CHAPTER III

RESULTS

1. Dose-response bioassay test of mosquito larvae

The results of larval susceptibility test of the PMD and PMD-R strains to permethrin with and without synergists, PBO (an oxidase inhibitor) and BNPP (an esterase inhibitor) were presented in Table 7 and Figure 10. No mortality was observed in the control group. Without synergist, the LC_{50} value of the PMD-R (25.42 ppb) was ~ 25-fold higher than the PMD (1.02 ppb). Addition of esterase inhibitor (BNPP) appeared to have no effect on the LC_{50} value of the PMD (1.30 ppb), but a small effect on the PMD-R was found (19.58 ppb). By contrast, when the oxidase inhibitor (PBO) was added, the LC_{50} values were significantly reduced 3.03-fold in the PMD-R (8.39 ppb) and 2.27-fold in the PMD (0.45 ppb) compared with the original levels. The synergism of permethrin by PBO has suggested that oxidative metabolism be involved in permethrin resistance in both PMD and PMD-R strains.

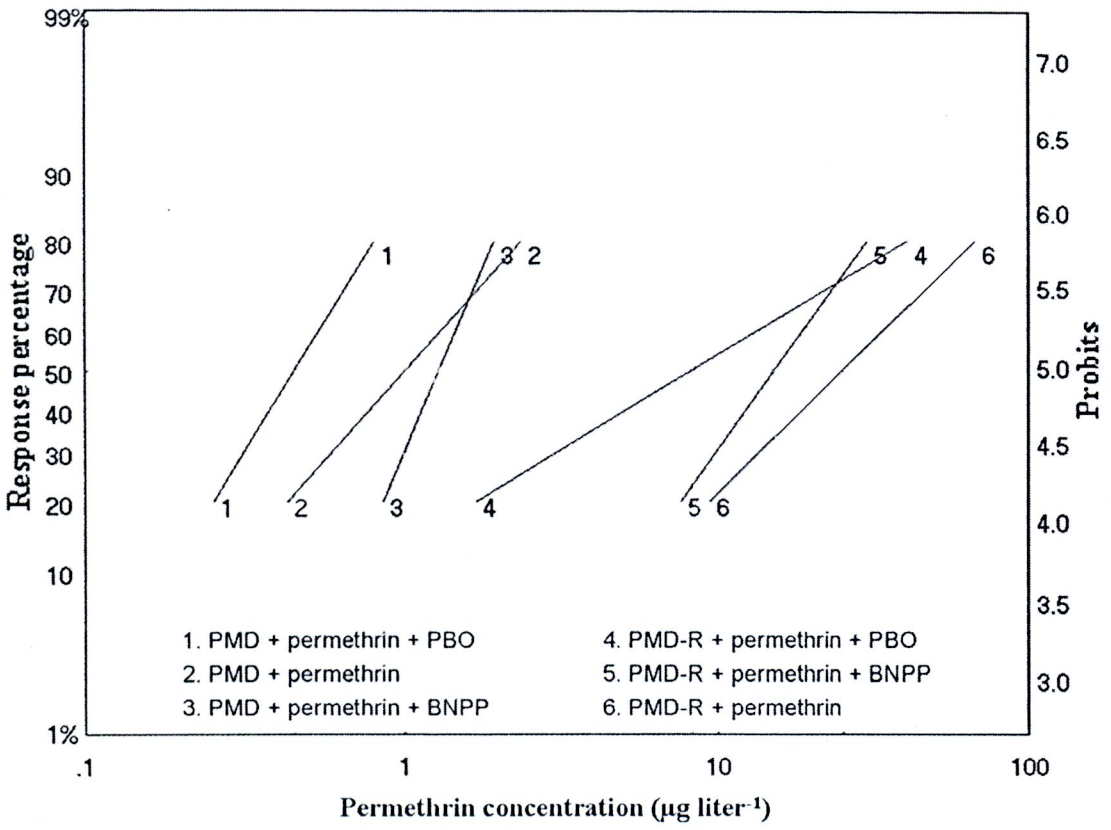

Figure 10 The log dose-probit mortality lines of the PMD and PMD-R strains after exposure to permethrin with and without synergists (PBO and BNPP).

Table 7 Susceptibility of PMD and PMD-R larvae to permethrin in the presence of PBO or BNPP after 24-h exposure

Strains	Permethrin		Permethrin + BNPP			Permethrin + PBO		
	LC_{50} (95%CI)	Slope(SE)	LC_{50} (95%CI)	Slope(SE)	SR	LC_{50} (95%CI)	Slope(SE)	SR
PMD	1.02(0.87-1.29)	2.26(0.28)	1.30(1.18-1.45)	4.73(0.42)	0.78	0.45(0.41-0.50)	3.32(0.28)	2.27
PMD-R	25.42(21.38-30.29)	1.98(0.16)	19.58(16.26-23.51)	1.83(0.15)	1.30	8.39(6.85-10.31)	1.22(0.08)	3.03

Values are parts per billion.

SE = standard error

SR = synergist ratio (LC_{50} observed in the absence of synergist/LC_{50} observed in the presence of synergist)

CI = confidence interval

2. Determination of P450s levels

The equivalent units of total P450s levels of three enzyme fractions, crude supernatant, cytosol and microsomes of PMD and PMD-R were determined by indirect assay with tetramethyl benzidine (TMBZ) (Penilla *et al.*, 2007). The total P450s levels were measured using the cytochrome C standard curve (Table 8, Figure 11).

Since the dithiothreitol (DTT), reducing agent, which used in the enzyme preparation had interfered with the amount of P450s; therefore, three enzyme fractions of PMD and PMD-R were purified by PD-10 desalting column before determination. The results showed significantly increasing of P450s levels in all enzyme fractions in both strains after purification ($p < 0.05$) (Table 9). In PMD, the P450s levels were increased from 0.216±0.03, 0.320±0.02 and 0.383±0.03 to 0.562±0.02, 0.718±0.03 and 1.493±0.03 equivalent units of cytochrome P450/mg protein in crude supernatant, cytosol and microsome, respectively (Table 9). Similarly in PMD-R, the P450s levels were increased from 0.223±0.02, 0.205±0.03 and 0.537±0.05 to 0.526±0.03, 0.639±0.01 and 1.466±0.04 equivalent units of cytochrome P450/mg protein in crude supernatant, cytosol and microsome, respectively (Table 9).

Comparison between the P450s contents in each enzyme fraction after PD-10 desalting purification, the levels of P450s in crude supernatant had as much as in cytosol while the P450s levels were significantly higher in microsomal fraction. However, the P450s levels were no significant difference between the PMD and PMD-R strains within each fraction ($p > 0.05$) (Figure 12). The microsomal fractions were used for further study in vitro permethrin metabolism.

Cytochrome C standard curve

Five concentrations of standard cytochrome C were prepared and the absorbances were read at 650 nm (Table 8).

Table 8 Absorbance values of 5 concentrations of standard cytochrome C

Cytochrome C (nmol/20 µl)	Absorbance (650 nm)
0.0025	0.020
0.005	0.048
0.01	0.263
0.02	0.799
0.04	2.229

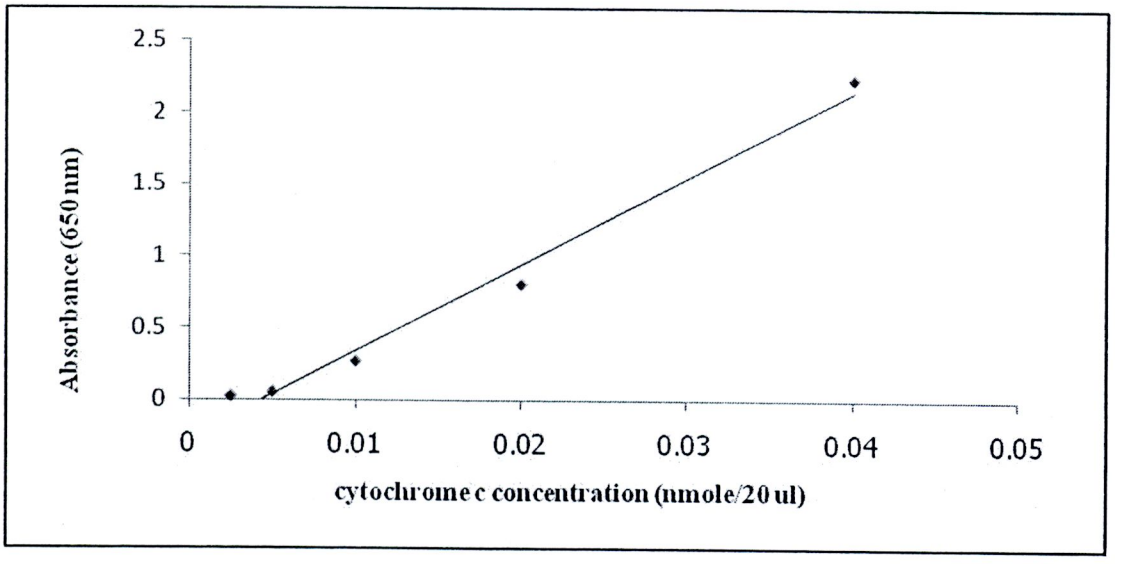

Figure 11 Cytochrome C standard curve. The cytochrome C standard curve was used to measure the P450s levels. The curve had an R^2 value of 0.985 and the equation used was $y = 60.21x - 0.261$.

