyzed ones, suggesting that glucuronide and/or other conjugates of 3-PBA are formed in larger portion than the free metabolite. A hydrolysis step to form free 3-PBA in urine is necessary for the evaluation of human exposure. With non-hydrolyzed urine, a C18-SPE using methanol for

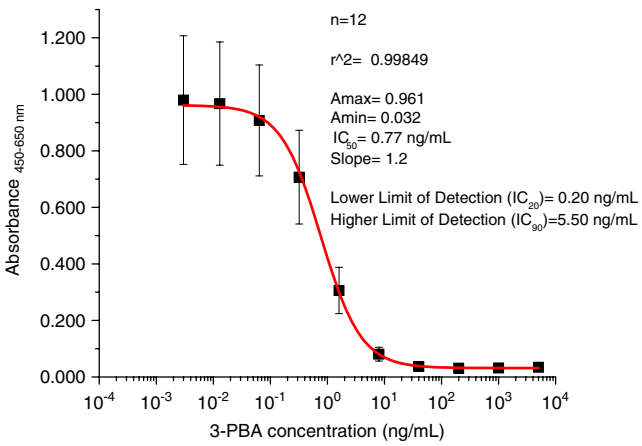

Fig. 1 Representative calibration curve for 3-PBA. Calibration standards were prepared in PBST. The *calibration curve* represents the average of 12 curves (± standard deviation) plotted from data obtained for assay period. *Calibration curves* are prepared on each day and sample data compared to the curve for that day

Table 1 3-PBA concentrations (μg/L) before and after hydrolysis of a subset of urine samples, determined by ELISA

Urine sample	Before hydrolysis	After hydrolysis
A	< LOQ	4.09±0.08
B	< LOQ	< LOQ
C	< LOQ	4.78±0.70
D	< LOQ	6.53±1.31
E	< LOQ	20.89±1.40
F	< LOQ	3.70±0.40

LOQ is 2 μg/L

elution and/or an additional liquid–liquid clean-up step required 20–50 times dilution to reduce interferences in the immunoassay [17]. The method did not increase the assay sensitivity much as the LOQ value was 5 μg/L. Urine hydrolyzed by heating with a strong acidic solution made the samples deteriorate becoming aromatic and dark. However, as seen in Table 2, the use of the mixed-mode SPE and an acidic mixture of ethyl acetate and hexane (30:70, *v*/*v*) for elution of 3-PBA and the 5-fold dilution for the ELISA completely removed urine interferences and provided a lower limit of quantitation of 2 μg/L. The mixed-mode SPE that strongly bound 3-PBA allowed water and methanol to wash away salts, as well as neutral and basic interferences in urine hydrolyzed by strong acid. In addition, the selection of an acidic non-polar mixture of ethyl acetate and hexane eluted 3-PBA more selectively than acidic methanol, suggesting that the lower LOQ value was due to the ability to dilute less because there were fewer co-eluted interferences. As shown in Table 2, there was no significant difference between the levels of 3-PBA in unspiked samples and those with 3-PBA subtracted from the spiked samples, suggesting that the sample preparation method and this ELISA were optimal.

Accuracy and precision The precision and accuracy of the whole analytical system for the spiked samples, including the SPE clean-up and ELISA, are presented in Table 3. For

Table 2 Comparison of 3-PBA concentrations before and after addition of 3-PBA

Urine sample	SPE/10-fold dilution/ELISA	
	Before addition	After addition (10 μg/L)
001	< LOQ	12.47±0.26
002	< LOQ	11.63±0.11
003	8.04±0.09	17.12±0.30
004	16.52±0.19	27.13±0.36

LOQ is 2 μg/L

Table 3 Day-to-day variation in the recovery of 3-PBA spiked into urine samples using a mixed-mode SPE clean-up

