CHAPTER 3

EXPERIMENTAL

3.1 Materials and Chemicals

3.1.1 Chemicals and reagents

3.1.1.1 Chemicals

Acetic acid ($C_2H_4O_2$), Disodium hydrogen phosphate dihidrate ($Na_2HPO_4.2H_20$), Hydrogen chloride (HCl), 30% Hydrogen peroxide (H_2O_2), Monosodium dihydrogen monophosphate dihidrate ($NaH_2PO_4.2H_20$), Potassium chloride (KCl), Potassium Dihydrogen Phosphate (KH_2PO_4), Sulfuric Acid (H_2SO_4)Sodium bicarbonate ($NaHCO_3$), Sodium carbonate (Na_2CO_3), Sodium chloride (NaCl) and Sodium hydroxide (NaOH),

All above chemicals were reagent grade or better from Merck (Darmstadt, Germany).

Acetonitrile (Fisher Scientific, Pittsburgh, PA, USA)

Acetylthiocholine iodide, Catalog No. A5751 (Sigma Chemical Co., St Louis, MO, USA)

Butyrylthiocholine iodide, Catalog No. 203989 (Calbiochem Billerica, MA USA)

5,5'-dithiobis-2-nitrobenzoic acid (DTNB), Catalog No. D8130 (Sigma Chemical Co., St Louis, MO, USA)

DMSO, Catalog No. 67-68-5 (Fisher Scientific, Loughborough, UK)

Eserine, Catalog No. E8625 (Sigma Chemical Co., St Louis, MO, USA)

Ethyl acetate, Catalog No. 9280-03(J.T. Baker, Mallinckrodt baker, Inc., Phillipsburg , USA)

Fetal bovine serum, Catalog No. A-7900 (Sigma Chemical Co., St Louis, MO, USA)

Hexane, Catalog No. 9309-03 (J.T. Baker, Mallinckrodt baker, Inc., Phillipsburg ,USA

Methanol, Catalog No. 9693-68 (J.T. Baker, Mallinckrodt baker, Inc., Phillipsburg USA)

Sodium acetate, Catalog No. 567446 (Calbiochem Billerica, MA, USA)

3,3’5,5’-tetramethyl benzidine (TMB), Catalog No. T2885 (Sigma Chemical Co., St Louis, MO, USA)

Trifluoroacetic Acid (Thermo Scientific, Rockford, IL, USA)

Tween-20, Catalog No. 63158 (Sigma Chemical Co., St Louis, MO, USA)

3.1.1.2 Immunological reagents

Goat anti-rabbit IgG-horseradish peroxidase conjugate, Catalog No. A6154 (Sigma Chemical Co., St Louis, MO, USA)

Bovine serum albumin, Catalog No. A-7900 (Sigma Chemical Co., St Louis, MO, USA)

3.1.1.3 Disposable products

Immuno plate Maxisorp 96F, Catalog No. 442404, NUNC, Denmark

Microwell 96F, Catalog No. 167008 (NUNC, Roskilde, Denmark)

3.1.2 Equipments

Micro plate spectrophotometer, Spectra MR, DYNEX Technologies, Inc.

Centrifugation, Kubota 5200

High-performance liquid chromatography/time-of-flight mass spectrometry (HPLC/TOFMS), Micromass, Manchester, UK

3.2 Methods

3.2.1 Organophosphate and carbamate pesticide experimental

3.2.1.1 Study site and population characteristics

The present study was a cross-sectional study designed to assess the exposure to OP and carbamate pesticides among consumers and farmers in an agricultural area in northern Thailand. Farmer volunteers were considered as occupationally exposed subjects. The Fang district of Chiang Mai, one of the intensive agricultural areas in northern Thailand, is composed of 8 sub-districts.

Firstly, we selected top 4 sub-districts of the most intensive agriculture in Fang district and selected randomly the households using the household numbers in each sub-district. Then, we offered enrollment to each selected household's occupants either being farmers or non- farmers (consumers). If they declined, we offered to next selected households until we had the target numbers of volunteers.