Table 9 Equivalent units of cytochrome P450s content in different enzyme fractions of PMD and PMD-R strains compared between with and without PD-10 desalting purification

Strains	Equivalent units of cytochrome P450/mg protein		
	Crude supernatant	**Cytosol**	**Microsome**
No purification			
PMD	0.216±0.03	0.320±0.02	0.383±0.03
PMD-R	0.223±0.02	0.205±0.03	0.537±0.05
Purified with PD-10 desalting column			
PMD	0.562±0.02	0.718±0.03	1.493±0.03
PMD-R	0.526±0.03	0.639±0.01	1.466±0.04

Values are represented as means±SD (n=3).

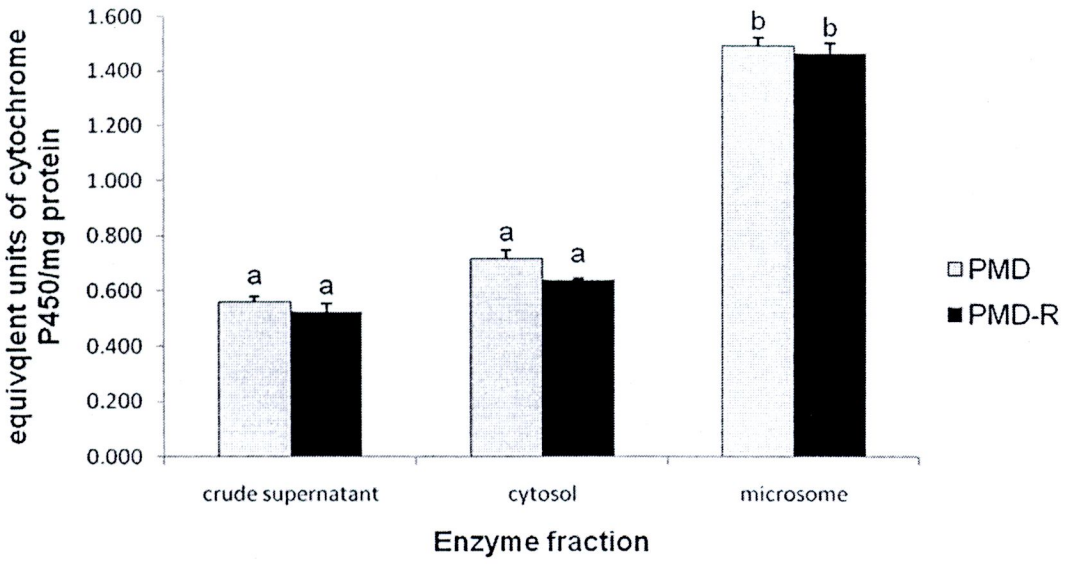

Figure 12 The equivalent units of total P450/mg protein in the PMD and PMD-R strains after PD-10 desalting purification. The different letters (a and b) indicate a significant difference ($p < 0.05$) among the enzyme fractions.

3. HPLC method validation

3.1 Linearity

The linearity of permethrin, PBOH, PBCHO and PBCOOH under the range of seven concentrations (2.5 - 80 ng/10 µl) and their peak areas were shown in Tables 10-13 and Figures 13-16. The regression equations and the correlation coefficient values (R^2) were presented in Table 14. The results showed all standard samples had the R^2 values > 0.99 which proved the acceptable linearity between peak areas and concentrations.

3.2 Accuracy and Precision

Ten repeatabilities of individual of PBOH, PBCHO and PBCOOH were determined for accuracy and precision of the method (Table 15). The percentages of recovery of each repeatability were in acceptable range (80 - 120%), % RSD of each sample was not exceeded 20% and the HORRAT values also in range (< 2). These results suggested that this method had an acceptable accuracy and precision.

3.3 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ values were calculated from 3SD and 10SD of the blank. The LOD and LOQ values of PBOH, PBCHO and PBCOOH were shown in Table 15.

Table 10 Detected peak areas of seven concentrations of standard permethrin

Repeatability	Peak areas of permethrin concentrations (ng/10 µl)						
	2.5	**5**	**7.5**	**10**	**20**	**40**	**80**
1	7575	19126	40356	92465	208464	239129	547308
2	9119	18223	42047	93944	202224	254322	539113
3	10026	19486	44572	92831	206377	254310	544634
Mean	8906.6	18945.0	42325.0	93080.0	205688.3	249254.0	543685.0

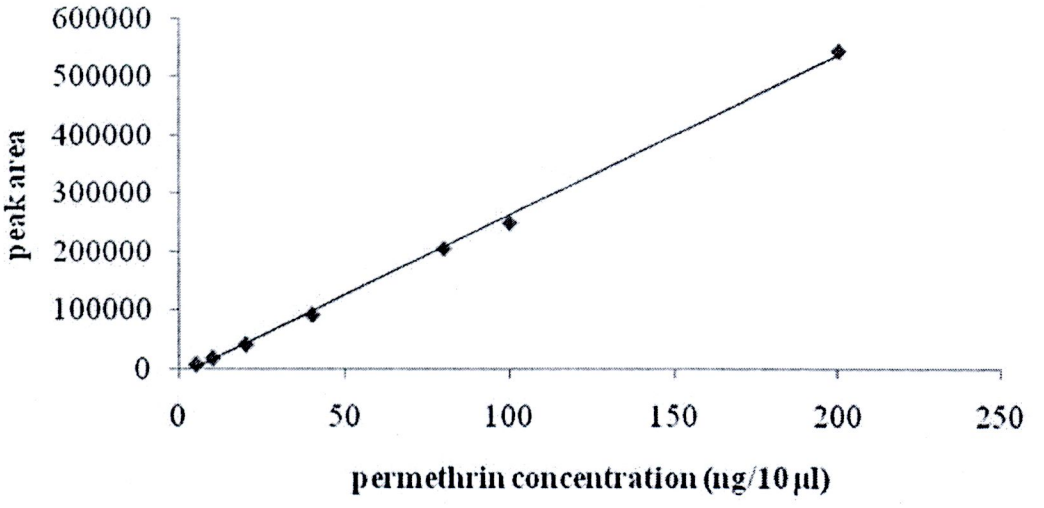

Figure 13 Linearity curve of permethrin concentrations and peak areas. The curve had the R^2 value of 0.9986 and the equation used was y = 2742.6x – 12287.