3-PBA spiked (μg/L)	No. of samples	Recovery (%)					CV (%)
		Day 1	Day 2	Day 3	Day 4	Mean	
1	4	101.0±13.44	95.0±3.44	99.0±24.74	133.0±14.30	107.00±17.52	16.4
3	4	109.7±12.02	93.7±3.30	106.7±18.60	100.7±20.74	102.7±7.07	6.9
5	4	97.4±6.65	112.6±0.57	100.0±6.36	117.4±17.25	106.85±9.67	9.1
10	4	112.9±1.77	103.0±2.26	116.2±7.00	136.5±6.57	117.15±14.06	12.0
50	4	116.0±1.88	115.1±4.16	109.4±2.30	121.9±1.41	115.6±5.12	4.4

quality assessment, pooled urine samples were spiked at four different concentrations (1–50 ng/mL), and the samples were analyzed on multiple days. The intra-assay (intraplate) precision of ELISA, expressed as a coefficient of variation (CV %) between three replicates was <25%, and in most cases <10%. The inter-assay (interplate) precision measured from the same sample solutions during 4 different days did not exceed 16%. The average recovery efficiency and CV for all tested concentrations were 109.86% and 9.8%, respectively. Table 3 shows that 3-PBA levels between 1 and 50 μg/L are recovered within acceptable levels each day. The day-to-day variability was also acceptable. From these data, after a SPE clean-up with a mixed-mode column, the ELISA was able to detect 1 ng 3-PBA per milliliter urine sample.

Validation To verify that the interferences were minimized, the validation of this ELISA was achieved over a wide range of concentrations of 3-PBA spiked into the sample. The linear regression of the results showed a highly positive correlation (r=0.999) with a slope of 1.16 when comparing spiked levels with levels determined by ELISA (Fig. 2a). A subset of urine samples of the forest workers were analyzed by both LC/MS/MS and immunoassay. Figure 2b shows that the two methods correlate well, indicating that matrix interferences were essentially removed. The ELISA and LC/MS/MS methods provided consistent measurements for analysis of 3-PBA in urine. The agreement between the ELISA and the LC-MS/MS results for field samples was good (r=0.985) with slope = 0.95 and intercept = 1.04. The inset in Fig. 2b shows, even at the lower concentration range (<20 ug/L), there was good agreement (slope value of 1.14, (r=0.964)) between methods.

Determination of the method detection limit An estimate of the method detection limit was made by identifying the concentration of 3-PBA that resulted in a 20% inhibition (IC_{20}) and multiplying by the final 10-fold dilution in a well of the original sample. The estimated value was 2 μg/L. The recovery data in Table 2 demonstrated empirically that a method detection limit of 1 μg/L is achievable. For this study, the method detection limit or lower LOQ was conservatively set at 2 μg/L. Urine samples containing over 55 μg/L 3-PBA out of the detection range (2–55 μg/L) were remeasured after 10- and 20-fold dilution.

Study sample results Creatinine analysis. Creatinine is a muscle breakdown product eliminated in the urine at a constant rate under normal conditions. With renal dysfunction, creatinine clearance increases [20]. Pesticide exposure studies routinely normalize urinary pesticide metabolite concentrations to creatinine output since the mass of creatinine cleared is constant with time while volume of urine can change dramatically [21]. The assay is based on the reaction of creatinine with picrate under basic conditions forming a yellow/orange complex that is detected at 490 nm.

For the creatinine assay, the % CV of the triplicate absorbance values for each standard was less than 5%. The % CV of the triplicate absorbance values for each diluted sample was less than 25%. The control was a sample from the test set that was analyzed each day on each plate. The concentration of creatinine for the control was 2.09± 0.11 mg/mL (n=27) run on a total of 5 days. The average creatinine content for all samples was 1.65±0.88 mg/mL.

The World Health Organization (WHO) suggests that for occupational exposure monitoring, sample creatinine values should be between 0.3 and 3 mg/mL [22]. For samples that are above or below this range, WHO suggests that another sample should be taken for analysis of creatinine and the target analyte. In this study, and for similar field-based studies in which a study monitor may not be present at the time of collection, or the laboratory facility or testing method readily available, another sample is not possible. In addition, Barr et al. [23] determined that age group, sex, race/ethnicity, body mass index, and fat-free mass are all significant predictors of creatinine concentration; thus, it is possible that when using the WHO criteria, data from a significant portion of certain working adult populations would be excluded. For all race and ethnicity, males and females, between the ages of 20 and 60, the percentage of those with spot urine creatinine values <0.3 mg/mL ranges