The four sub-districts were selected as sites of study, including Mae Kha, Mae Ngon, Wiang, and Mae Ka sub-districts. These 4 sub-districts contained 56% (63,879) of the total Fang district population (112,402) (The department of provincial administration comparison of ratio between total population and consumer group in each province in

Thailand, 2010). The subjects included 100 consumers (50 females, 50 males) and 100 farmers (50 females, 50 males).

Farmers and consumers in these selected sub-districts were sampled using a random number of their household numbers in order to provide the same probability of being selected for the study. They were also 100 proportionally recruited consumers from 4 sub-districts, and each sub-district was recruited as follows: Mae Kha (n = 37), Mae Ngon (n = 19), Wiang (n = 26), and Mae Ka (n = 18). One hundred farmers were also randomly recruited as follows: Mae Kha (n = 20), Mae Ngon (n = 15), Wiang (n = 36), and Mae Ka (n = 29). Numbers of farmers and consumers participated in the study described by sub-district is shown in Table 3.1. This study was approved by the Human Experimentation Committee, Research Institute for Health Sciences (RIHES), Chiang Mai University (N0.32/2006).

Table 3.1 Participant distribution through 4 sub-districts in the Fang district of Chiang Mai, Thailand

Sub-districts	Consumer (n)	Farmer (n)
Mae Kha	37	20
Mae Ngon	19	15
Wiang	26	36
Mae Ka	18	29
Total	100	100

Consumer and farmer groups consisted of 50 males and 50 females.

3.2.1.2 Sample collection and preparation

A consent form was introduced and signed by all participants prior to the collection of blood, saliva, and personal data. The personal data included health status i. e. hypertension, hyperlipidemia, diabetes and taking of the contraceptive pill. Volunteers who take medicines were identified regarding their health status. The process for sample collection was the following:

(1) Unstimulated whole saliva (approximately 5 mL) was collected with cotton wool from volunteers for 5 min after rinsing their mouth with water five times. Sample collection using cotton wool didn't cause any problems with adhesion of molecules, including SChE. As SChE activities measured in spat saliva samples and the cotton wool-collected saliva samples in the present study were significantly correlated for SAChE ($r = 0.763$, $P < 0.01$, $n = 20$) and SBChE ($r = 0.803$, $P < 0.01$, $n = 20$) in Figure 3.1.These samples were collected in the morning between 8.00 and 11.30 h in order to minimize the possibility of diurnal variation effects on activity (Henn *et al.*, 2006; Ryhanen *et al.*, 1983). (2) These samples were collected in ziplock plastic bags and kept in an ice bath during sample collection. The samples were pressed out of the cotton wool until dry and centrifuged at 3000g for 15 min at 4ºC to remove the debris. Then the clean saliva sample was divided into 1 mL aliquots and kept in microcentrifuge tubes at -20ºC in the freezer (Breen, 2002). (3) Approximately 10 mL of blood sample was obtained by venous puncture and collected in heparinized tubes (for red blood cell (RBC) and plasma isolation). (4) Each sample was centrifuged at 2500 rpm for 15 min at 4ºC to separate the plasma and RBC. Then the RBC was washed twice in phosphate buffer saline. The RBC and plasma were aliquoted into 1 mL microcentrifuge tubes and stored at-20ºC in the freezer (Dyer *et al.*, 2001). (5) Structured questionnaires were used to collect

demographic information, factors affecting variability and protection of pesticide exposure among farmers.

3.2.1.3 Development of human salivary cholinesterase assay

The optimal conditions for ChE measuring in saliva and blood are shown in Table 3.2. SAChE and SBChE activities were assessed at 37ºC in a water bath shaker using a slight modification from previous study (Breen, 2002).