Table 11 Detected peak areas of seven concentrations of standard PBOH

Repeatability	Peak areas of PBOH concentrations (ng/10 µl)						
	2.5	**5**	**7.5**	**10**	**20**	**40**	**80**
1	4170	8379	16283	18568	46640	97358	201928
2	4968	7885	17234	23726	42006	98058	195158
3	3964	10341	17161	21566	46216	99578	202724
Mean	4367.3	8868.3	16892.7	21286.7	44954.0	98331.3	199937.0

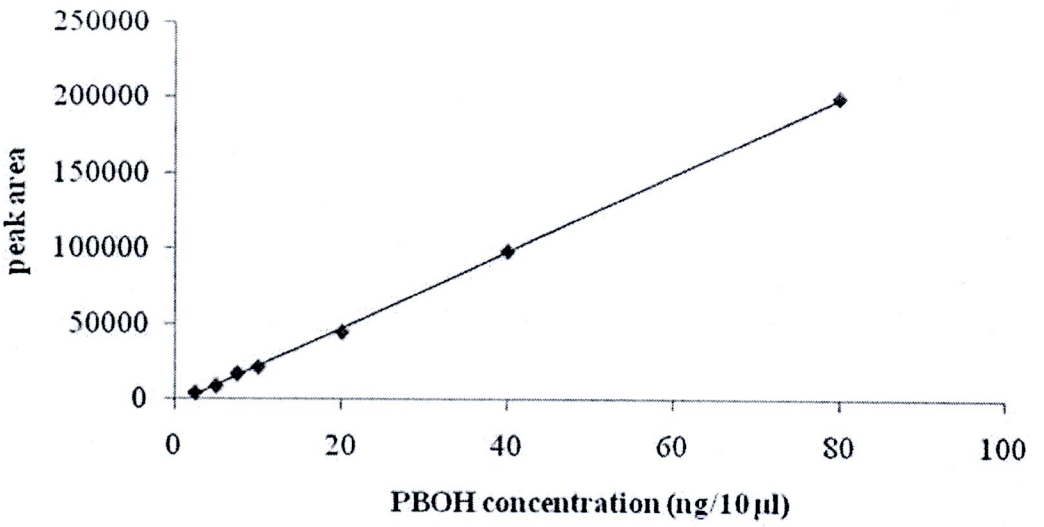

Figure 14 Linearity curve of PBOH concentrations and peak areas. The curve had the R^2 value of 0.9997 and the equation used was $y = 2537.1x - 2436.8$.

Table 12 Detected peak areas of seven concentrations of standard PBCHO

Repeatability	Peak areas of PBCHO concentrations (ng/10 µl)						
	2.5	**5**	**7.5**	**10**	**20**	**40**	**80**
1	3831	8714	27299	22564	157640	308985	444491
2	6958	10272	33208	75593	92091	359490	411685
3	4676	11441	36404	55905	140366	318804	454754
Mean	5155.0	10142.3	32303.7	51354.0	130032.3	329093.0	436977.0

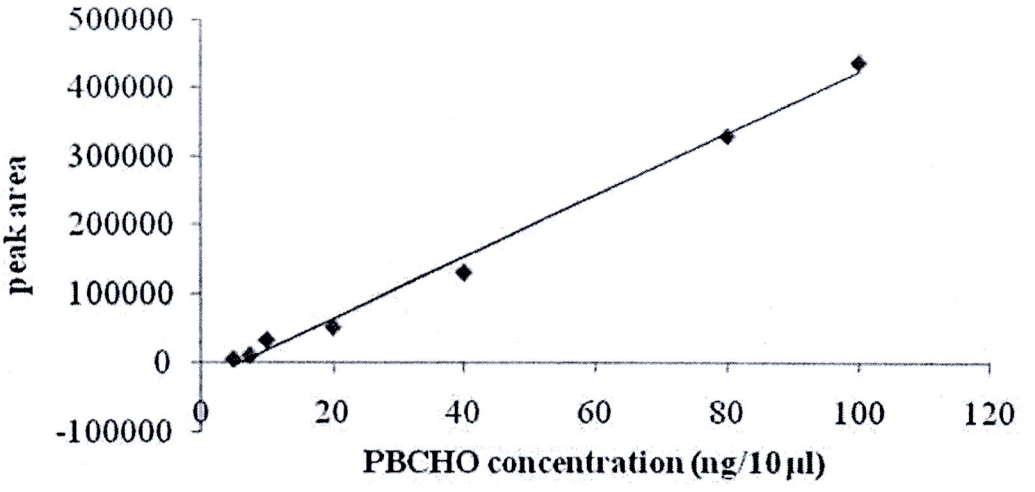

Figure 15 Linearity curve of PBCHO concentrations and peak areas. The curve had the R^2 value of 0.9934 and the equation used was $y = 4505.1x - 26792$.

Table 13 Detected peak areas of seven concentrations of standard PBCOOH

Repeatability	Peak areas of PBCOOH concentrations (ng/10 µl)						
	2.5	**5**	**7.5**	**10**	**20**	**40**	**80**
1	20243	34297	32164	59255	123576	291946	412273
2	25799	28160	36655	53661	119324	294050	396559
3	20220	25671	31045	59712	137657	298069	402067
Mean	22087.3	29376.0	33288.0	57542.7	126852.3	294688.0	403633.0

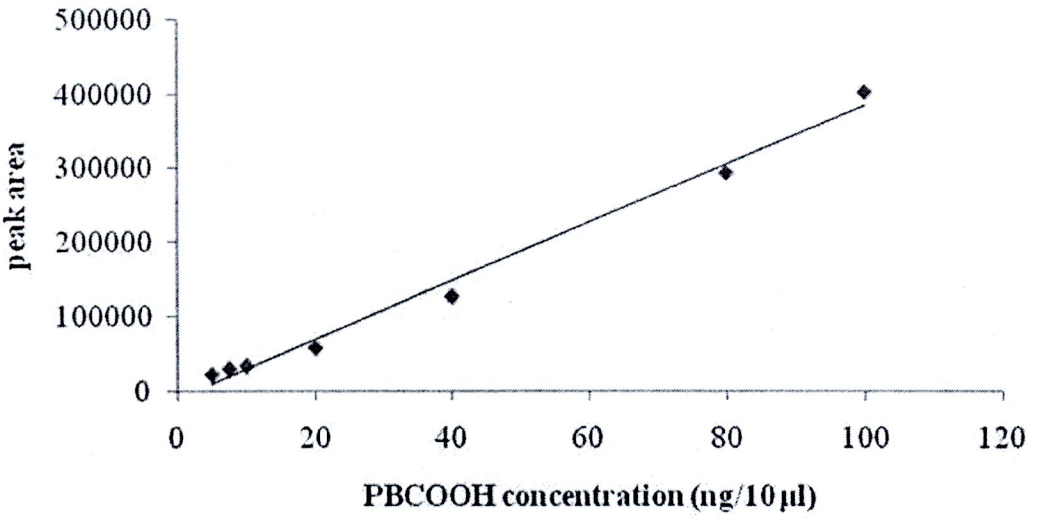

Figure 16 Linearity curve of PBCOOH concentrations and peak areas. The curve had the R^2 value of 0.9905 and the equation used was $y = 3953.9x - 10060$.

Table 14 The regression equation and the correlation coefficient of permethrin, PBOH, PBCHO and PBCOOH from linearity calibration curve

Compounds	**Regression equation**	**Correlation coefficient (R^2)**
Permethrin	y = 2742.6x - 12287	R^2 = 0.9986
PBOH	y = 2537.1x - 2436.8	R^2 = 0.9997
PBCHO	y = 4505.1x - 26792	R^2 = 0.9934
PBCOOH	y = 3953.9x - 10060	R^2 = 0.9905

Table 15 Concentrations of standard PBOH, PBCHO and PBCOOH after fortified with 2.5 ng/10 µl

Repeatability	Detected concentration (ng/10 µl)			% recovery
	PBOH	PBCHO	PBCOOH	
1	1.65	1.70	1.77	85.9
2	2.01	1.77	1.93	92.1
3	1.94	1.83	1.96	89.4
4	1.79	1.86	1.84	85.9
5	1.84	1.75	2.05	87.5
6	1.95	1.95	1.77	94.1
7	1.93	1.97	1.82	89.6
8	1.88	1.76	2.09	89.9
9	1.76	1.88	2.11	89.1
10	1.76	1.67	1.92	85.1
Mean	1.85	1.81	1.93	
SD	0.11	0.10	0.13	
%RSD	5.95	5.52	6.73	
C (concentration)	1.85×10^{-9}	1.81×10^{-9}	1.93×10^{-9}	
Predicted Horwitz RSD	27.29	27.29	27.05	
HORRAT	0.22	0.20	0.25	
LOD	0.33	0.30	0.39	
LOQ	1.10	1.10	1.30	

%RSD = SD x 100 / Mean

Predicted Horwitz RSD = $0.66 \times 2^{(1-0.5\log C)}$

HORRAT = %RSD / Predicted Horwitz RSD

Limit of detection (LOD) = 3SD

Limit of quantification (LOQ) = 10SD

Preliminary study of permethrin metabolism by microsomal enzymes

Experiment I: Optimization of microsomal protein

The purpose of this experiment was to optimize the microsomal protein for the in vitro permethrin metabolism. Permethrin and its metabolic products were determined after incubation with different amount of microsomal protein, 0.2, 0.4 and 0.8 mg of microsomes (Table 16) (Choi *et al.*, 2002). The results showed that very small amounts of metabolites, i.e. phenoxybenzoic acid (PBCOOH) were detectable. Nevertheless, PBCOOH formations were much produced when incubated with more microsomal protein. PBCOOH formations were approximately equal detected (0.24±0.02 nmole/min/mg protein) when incubated with 0.2 or 0.4 mg of microsomes, but there were higher detected (0.40±0.03 nmole/min/mg protein) when incubated with 0.8 mg of microsomal protein. Addition of NADPH regenerating system in incubation with 0.2 or 0.4 mg of microsomes, PBCOOH was slightly increased (approximately 0.28±0.05 nmole/min/mg protein), while there were significantly increased when incubation with 0.8 mg of microsomes (0.60±0.17 nmole/min/mg protein). However, incubation permethrin with 1 mg of microsomes had been done, but it could not be extracted by chloroform. Therefore, the 0.8 mg of microsomes was the optimum use for in vitro permethrin metabolism.

