CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quality of longan honey

The longan honey sample had L*, a* and b* values of 37.27±0.47, 6.22±0.05 and 23.21±0.86, respectively. It had pH, total soluble solids and water activity (a_w) of 3.97, 80.6^{o}Brix and 0.55, respectively. Yeast, mold and total bacteria counts were lower than 10 cfu/g and 100 cfu/g, respectively.

The negative longan honey had quality conforming to Thai standards (Ministry of Public Health, 2000). Microorganisms in honey may influence quality or safety. Due to the natural properties of honey and control measures in the honey industry, honey is a product with minimal types and levels of microbes. Microbes of concern in post-harvest handling are those that are commonly found in honey (i.e., yeasts and spore-forming bacteria), those that indicate the sanitary or commercial quality of honey (i.e., coliforms and yeasts) and those that under certain conditions could cause human illness (Snowdon and Cliver,1996).

4.2 Optimal method for reduction of inhibines in honey

The honey was diluted in sterile deionized water to different concentrations of 10-50.0% (v/v) and 100.0% honey and incubated at 30^{o}C for 5 minutes, 1 hour, 3 hours, 65^{o}C for 5 minutes, 3 hours, 80^{o}C for 3 hours, 85^{o}C for 5 minutes and 95^{o}C for 5 minutes. H_2O_2 was found in 10-50% concentration of honey at only 30^{o}C except in 10% concentration of honey at 30^{o}C for 5 minutes. H_2O_2 was not found in 10-50% concentration of honey at 65, 80, 85 and 95^{o}C and also was not found in 100% concentration of honey at 30, 65, 80, 85 and 95^{o}C (Table 4.1).

Table 4.1 Effect of temperature, time and concentration of honey on H_2O_2 (mg/peroxide)

concentration of honey (%)	30°C 5 min	30°C 1 hour	30 °C 3 hours	65°C 5 min	65°C 3 hours	80°C 3 hours	85°C 5 min	95°C 5 min
10	0	2	5	0	0	0	0	0
20	2	5	5	0	0	0	0	0
30	5	5	10	0	0	0	0	0
40	5	5	10	0	0	0	0	0
50	5	5	10	0	0	0	0	0
100	0	0	0	0	0	0	0	0

In 100 % of concentration of honey, whether it was treated with heat or not, the bacteria could not grow because of high osmotic pressure. Osmosis is the movement of water across a membrane towards a more concentrated solution. Solutions of high osmolarity cause removal of water molecules from cells. H_2O_2 has been found to be practically inactive in full-strength honey.

H_2O_2 was not found in 10% at 30°C for 5 minutes. The presence of hydrogen peroxide generated by the enzymatic activity of glucose oxidase in dilute honey also contributes to its antibacterial activity at optimal condition (Molan, 1992a). However, the hydrogen peroxide concentration produced in honey activated by dilution is typically around 1 mmol/l, about 1000 times less than in the 3% solution commonly used as an antiseptic (Molan, 1992a).

In addition, *Geobacillus stearothermophilus* can produce catalase enzyme (Yasuichiroa *et al*., 1996). Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al.*, 2004). Hydrogen peroxide is destroyed when honey is exposed to heat and light (Molan, 1992b), therefore, in

unheated and heated 10-50% (v/v) and 100% honey, hydrogen peroxide and bacterial growth were not found.

The antibacterial activities were studied by well diffusion method against *Geobacillus stearothermophilus* (Figure 4.1) and after incubation of 18-24 h at 65°C, zone around the well was measured as inhibition of growth (Table 4.2).