The reaction was started by the addition of 750 mL of a 0.1 M phosphate buffer, pH 8, 25 mL of 10 mM 5,5'dithiobis-2-nitrobenzoic acid (DTNB), pH 7, and 100 mL of saliva in test tubes. The test tubes were put on ice to minimize enzyme denaturation in the sample. Then the mixture was incubated for 10 minutes at 37ºC in a water bath shaker before adding 40 mL of 75 mM acetylthiocholine iodide (ATC) for AChE or 40 mL of 75 mM butyrylthiocholine iodide (BTC) for BChE (instantly captured at 9 times). After 30 minutes of incubation, the reaction was stopped by adding 25 mL of 12 mM eserine. The reaction between DTNB and the thiocholine component of the molecules ATC and BTC produces a yellow nitrobenzoate product (see equation below). The changes in colour intensity were measured using an ELISA plate reader at a wavelength of 405 nm. The mixture solution was incubated at 37°C because it is the average internal temperature in humans.

Acetyl/Butyrylthiocholine iodide =======> thiocholine iodide + acetate/butyrate

thiocholine + dithiobisnitrobenzoate ======> yellow colored products*

* Products of the reaction are 2-nitrobenzoate-5 mercaptothiocholine and 5-thio-2-nitrobenzoate (The latter is the yellow product)

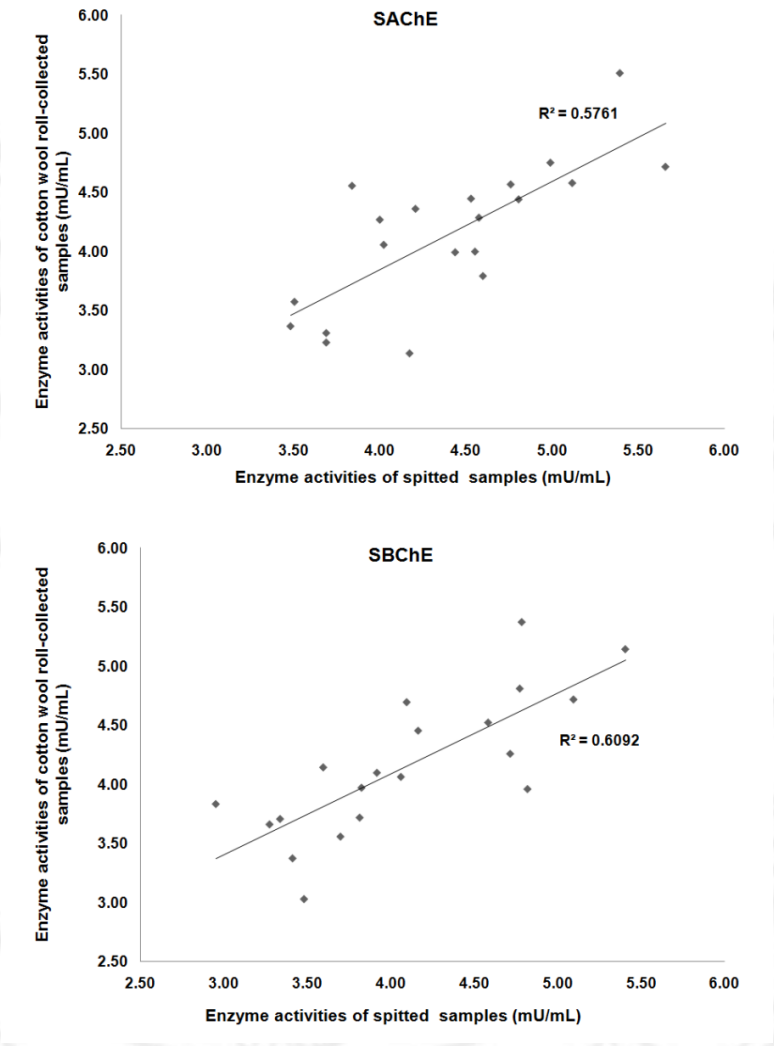

Figure 3.1 Bivariate scattergram of SAChE and SBChE activities between using the spitted samples and the cotton wool-collected samples were significantly correlated for SAChE ($r = 0.763$, $P < 0.01$, $n = 20$) and SBChE ($r = 0.803$, $P < 0.01$, $n = 20$).