Experiment II: Purified microsomal fraction with PD-10 desalting column

From the study of P450s levels, the results revealed that the amount of P450s was interfered with the dithiothreitol (DTT). This experiment was to clarify the interference of DTT to P450s activity, and hence the microsomal fractions were expected to have induced P450s activity after purification with PD-10 desalting column. The metabolite products were detected after incubating the substrates i.e. permethrin, PBOH and PBCHO with 0.8 mg of PMD-R microsomes compared between with and without PD-10 desalting column purification. The results showed clearly higher metabolite products whether permethrin, PBOH or PBCHO were incubated with purified microsomal fractions (Table 17).

When permethrin was used as a substrate, the metabolic product PBCOOH was increased from 1.58±0.31 to 2.76±0.12 nmole/min/mg protein after incubated with purified microsomal fractions while PBOH and PBCHO were rarely detected (Table 17, Reactions 1, 4). It obviously demonstrated when PBOH or PBCHO were used as substrates, in which PBCOOH was increased from 13.44±0.24 to 27.28±0.76 and 29.87±2.10 to 41.71±4.60 nmole/min/mg protein, respectively. From these results, microsomal fractions were purified with PD-10 desalting column to reduce the interference of DTT before using in the in vitro permethrin metabolism.

Table 16 Permethrin and its metabolite products, PBOH, PBCHO and PBCOOH formations after incubation with different PMD-R microsomal protein (Mic) in the presence of NADPH

Reactions	Permethrin*	Metabolite products*			% recovery
		PBOH	PBCHO	PBCOOH	
0.2 mg microsomal fraction					
1. Mic(PMD-R) + Permethrin	116.12±2.67	0.12±0.00	No peak	0.23±0.02	91.3
2. Mic(PMD-R) + Permethrin + NADPH	117.73±0.66	0.14±0.00	No peak	0.23±0.08	86.7
0.4 mg microsomal fraction					
3. Mic(PMD-R) + Permethrin	110.70±3.49	0.10±0.01	No peak	0.26±0.02	89.9
4. Mic(PMD-R) + Permethrin + NADPH	105.09±0.62	0.13±0.03	0.03±0.01	0.34±0.03	92.7
0.8 mg microsomal fraction					
5. Mic(PMD-R) + Permethrin	109.78±2.44	0.07±0.00	0.10±0.01	0.40±0.03	91.4
6. Mic(PMD-R) + Permethrin + NADPH	109.10±0.51	0.12±0.00	0.12±0.01	0.60±0.17	93.2

*amount of permethrin, PBOH, PBCHO and PBCOOH represented nmole/min/mg protein.

Table 17 Comparison of metabolite products after incubation the substrates, permethrin, PBOH and PBCHO with PMD-R microsomal fractions (Mic) with and without PD-10 desalting purification

Reactions	Permethrin*	Metabolite products*			%recovery
		PBOH	PBCHO	PBCOOH	
No purification					
1. Mic(PMD-R) + Permethrin	89.87±5.35	0.10±0.00	0.10±0.04	1.58±0.31	104.5
2. Mic(PMD-R) + PBOH		138.36±5.42	68.32±0.94	13.44±0.24	106.4
3. Mic(PMD-R) + PBCHO		0.61±0.03	460.46±19.32	29.87±2.10	103.3
Purified with PD-10 desalting column					
4. Mic(PMD-R) + Permethrin	79.64±4.16	0.21±0.01	No peak	2.76±0.12	98.7
5. Mic(PMD-R) + PBOH		152.13±2.41	37.13±0.13	27.28±0.76	106.5
6. Mic(PMD-R) + PBCHO		1.49±0.03	424.69±16.92	41.71±4.60	105.6

0.8 mg of microsomal protein per reaction

*amount of permethrin, PBOH, PBCHO and PBCOOH represented nmole/min/mg protein.

4. In vitro metabolism of permethrin, PBOH, and PBCHO by microsomal enzymes

4.1 Permethrin metabolism

The in vitro metabolism of permethrin incubated with 0.8 mg protein of the microsomes of the fourth instar larvae of the PMD and PMD-R strains was shown in Table 19. The data presented in this study were based on the principle of oxidoreduction catalysed by in vitro activity of P450s, which was dependent on the presence of NADPH as a cofactor. Permethrin and its metabolite products, PBOH, PBCHO and PBCOOH were observed using standardized HPLC chromatogram at retention times 13, 4.5, 8.5 and 5.3 (Table 18, Figure 17).

When permethrin was used as the substrate, the metabolite product, PBCOOH, was detected equally in both strains, irrespective of the presence of an exogenous NADPH regenerating system (Table 19, Reactions 1, 5) (Figure 18). This indicates that the endogeneous NADPH within the microsome preparation is sufficient for the reaction assay. The intermediate products, PBOH and PBCHO, were also detectable, but were not detected in the absence of the microsomes or in the absence of substrates (data not shown). Addition of either BNPP or PBO resulted in a significant reduction of PBCOOH formation in both strains. The inhibition effect was significantly lower ($p < 0.05$) in PMD-R which produced more metabolite. The difference in inhibition to enzyme activity suggests the qualitative difference of the enzymes. The enzyme activity in microsomes was clearer demonstrated when PBOH or PBCHO were used as substrates.

Table 18 Reaction mixtures for standard preparation

Standard chemicals	Stock conc. (ng/ml)	final conc. (ng/10 µl)	volume (µl)
Permethrin	$2x10^6$	2000	100
PBO	$1x10^6$	1000	100
PBCHO	$1x10^6$	800	80
PBOH	$1x10^5$	400	400
PBCOOH	$1x10^5$	500	500
Pyrene	$9x10^5$	450	50
Acetonotrile			50

Preparation of permethrin, PBO and PBCHO was dissolved with ethanol.

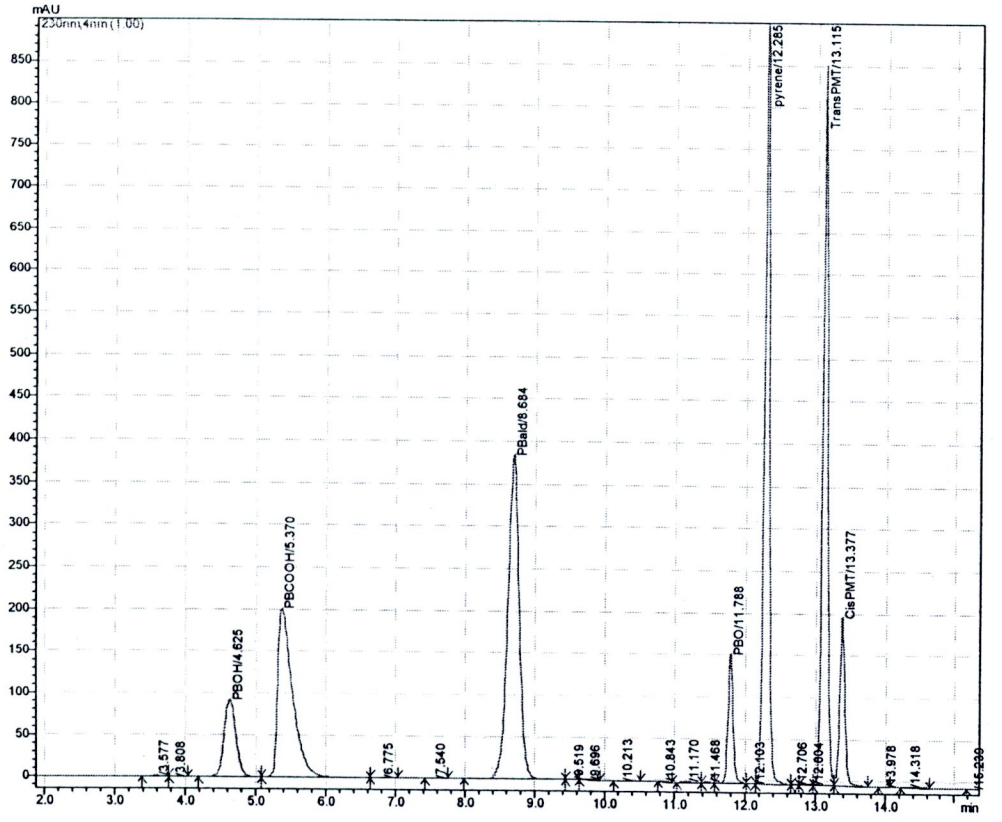

Figure 17 HPLC chromatogram of the standard chemicals. RT = retention time in minutes. Standard chemical concentrations are indicated as nanogram per injection.