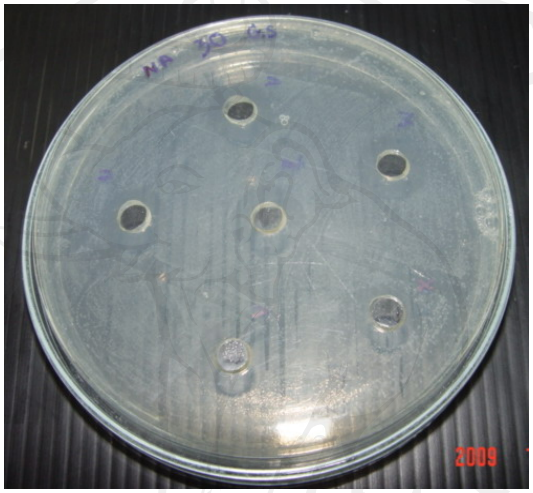

Figure 4.1 An agar well diffusion assay on nutrient agar

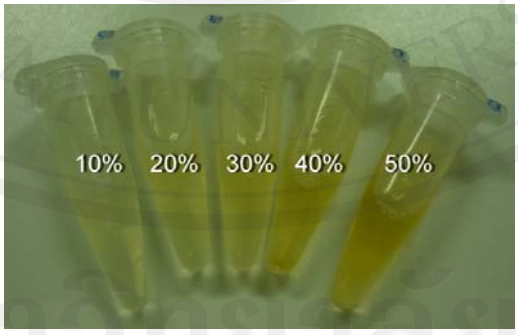

Figure 4.2 10-50% concentration of honey in micro vial

Table 4.2 Effect of pH and concentration of honey on inhibition zone diameter

concentration of honey (%)	pH							
	2.50	3.00	3.25	3.75	4.00	5.00	6.00	7.00
10%	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
20%	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
30%	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
40%	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
50%	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
100%	16±0	16±1	15±1	15±1	14±0	15±0	15±0	14±0

NZ = No zone of inhibition

Zone of inhibition (in mm diameter) including the diameter of well (5 mm)

The activity was shown as the diameter (mm), ± Standard deviation), of the clear zone obtained in an agar well diffusion.

10-50% concentration of honey (Figure 4.2) with adjusted pH values between 2.5-7.0 did not show zone of inhibition but zone of inhibition could be observed at 100% concentration of honey because it had high osmotic pressure (Table 4.2). An osmotic effect draws water from bacterial cells and dehydrates them. From data on studying Table 4.1 and Table 4.2 show that antibacterial components “inhibines” in honey were naturally-occurring, could be destroyed by heat and dilution. Dilution to concentrations below 50% and/or heating at 65^{o}C or higher could affectively eliminate natural inhibine effects of honey.

4.3. Production and test of test kit

4.3.1 Media formulation of test kit

Each of formula of media (Table 3.1) was mixed with bromocresol purple and sterilized at 121^{o}C for 15 minutes. It was found that: Formula of medium A

changed colour from purple to yellow after sterilization. Autoclaving at 15 pounds pressure causes hydrolysis of maltose to glucose and destruction of the glucose with the production of acid (Smith, 1932). Media B, C, D and E changed colour from purple to light purple after sterilization. Autoclaving at 15 pounds pressure causes hydrolysis of sucrose to glucose and slow destruction of the glucose with the production of acid (Smith, 1932). Autoclaving the culture media without agar produced significant changes in pH value. Similar changes in pH value were observed, only at a slower rate, when the solutions stood for some time. These results were equally applicable to ordinary nonsterilized culture media (Emil and Went, 1949). Medium E had much precipitation. Media A-E were solution media. So media A, B, C, D and E were not used because of difficulty to see colour change after test with negative and positive control or difficult to adjust pH of media after sterilization. Media F-U did not change colour (purple) after autoclaving. So media F-U were prepared to produce test kit. The next step of this research used media F1-F8 that were tested for negative and positive control in order to study appeared colour in media with or without pH buffer and different concentration indicator.

4.3.2 Result of negative control

After adding 0.1 ml of negative control sample at 10-40% concentration of honey (Figure 4.3) with F1-F8 test kit. Number of F2 test kit found to have complete yellow colour (Figure 4.4) after incubating for 150 minutes was more than other test kit (F1, F3-F8 test kit). A diluent A (sterile deionized water on pH 5) used to diluted 10-30% concentration of honey resulted complete very clear yellow colour (Figure 4.4), and incomplete yellow colour at 40% concentration (Figure 4.5) after incubating for 150 and 180 minutes (Table 4.3) because high osmotic pressure could inhibit the growth of bacteria.