RAChE and PBChE activities were assayed at 25ºC in a water bath shaker using a slight modification of previous study (Ellman *et al.*, 1961). The reaction was started by the addition of 3mL of 0.5 mMDTNB, pH 7.7 and 100 mL of the diluted sample (RBC : water = 1 : 100, plasma : water = 1 : 50). The test tubes were put on ice to minimize enzyme denaturation in the sample.

Then the mixture was incubated for 10 minutes at 25ºC in a water bath shaker before adding 10 mL of 156 mM ATC for RAChE or 40 mL of 156 mM BTC for PBChE (instantly capture at times). After 30 minutes of incubation, the reaction was stopped by adding 25 mL of 12 mM eserine, and the absorbance at 405 nm was read by a spectrophotometer. The mixture was incubated at 37ºC because this condition reduced color intensity from RAChE compared to 37ºC.

The activity of the enzyme in the sample was calculated from the equation below (Ellman *et al.*, 1961):

$$\text{Activity (U/mL)} = \frac{\Delta \text{ Abs} \times \text{Total volume (mL)} \times \text{Dilution factor} \times 10^3}{\varepsilon \times \text{Sample volume (mL)} \times \text{Incubation time (minutes)}}$$

$$\Delta \text{ Abs} = \Delta \text{ Abs}_{\text{test}} - \Delta \text{ Abs}_{\text{sample blank}}$$

$$\varepsilon \text{ (Extriction Coefficient at 405 nm)} = 13{,}600$$

3.2.1.4 Quality assurance and control

3.2.1.4.1 The intra- and inter-assay coefficients of variation (%CV)

Prior to analysis of the samples, saliva and blood controls (n =20) were used to calculate the optimum condition variance (OCV) range (mean±2 standard deviation (SD)). The accuracy of the methods was monitored during analysis of the samples. In each batch of samples (n = 10), there were 1 blank sample and 3 controls (pooled saliva or blood) that were measured first, in the middle, and last, and all were run in duplicate and analyzed in the intra-assay to obtain within-day %CV. The inter-assay of the samples was analyzed between days from all batches (n = 20) to obtain between-day %CV calculated from the mean concentration divided by the SD from all controls.

3.2.1.4.2 Limit of blank, limit of detection and limit of quantitation

The sensitivity of methods was measured using the limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) as terms to describe the smallest concentration of a measurement that can be reliably measured by an analytical procedure (Armbruster *et al.*, 2008). The activities of all ChE in the present study will be higher than the LOQ.

The LOB is the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested.

The LOD is the lowest analyte concentration likely to be reliably distinguished from the LOB and at which detection is feasible. The LOD is determined by utilising the measured LOB and test replicates of a pooled sample (control) which has a low concentration of the analyte.

$$LOD = LOB + 1.645(SD_{control})$$

The LOQ is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LOQ may be equivalent to the LOD or it could be at a much higher concentration.

$$LOB = mean_{blank} + 1.645(SD_{blank})$$

Table 3.2 The optimal condition for ChE measuring in saliva and blood

Chemicals	Saliva	blood
Phosphate buffer, pH 7-8	0.1M (750 µL)	0.5 mM (3 mL) of DTNB in 5 mM buffer
DTNB, pH 7	10mM (25 µL)	
Sample	100 µL of saliva	100 µL of diluted sample (RBC: water = 1 : 100, Plasma : water = 1 : 50)
ATC (AChE)	75 mM (40 µL)	156 mM (10 µL)
BTC (BChE)	75 mM (40 µL)	156 mM (40 µL)
Incubation	30 minutes at 37°C in a shaker	15 minutes at 25°C in a shaker
Eserine	12 mM (25 µL)	12 mM (50 µL)
Wavelength	405 nm	405 nm