Table 19 Permethrin and its metabolite products, PBOH, PBCHO and PBCOOH formations after incubation of the PMD and PMD-R microsomal fractions (Mic) in the presence or absence of co-enzyme and inhibitors (NADPH, BNPP and PBO)

Reactions	NADPH	BNPP	PBO	Permethrin*	Metabolite products*			%recovery
					PBOH	PBCHO	PBCOOH	
PMD-R								
1. Mic(PMD-R) + Permethrin	-	-	-	107.30±3.13	0.05±0.01	No peak	1.18±0.07	100.2
2. Mic(PMD-R) + Permethrin	-	+	-	109.68±1.40	No peak	No peak	0.77±0.04	99.4
3. Mic(PMD-R) + Permethrin	+	-	-	107.52±1.92	0.07±0.01	0.20±0.00	1.17±0.03	99.4
4. Mic(PMD-R) + Permethrin	+	-	+	108.21±1.27	0.09±0.00	0.44±0.02	0.61±0.04	100.1
PMD								
5. Mic(PMD) + Permethrin	-	-	-	110.40±0.70	0.10±0.01	0.09±0.01	1.13±0.00	97.8
6. Mic(PMD) + Permethrin	-	+	-	107.58±0.97	No peak	0.02±0.00	0.58±0.01	100.04
7. Mic(PMD) + Permethrin	+	-	-	110.95±0.36	0.14±0.06	0.09±0.02	1.18±0.11	98.7
8. Mic(PMD) + Permethrin	+	-	+	116.37±3.20	0.10±0.01	0.08±0.00	0.31±0.01	98.1

0.8 mg of microsomal protein per reaction
*amount of permethrin, PBOH, PBCHO and PBCOOH represented nmole/min/mg protein.

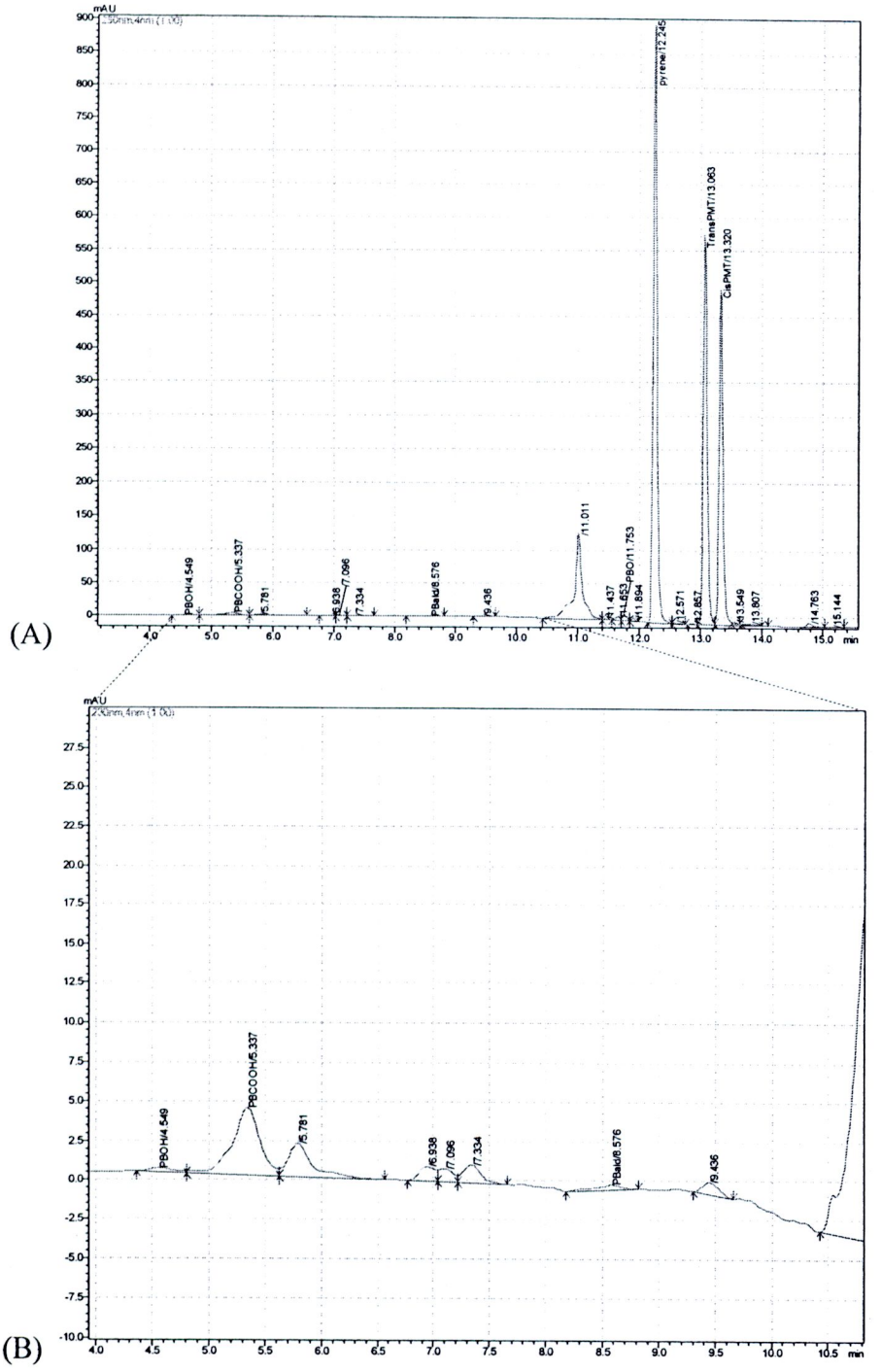

Figure 18 HPLC elution profile of permethrin metabolites separated in a C-18 reverse phase column. (A) Permethrin and its metabolites, PBOH, PBCHO and PBCOOH peaks were detected at retention times 13.0, 4.5, 8.5 and 5.3. (B) The peak area of PBOH, PBCHO and PBCOOH was magnified in order to visualize metabolites produced as a result of the permethrin metabolism.

4.2 Oxidase activity of microsome preparation toward PBOH

The oxidase activity toward PBOH was examined. PBCHO and PBCOOH were formed from PBOH after incubating with PMD or PMD-R microsomal fractions. PBOH and its metabolites, PBCHO and PBCOOH were observed with HPLC chromatogram at retention times 4.5, 8.6 and 5.2, respectively (Figure 19). When PBOH was used as the substrate, much more of the PBCOOH was detected than when using permethrin, with a higher quantity in the PMD-R than the PMD samples. The oxidase activity for PBOH metabolizing in PMD-R was about 2.55-fold higher than in PMD in which more PBCOOH formed were detectable, 46.17±0.21 in PMD-R and 18.06±1.76 nmole/min/mg protein in PMD, though the equal quantity of PBCHO were observed (Table 20, Reactions 1, 7). Addition of BNPP did not show much effect on the esterase activity on PBOH metabolism in both PMD and PMD-R (Table 20, Reactions 2, 8).