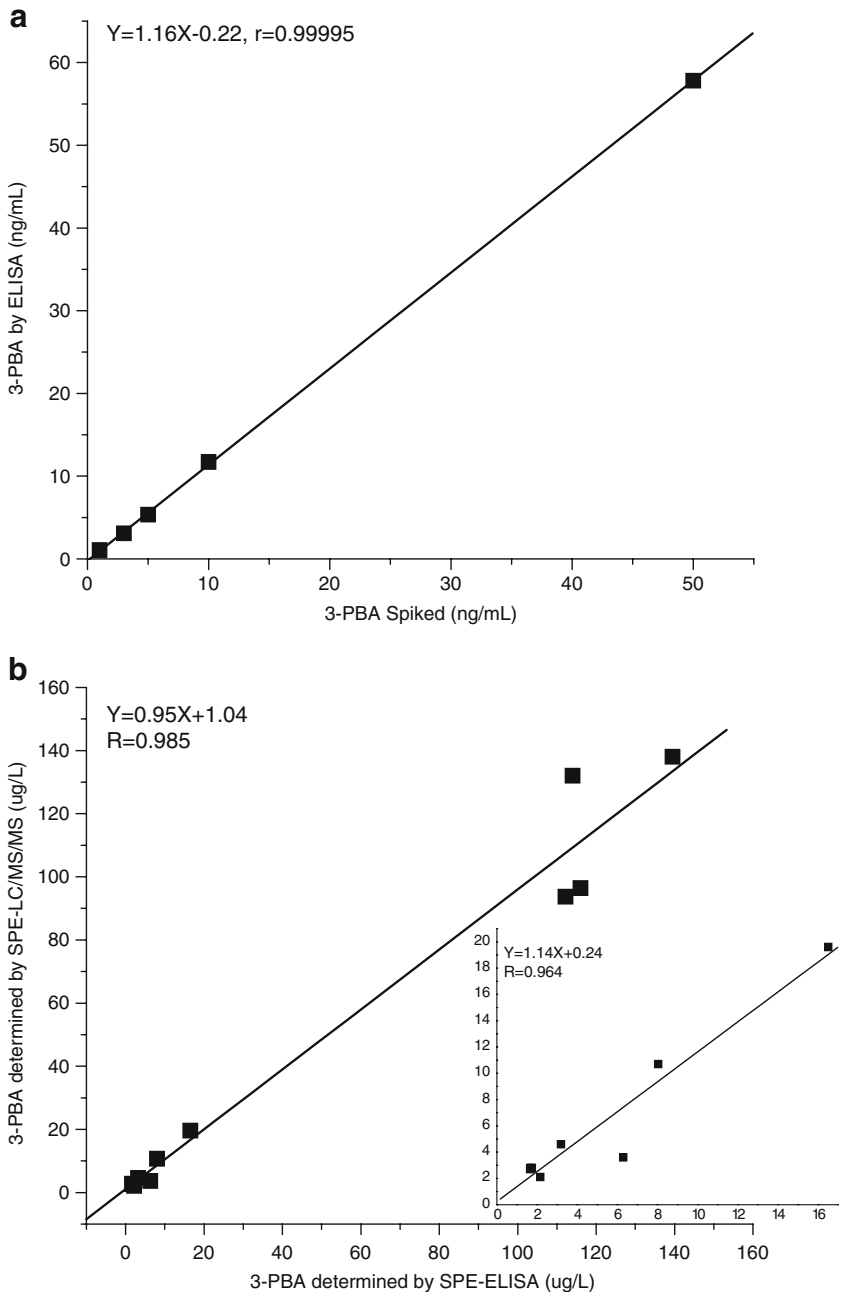

Fig. 2 Correlations between the spiked and measured concentrations of 3-PBA by ELISA (**a**) and LC/MS/MS (**b**). The ELISA and LC/MS/MS analysis were performed after the mixed-mode SPE sample preparation. The *inset* shows the scale expanded between 0 and 20 μg/L