DTNB = 5,5'-dithiobis-2-nitrobenzoic acid, ATC = acetylthiocholine iodide, BTC = butyrylthiocholine iodide, RBC = red blood cell, RT = room temperature

3.2.1.5 Statistical analysis

Data from laboratory results and questionnaires were analyzed using non-parametric methods by the Statistical Package for the Social Sciences (SPSS) version 17 as follows;

(1) the median for ChE activities were computed, (2) the correlation of AChE and BChE activities between saliva and blood was analyzed using the Spearman test, (3) the correlation of SAChE and SBChE activities between using the spat and the cotton wool-collected samples were analyzed using the Spearman test, (4) correlation of the disease status, i. e. hypertension, hyperlipidemia, diabetes and taking of the contraceptive pill, and all ChE activities were analyzed using the Spearman test.

3.2.2 Synthetic pyrethroid pesticides experimental

3.2.2.1 Study site and population characteristics

Study site and population characteristics are the same as in 3.3.1

3.2.2.2 Developing of sample preparation step and sensitivity ELISA method

3.2.2.2.1 Sample collection

For synthetic pyrethroid pesticides, the volunteers were asked to collect blood and urine. The methodology for blood collection is the same as in 3.3.2 but urine collection following; the morning urine was collected in 50 mL polypropylene bottom. Samples were immediately labeled and stored in a cooler with frozen ice packs for transportation to the RIHES. The urine was shaken vertically for 30 seconds to complete homogenize the sample and left to stand for about 2 hours in cold room (10°C). The supernatant was aliquot into a 15-mL polypropylene tube, labeled, and stored at −20°C until analysis.

3.2.2.2.2 Plasma sample preparation

Alkaline hydrolysis: A 0.1 mL aliquot of 6N sodium hydroxide (NaOH) was added to 0.5 mL of homogenized plasma and placed in a heating block at 100°C for 1 hr.

Liquid-liquid extraction (LLE): In Figure 3.2, 1 mL of 0.2 M sodium acetate buffer, pH 4.5 was added to adjust the pH to around 12 to obtain the ionic form (hydrophilic) of 3-PBA. For the cleanup step, 2 mL of ethyl acetate was added to the sample. The ethyl acetate removes organic contaminants while the 3-PBA remains in the aqueous phase. After shaking vigorously for 10 min and centrifugation, the aqueous phase (lower phase) was collected.

The remaining aqueous phase was acidified (pH around 3) to produce the nonionic form of 3-PBA (hydrophobic) by adding 120 µL of 6N hydrochloric acid (HCl). It was extracted twice with 2 mL of ethyl acetate. Following shaking and centrifugation, the organic phase (upper phase) was combined and evaporated to dryness with a gentle stream of nitrogen. The residue was dissolved in 200 µL of methanol. Two milliliters of 0.2 M sodium acetate buffer was added to adjust pH to around 5 and mixed thoroughly.

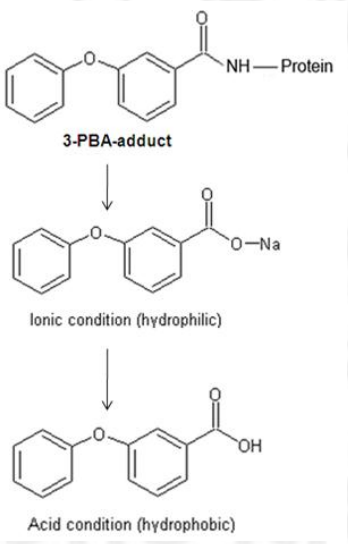

Figure 3.2 Forms of 3-PBA in the plasma sample during alkaline hydrolysis and the LLE method