On incubation with NADPH regenerating system, PBCOOH formations were significantly increased in PMD, but only slightly increased it in PMD-R (Table 20, Reactions 3, 9). These results also noticed that in presence of NADPH, not only PBCOOH was much more detected, PBOH was also increased while the intermediate product PBCHO was significantly reduced (Figure 20). However, the reason for this occurrence still remains unclear. Addition of PBO in the presence of NADPH resulted in a significant reduction both of PBCHO and PBCOOH formations in both strains (Table 20, Reactions 4, 10). These suggested that PBO showed the inhibition effect on the oxidase activity on PBOH metabolism. In addition, the highest amount of PBCOOH formations was detected in the presence of NAD^+ (Figure 21). The NAD^+-linked oxidase activity in PMD-R was higher than PMD (Table 20, Reactions 5, 11).

From these results, the product was significantly increased in the presence of NADPH but a much greater quantity was detected when NAD^+ was added. These suggested that both cytochrome P450 and dehydrogenase enzyme systems are involved in the oxidation of PBOH to PBCHO and PBCOOH.

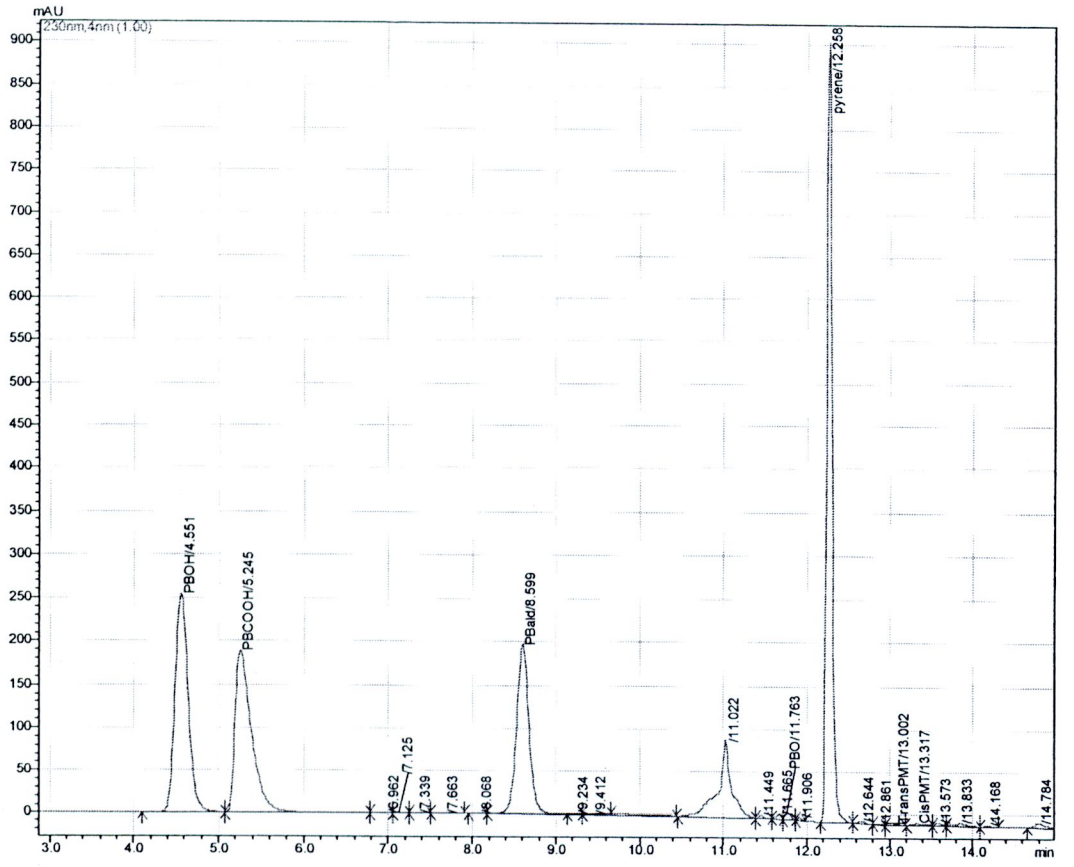

Figure 19 HPLC elution profile of PBOH metabolites separated in a C-18 reverse phase column. PBOH and its metabolites, PBCHO and PBCOOH peaks were detected at retention times 4.5, 8.6 and 5.2.

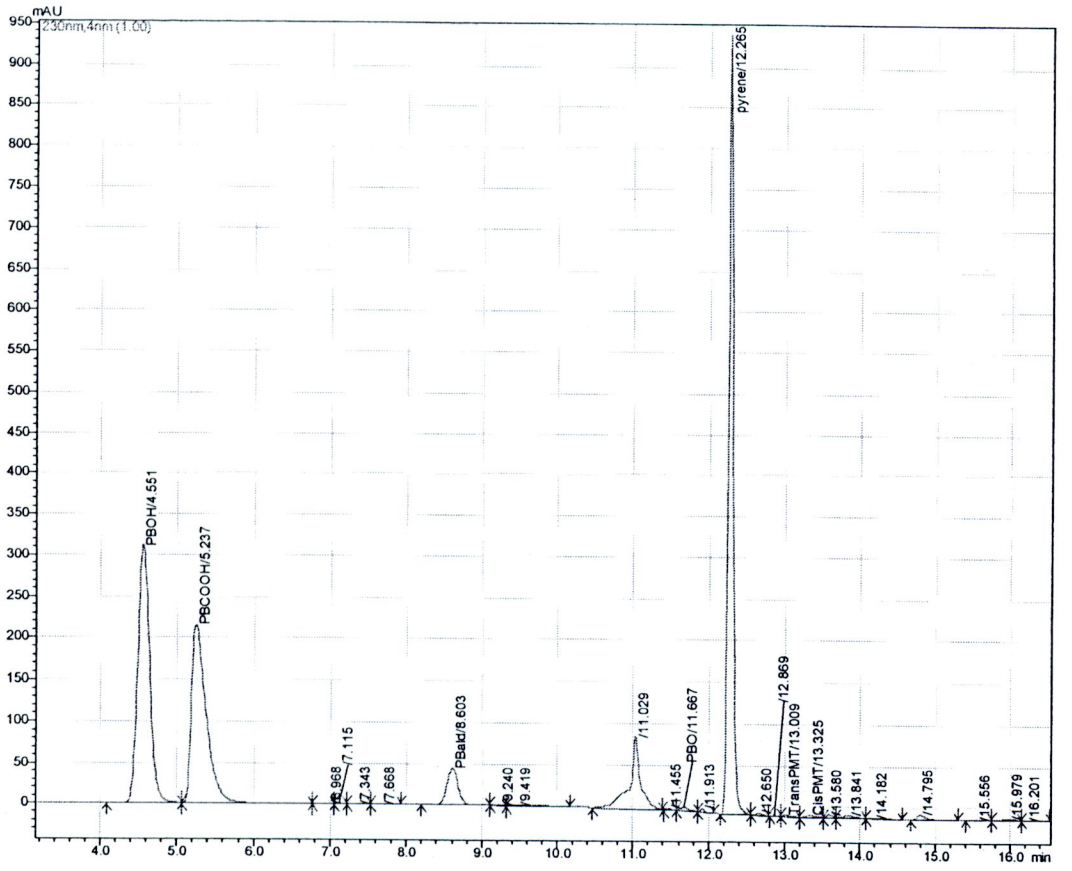

Figure 20 HPLC elution profile of PBOH metabolites in the presence of NADPH separated in a C-18 reverse phase column. PBOH and its metabolites, PBCHO and PBCOOH peaks were detected at retention times 4.5, 8.6 and 5.2.