Table 4 3-PBA in the urine of forestry worker and control groups

Exposure group	No Subjects	*n*	Avg μg/L	SD	Avg μg/g creatinine	SD	Geometric mean μg/L	Range	% samples below LOQ
High	48	200	6.40	9.60	3.88	4.65	4.41	LOQ-116.29	16
Low	14	52	4.94	5.34	4.44	4.11	2.19	LOQ-26.79	35
None (Field)	10	34	3.18	2.73	2.37	1.42	1.15	LOQ-11.68	44
None (Laboratory)	10	10	4.38	4.82	NM	–	2.50	LOQ-16.52	30

n individual urine samples analyzed, *NM* creatinine was not measured in these samples

between 5.2% and 12% and those with spot urine creatinine values >3.0 mg/mL was 0.6–6.9%. For this study, 1.3% was below 0.3 μg/L and 6% was above 3.0 mg/mL. Spot urine samples in this study were taken from individuals on at least four different days. Comparison of values for creatinine among the days for an individual generally did not show large variations. Creatinine values for an individual may vary depending upon degree of hydration, diet, physical work performed or health status. The data for 3-PBA in Table 4 are expressed both as analyte concentration and analyte concentration adjusted for creatinine so that both approaches are available for evaluation.

3-PBA analysis Each study sample was independently processed through the hydrolysis and SPE in triplicate. A reported value for an individual is the mean±standard deviation of the three independent replicates. The variation among replicates was <30%. Values that could be calculated from the absorbance, but were <2.0 μg/L are reported as <LOQ. Around 77% of all urine samples collected contained detectable urinary 3-PBA concentrations. The highest 3-PBA concentrations measured in the urine samples were around two orders of magnitude higher than the LOQ. In general, there was a trend toward fewer samples below the LOQ as the as the putative exposure risk rose (Table 4).

Forestry workers were classified by work task. Those handling cones, either picking from trees or the ground, were assigned to the high exposure group. Subjects conducting ancillary tasks such as loading bins and driving equipment in the orchard were classified as low exposures. Supervisors and office workers comprised the no exposure group. Table 4 shows the concentrations of 3-PBA in the urine by exposure group. Initial statistical evaluation showed no effect of orchard or day, but a significant effect by exposure group. Pair-wise comparison of the differences in least squares means of 3-PBA concentrations showed that only the difference between high and no exposure was significant. When 3-PBA excretion was normalized by creatinine excretion, the group difference was apparent, but less significant; and there was no significance between the high and no exposure groups, although the general trend in concentrations was still apparent. In comparing the data from the forestry workers and those of the no exposure group from laboratory samples (Table 4), there does not appear to be a significant difference in 3-PBA concentrations. The National Report on Human Exposure to Environmental Chemicals measured 3-PBA in their population-based study that includes a wide cross-section of the US population including children. In this study, the geometric mean was 0.321 μg/L (95% confidence interval) and ranged from 0.1 to 11.5 μg/L. They report that much of the population exposure to pyrethroids occur in the diet or residential use of the pesticides [6].

Conclusion

An easy, fast, inexpensive, and sensitive ELISA method was performed for the analysis of 3-PBA in urine. The sample preparation before analysis consisted of hydrolysis and removal of interfering substances using a mixed-mode SPE column. The method provided excellent precision and accuracy for human exposure samples with low levels of 3-PBA as a high-throughput tool for a large number of samples. One ELISA plate can generate 96 data points within 2.5 h, compared to 12 by the LC-MS/MS. Based on calculation of the sample preparation and immunoassay performance it takes a skilled person 1.25 months for 100 urine samples. Each sample costs about $12.00 to run compared to the cost ($50–150 per sample) of instrumental analysis such as LC/MS/MS. With this ELISA method, the LOQ for 3-PBA in urine was 2 μg/L, which is only 2× higher than the reported instrumental analytical methods using GC/MS and LC/MS/MS. Although there is good concordance with LC/MS/MS data for spike samples, further reproducibility studies are needed before it can be used as a stand-alone method. The method provided a simple, rapid assessment of urine samples from forestry workers. Results of the forestry worker study indicate that some work tasks may result in higher exposures, and further study is warranted.

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