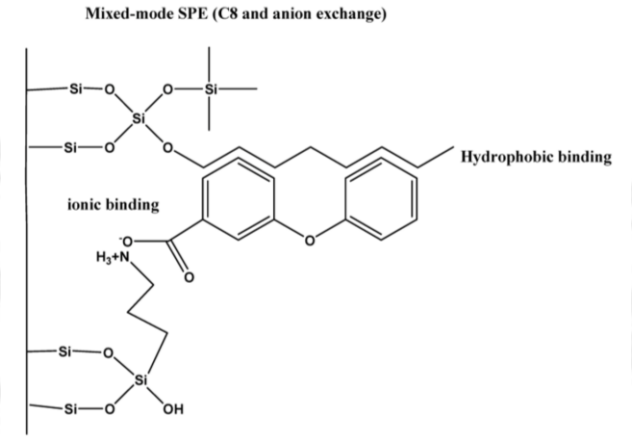

Figure 3.3 The target molecule (3-PBA) linked to dual mode SPE (C8 and anionic exchange) cartridge

Solid phase extraction (SPE): The SPE method was modified from a previously reported method (Ahn *et al.*, 2007) using mixed-mode solid phase extraction (C8+anion exchange). The SPE cartridge used was the Strata screen-A (55 µm, 70 Å), 100 mg / 1 mL containing the mixed mode silica-based sorbent. The mixed-mode SPE hydrophobic (C8) and ionic (anion exchange) phases are shown in Figure 3.3. A set of SPE tubes was placed into the vacuum manifold. The liquid was allowed to flow through under gentle vacuum at a flow rate of about 2-3 mL/min for each addition of liquid to the tube.

The mixed mode cartridge was preconditioned with 1 mL of methanol, 1 mL of water and 1 mL of 0.2 M sodium acetate buffer (pH 4.5). The extracted plasma was loaded onto the cartridge and subsequently washed with 1 mL of water and 1 mL of methanol, sequentially to reduce matrix effects. The cartridge was dried under high vacuum (10 mmHg) for 5 min. After the test tube was replaced, the 3-PBA was eluted with 1.5 mL of 1% acetic acid in a mixture of hexane and ethyl acetate (70 : 30 v/v). The eluate was dried under nitrogen. The residue was re-dissolved in methanol (0.25 mL)

followed by addition of 2.25 mL of phosphate buffered saline (PBS) to make a 5 fold dilution based on the original plasma amount (500 µL) prior to ELISA analysis.

3.2.2.2.3 Urine sample preparation

The method was slightly modified from the previous report (Ahn *et al.*, 2007; Kim *et al.*, 2009). Briefly, 0.5 mL aliquot of urine was added into a glass vial. The urine sample was hydrolyzed using 100 µL of 6 N hydrochloric acid (HCl) and heating at 100°C for 1 hr. The hydrolyzed urine was neutralized by adding 1 mL of 0.2M sodium acetate buffer (pH 4.5) followed by 100 µL of sodium hydroxide (6N) and mixing thoroughly to adjust the pH to around 4.5. To reduce the matrix effect of the hydrolyzed urine, the same SPE method as for the plasma 3-PBA was used**.**

3.2.2.2.4 Competitive indirect enzyme-linked immunosorbent assay

3.2.2.2.4.1 Immunoreagents

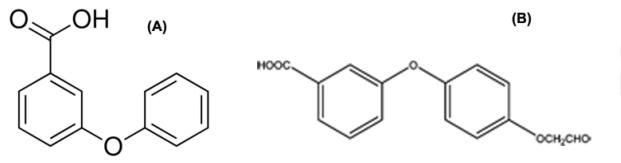

Figure 3.4 Comparison between 3-PBA (A) and hapten (B) in this study (Shan *et al.*, 2004)

The specific antibody and hapten for the target analyte (3-PBA) were previously described (Shan *et al.*, 2004). In hapten design, it is important to consider the structure of hapten that similar to the target compound in Figure 3.4 (Szurdoki *et al.*, 2002).