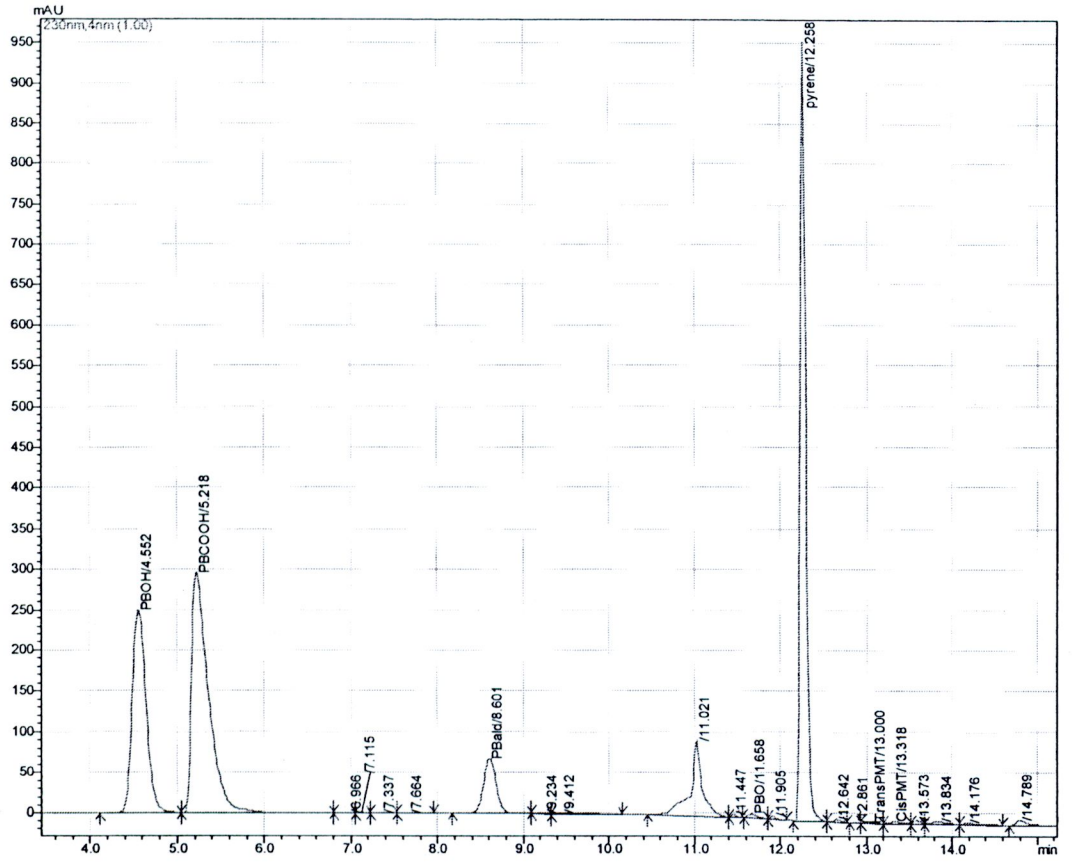

Figure 21 HPLC elution profile of PBOH metabolites in the presence of NAD^+ separated in a C-18 reverse phase column. PBOH and its metabolites, PBCHO and PBCOOH peaks were detected at retention times 4.5, 8.6 and 5.2.

Table 20 Amount of PBOH and its metabolite products, PBCHO and PBCOOH formations after incubation of the PMD and PMD-R microsomal fractions (Mic) in the presence or absence of co-enzymes and inhibitors (NADPH, NAD^+, BNPP and PBO)

Reactions	NADPH	NAD^+	BNPP	PBO	Substrate	Metabolite products*		% recovery
					PBOH*	PBCHO	PBCOOH	
PMD-R								
1. Mic(PMD-R) + PBOH	-	-	-	-	134.33±6.28	39.09±3.24	46.17±0.21	99.2
2. Mic(PMD-R) + PBOH	-	-	+	-	142.88±0.41	28.83±1.70	40.69±1.65	104.2
3. Mic(PMD-R) + PBOH	+	-	-	-	158.19±8.14	7.66±2.16	49.44±2.06	102.2
4. Mic(PMD-R) + PBOH	+	-	-	+	189.98±4.06	3.24±0.55	37.71±2.84	102.3
5. Mic(PMD-R) + PBOH	-	+	-	-	122.19±2.56	13.05±0.32	69.18±0.61	102.3
PMD								
7. Mic(PMD) + PBOH	-	-	-	-	181.94±4.70	42.76±0.37	18.06±1.76	96.8
8. Mic(PMD) + PBOH	-	-	+	-	177.34±1.22	43.97±1.22	19.75±0.97	97.3
9. Mic(PMD) + PBOH	+	-	-	-	209.07±0.86	11.89±0.00	26.79±1.41	98.1
10. Mic(PMD) + PBOH	+	-	-	+	216.18±4.55	4.37±0.25	22.77±0.56	101.1
11. Mic(PMD) + PBOH	-	+	-	-	166.78±2.06	4.93±0.41	49.06±0.42	100.1

0.8 mg of microsomal protein per reaction.
*amount of permethrin, PBOH, PBCHO and PBCOOH represented nmole/min/mg protein.

4.3 Oxidase activity of microsome preparation toward PBCHO

The oxidase activity toward PBCHO was further examined. PBCOOH was formed from PBCHO after incubating with PMD or PMD-R microsomal fractions. PBCHO and its metabolite, PBCOOH were observed with HPLC chromatogram at retention times 8.6 and 5.2, respectively (Figure 22). Similarly, the oxidase activity toward PBCHO metabolism in PMD-R was about 2-fold higher than in PMD in which much more PBCOOH formed were detectable, 60.03±4.38 and 29.73±0.27 nmole/min/mg protein in PMD-R and PMD respectively, though the PBOH were rarely observed (Table 21, Reactions 1, 5).

A similar tendency was observed when NADPH or NAD^+ was added. The PBCOOH formations were significantly generated in both strains in the presence of NADPH but a much greater quantity was detected when NAD^+ was added (Table 21, Reactions 2-8) (Figures 23, 24). Similarly, both PBCOOH and PBOH formations were increased when NADPH was added (Figure 23). Addition of PBO in the presence NADPH resulted in a significant reduction of PBCOOH formations in PMD-R (Table 21, Reactions 3, 7). These suggested that PBO showed the inhibition effect on the oxidase activity on PBCHO metabolism. Overall, the PMD-R microsomes produced more PBCOOH in all cases.

Based on these observations, I suggested that microsomal esterases play a little role in permethrin metabolism. However, using of the permethrin metabolite products, PBOH and PBCHO as substrates, the metabolite product PBCOOH were generated by oxidation of cytochrome P450, alcohol and aldehyde dehydrogenase enzymes.

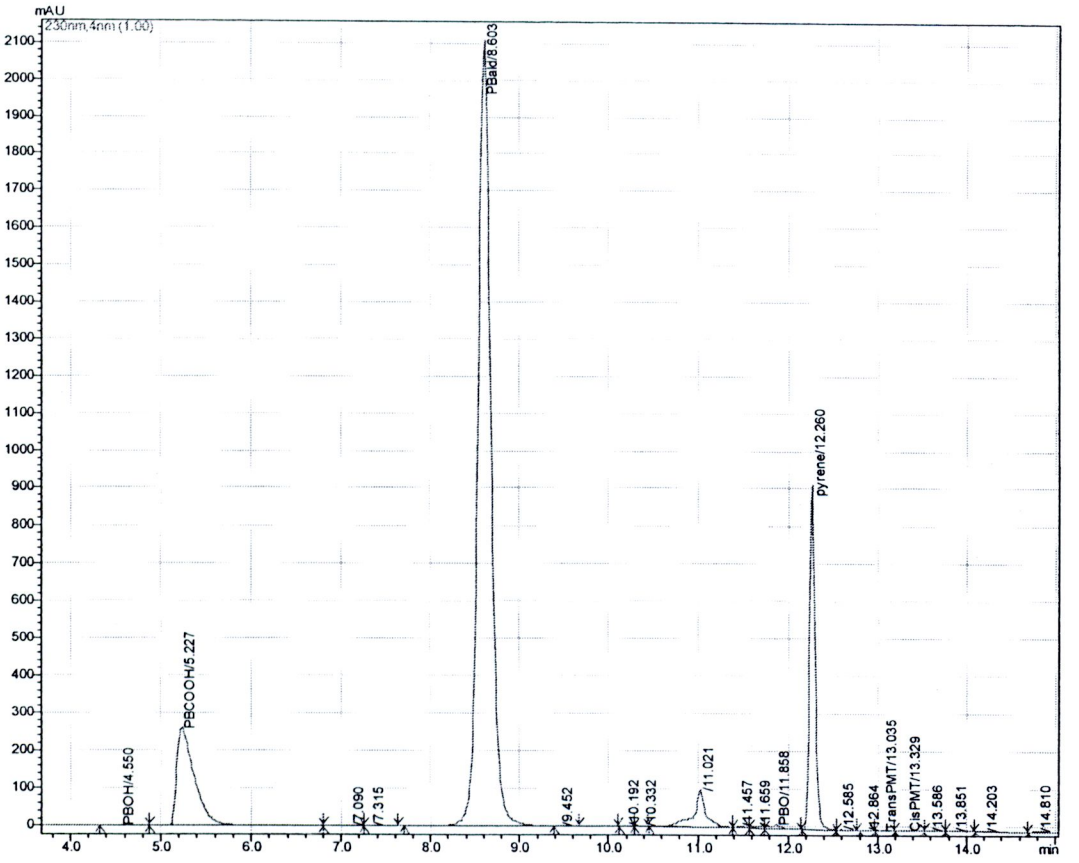

Figure 22 HPLC elution profile of PBCHO metabolites separated in a C-18 reverse phase column. PBCHO and its metabolite, PBCOOH peaks were detected at retention times 8.6 and 5.2.