Briefly, two New Zealand white rabbits were immunized with 3-[4-(3-carboxyphenoxy) phenoxy] N-thyroglobulin ethylamine. The antigen solution, 100 µg in PBS, were emulsified with Freund's complete adjuvant (1 : 1, v/v) and injected subcutaneously.

After 1 month, the animals were boosted with additional immunogen (100 µg) that was emulsified with Freund's incomplete adjuvant (1 : 1 v/v). Booster injections were given at 4 week intervals. The rabbits were bled about 10 days after each boost. The serum was isolated by centrifugation at 1500 rpm for 10 min at 4 °C. The results of antibody characterization were obtained from sera of terminal bleeds after four boosters. This terminal bleed of rabbit no. 294 was used for ELISA development.

3.2.2.2.4.2 Competitive indirect enzyme-linked immunosorbent assay

The specific antibody and hapten for the target analyte (3-PBA) and the preparation of coating antigen, the buffers and the procedure for the indirect competitive ELISA was previously described (Shan *et al.*, 2004). Briefly, 96-well plates were coated overnight at 4 °C with 100 µL of coating antigen, 3-PBA-BSA, (0.5 µg/mL). The following day, plates were blocked for 30 min with 200 µL 0.5% BSA in PBS (phosphate buffered saline) at room temperature. After 30 min the blocking solution was removed. Then 50 µL of extracted samples (plasma or urine) and 50 µL of antiserum (1 : 7000) were added to each well and mixed for 1 hr at room temperature. The plates were washed five times. The goat anti-rabbit IgG-horseradish peroxidase conjugate diluted 1/10000 (100 µL) was added for 1 hr. Following a wash, the TMB substrate (100 µL) was added for 15 min then the reaction stopped and absorbance measured at 450 nm.

The concentrations of unknown samples were calculated from the calibration curve using a 4-parameter fit equation and expressed as 3-PBA equivalents. Urinary 3-PBA

concentrations were normalized to creatinine levels to correct for dilution of urine and is often used to normalize when spot samples are taken rather than 24 hr samples (Barr *et al.*, 2005). The IC_{50} value is expressed as the sensitivity of the immunoassay and approximates the concentration of analyte giving 50% inhibition.

3.2.2.3 Quality assurance and control

For laboratory quality assurance and control, precision measurements were conducted prior to the analysis of real samples. Plasma and urine controls (10 tubes) were analyzed for day-to-day variation of samples to obtain an optimum condition variance (OCV) range (mean±2SD). Each plasma analysis batch consisted of 15 samples, 1 sample blank and 2 controls using pooled plasma that were placed in the first and last tubes. For urine, 25 samples were analyzed in each batch with 3 pooled urine controls placed in the first, middle and last tubes. The controls in each batch were analyzed for intra-batch variation of samples to obtain within-day CV (%). Inter-batch variation of samples was analyzed from controls in all batches analyzed (n = 14 and 9, respectively) to obtain between-day CV (%).

3.2.2.4 Statistical analysis

Data from laboratory results were analyzed by SPSS Statistical Software Package version 17 as follows; (1) the concentrations of 3-PBA were tested for normal distribution (parametric or nonparametric), (2) % detection, median and range of 3-PBA concentrations were computed, (3) the plasma 3-PBA and urinary 3-PBA concentrations were compared between consumers and farmers using the paired sample t-test, (4) the

correlation between plasma 3-PBA and urinary 3-PBA concentrations was analyzed using the Spearman test.

3.2.2.5 High-performance liquid chromatography/time-of-flight mass spectrometry (HPLC/TOFMS)

3.2.2.5.1 High resolution mass spectrometry

All the exact mass measurement experiments were performed in positive mode, on Micromass LCT an orthogonal acceleration-Time-of-Flight (oa-TOF) mass spectrometer (Micromass, Manchester, UK) configured with dual sprayer electrospray ion source, a standard Z-spray electrospray (ES) ionization source and a Lock-spray source that samples analyte and reference ions independently. The mass spectrometer has been equipped with a 4 GHz time-to-digital converter (TDC).