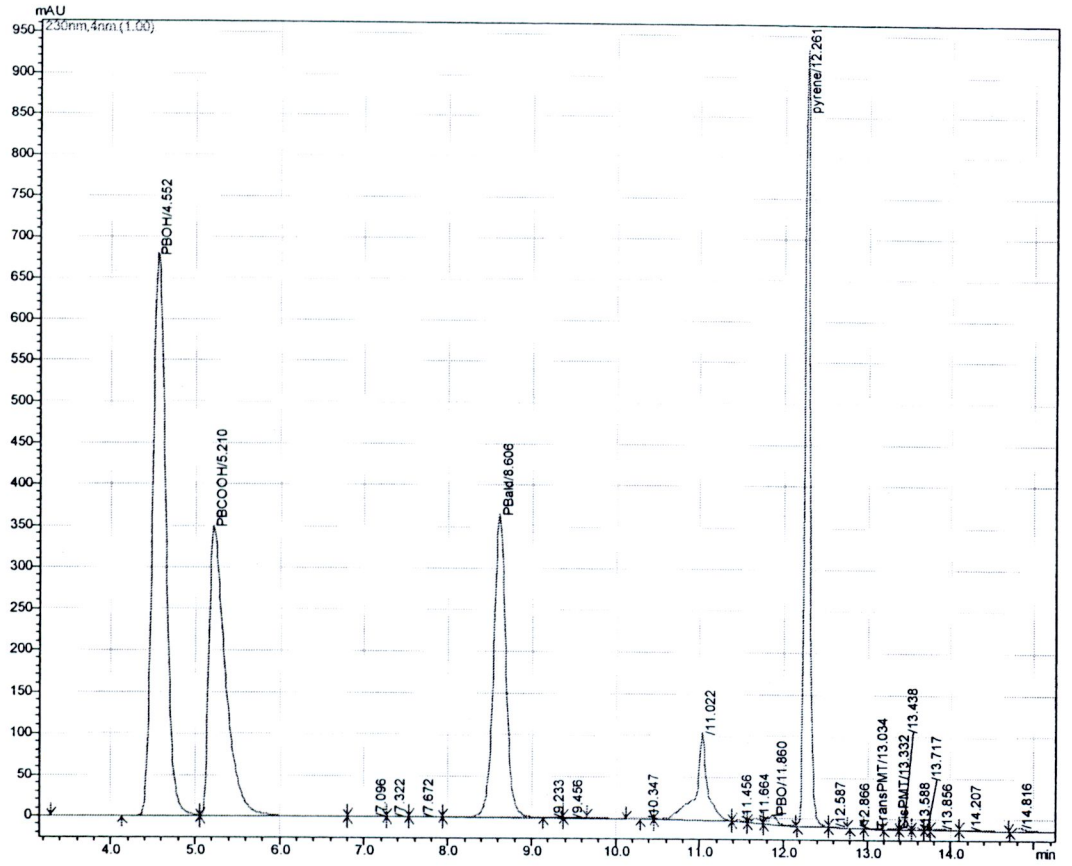

Figure 23 HPLC elution profile of PBCHO metabolites in the presence of NADPH separated in a C-18 reverse phase column. PBCHO and its metabolite, PBOH and PBCOOH peaks were detected at retention times 8.6, 4.5 and 5.2.

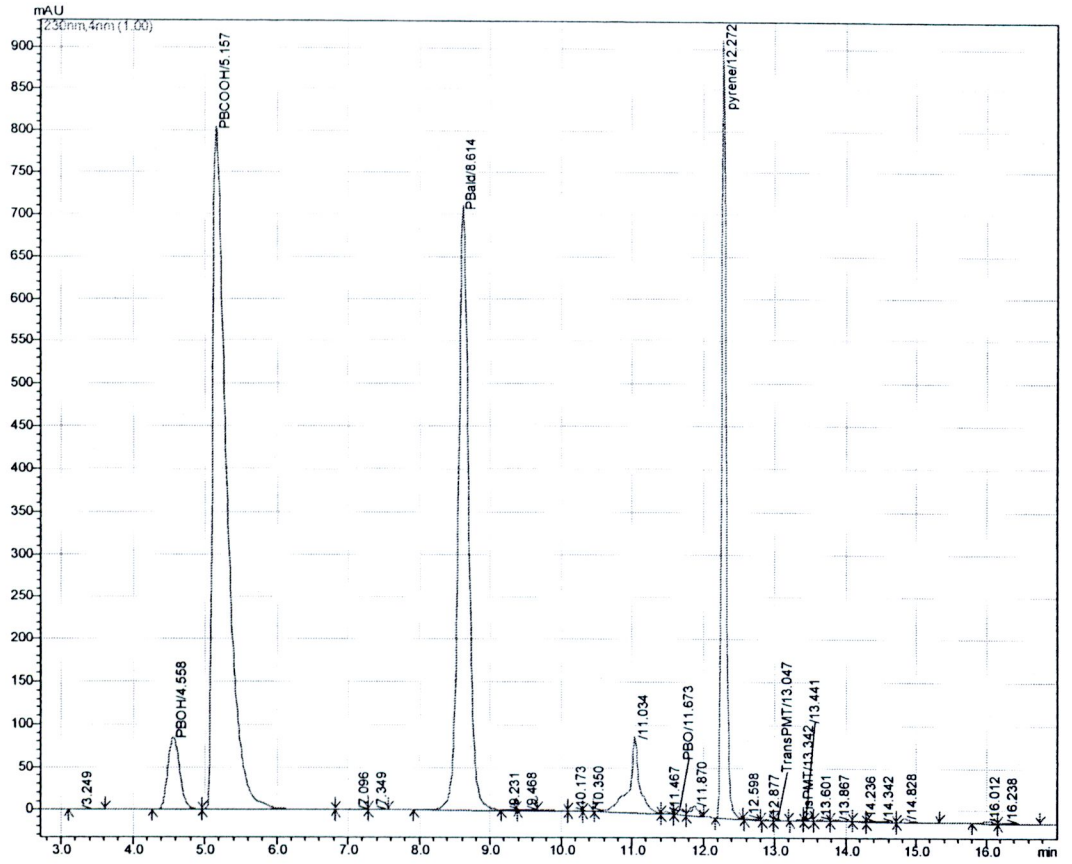

Figure 24 HPLC elution profile of PBCHO metabolites in the presence of NAD^+ separated in a C-18 reverse phase column. PBCHO and its metabolite, PBOH and PBCOOH peaks were detected at retention times 8.6, 4.5 and 5.2.

Table 21 Amount of PBCHO and its metabolite products, PBCOOH formations after incubation of the PMD and PMD-R microsomal fractions (Mic) in the presence or absence of co-enzymes and inhibitor (NADPH, NAD^+ and PBO)

Reactions	NADPH	NAD^+	PBO	Substrate	Metabolite products*		%recovery
				PBCHO*	PBOH	PBCOOH	
PMD-R							
1. Mic(PMD-R) + PBCHO	-	-	-	408.27±2.40	1.23±0.07	60.03±4.38	99.2
2. Mic(PMD-R) + PBCHO	+	-	-	70.69±1.89	336.54±2.54	82.17±2.26	102.6
3. Mic(PMD-R) + PBCHO	+	-	+	173.09±8.35	290.18±3.04	69.71±2.43	93.2
4. Mic(PMD-R) + PBCHO	-	+	-	133.34±13.42	42.45±0.20	198.32±2.51	101.5
PMD							
5. Mic(PMD) + PBCHO	-	-	-	439.10±4.71	0.29±0.00	29.73±0.27	99.6
6. Mic(PMD) + PBCHO	+	-	-	185.63±2.60	232.08±1.61	51.30±1.67	100.1
7. Mic(PMD) + PBCHO	+	-	+	204.71±7.54	222.79±1.47	53.14±2.30	99.1
8. Mic(PMD) + PBCHO	-	+	-	157.80±15.64	89.10±6.44	157.92±5.43	96.5

0.8 mg of microsomal protein per reaction
*amount of permethrin, PBOH, PBCHO and PBCOOH represented nmole/min/mg protein.