3.2.2.5.2 Ion source conditions

Ion source parameters were: capillary voltage 3200 V, sample cone voltage 35 V, extraction cone voltage 5 V, source temperature 110 °C and desolvation temperature 300 °C. Transfer optics settings were follows: rf lens 375 V, rf dc offset-1, 6.0 V, rf dc offset-2, 4.0 V aperture, 4.0 V, acceleration 200.0 V, focus 0.0 V and steering, -1.0 V. Analyzer settings were as follows: MCP (multichannel plate) detector 2700 V, ion energy 40.0 V, tube lens, -1.0 V, grid-2 5.0 V, TOF flight tube 4578 V and reflectron 1813 V.

The pusher cycle time was 75 μs, data files were acquired in continuum mode and spectra were stored from m/z 100 to 2100 with a 2.1 second scanning cycle consisted of a 2.0 second scan and a 0.1second inter-scan time. Each averaged spectrum stored to the

data system, represented an average of 2,000 individual spectra. Typically 20-30 individual spectra were summarized. The cone gas and desolvation gas was set to 12 and 730 L/hour, respectively. Lock spray parameters were identical to sample setting parameters. Lock spray sampling frequency mode was set to 4, i. e. every 4^{th} spectrum generated was the signal from the lock spray inlet. Mass resolution was set to 6000.

3.2.2.5.3 TOF calibration and lock-mass set up

Lteff (effective length of the flight tube) value has been set to 1125.0700 using molecular ions of Leucine Enkephalin at 556.2771 Th. This value is responsible to set the zero cut on the calibration (error) function (5^{th} order polynomial). System calibration was performed using Poly-D-L-alanine (P9003, Sigma, MO, USA) in both, positive and negative mode; a 50 ng/mL solution of Leucine Enkephalin (L9133, Sigma, MO, USA) has been infused at 5 μL/min into the lock spray (positive ion lock mass: 556.2771 Th) using ISCO μLC-500 micro flow pump.

To obtain accurate masses the following procedure was performed: Savitsky-Golay smoothing using ± 4 channel window, repeated twice and centering, using the center value at the 80% height of the peak. After data acquisition and signal averaging, spectra were individually corrected relative to the Leucine Enkephalin lock-mass calibration mass via standard procedure. Samples were introduced to the mass spectrometer via direct flow injection using Waters Alliance 2795 (Bedford, MA, USA) HPLC system was used for solvent delivery at the flow rate of 25 μL/min, mobile phase ACN/H_2O (1/1).

3.2.2.5.4 HPLC separation

Waters Alliance 2795 (Bedford, MA, USA) HPLC system was used for solvent delivery at the flow rate of 0.35 mL/min.

3.2.2.5.5 Mobile phase, gradient

Solvents- A: D.I. water 99.9, and trifluoroacetic Acid, 0.1 volume %, B: acetonitrile 99.9, trifluoroacetic acid 0.1 volume %. gradient: 0 -5 min 20 % B, 105 min 80% B, 110 min 100 % B, 116 min 100 % B, 117 min 20 % B, 120 min 20 % B. Sample temperature was kept on 20 °C.

3.2.2.5.6 Column

For separation Waters Symmetry300, C18, 250X4.6 mm, 5 μm HPLC column was used. Column temperature was kept on 20 °C. Instrument back pressure has not exceeded 150 bar.

3.2.2.5.7 UV-VIS detector

For UV-VIS signal detection Waters 996 PDA detector was used, wavelength range 210-550 nm, resolution 1.2 nm, with sampling rate of 1 spectrum/s.

3.2.2.5.8 Data processing

Data acquisition, instrument control was performed by MassLynx software version 4.0 sp 3, also used for data evaluation and visualization.