Diluent A and F2 test kit was a good diluent and test kit at 10-30% concentration of honey sample because a negative reaction showed propagation of

bacteria which changed the pH value of total culture medium more than other diluents and test kit (Table 4.3).

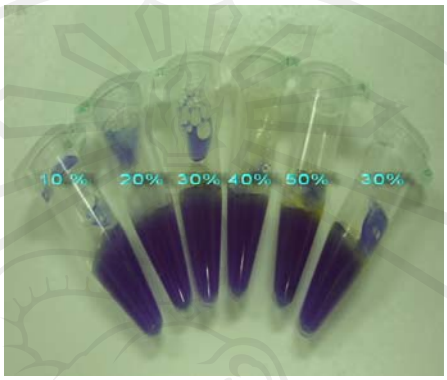

Figure 4.3 0.1 ml of 10-50 % concentration of honey in test kits

Figure 4.4 Complete yellow colour of test kit on negative reaction

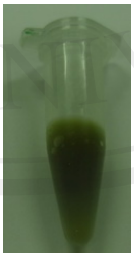

Figure 4.5 Incomplete yellow colour of test kit on negative reaction

Table 4.3 Effect on diluents and concentration of honey on a negative reaction

Diluent/ concentration of honey (%)	Number of test kit were found complete yellow colour at 150/180 minutes of incubation							
	F1	F2	F3	F4	F5	F6	F7	F8
A, 10%	5/5	5/5	0/5	5/5	5/5	0/5	0/5	0/5
B, 10%	3/5	5/5	3/5	0/5	5/5	0/3	0/3	0/3
C, 10%	3/5	5/5	2/3	3/5	5/5	0/5	0/5	0/3
D, 10%	5/5	0/3	2/5	0/5	5/5	0/5	0/3	0/5
E, 10%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
F, 10%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
A, 20%	5/5	5/5	0/5	0/3	0/5	0/5	3/3	0/3
B, 20%	0/5	0/5	4/5	0/3	0/5	0/2	0/0	0/0
C, 20%	2/5	0/5	3/5	0/0	0/0	0/0	0/0	0/0
D, 20%	2/5	0/5	2/3	0/0	0/0	0/0	0/0	0/0
E, 20%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
F, 20%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
A, 30%	2/5	5/5	3/5	0/0	3/5	0/5	0/0	0/0
B, 30%	3/3	0/5	4/5	0/0	0/0	0/0	0/0	0/0
C, 30%	0/5	0/0	0/0	0/0	0/0	0/0	0/0	0/0
D, 30%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
E, 30%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
F, 30%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
A, 40%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
B, 40%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
C, 40%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
D, 40%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
E, 40%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
F, 40%	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

Significant differences ($p \leq 0.05$) were found in honey solution that was diluted by diluent A that appeared to have pH values lower than by using other diluents. Honey was diluted, especially B-F diluents which were good buffered (Table 4.4). The pH slightly decreased from the beginning pH values of negative honey (3.97). In addition, the pattern of pH upon honey dilution were also studied with increasing honey concentration of non-buffered solution (Diluent A). From this results, the optimal diluent was a diluent that to be in agreement with decreased pH which can lead to increase the activity of tetracyclines (Increased pH has the opposite effect) (Hindler and Jorgensen, 2007). Thus, in undiluted honey the acidity is a significant antibacterial factor. But if honey is highly diluted, especially by buffered solution, the pH will not be so low than the beginning pH values and the acidity of honey may not be an effective inhibitor of many species of bacteria (Department of Biological Sciences, 2010). The non-peroxide antibacterial activity in honey was found to correlate significantly with the acid content of honey, but does not correlate with the honey pH (Bogdanov, 1997). From Table 4.3, diluent A was a good diluent because it was non-buffered solution and colour of media changed easily from purple to yellow follow the pH value.

Table 4.4 Effect of diluents and concentration of honey on pH values

Diluent	Concentration of honeys			
	10%	20%	30%	40%
A	3.34±0.04	3.43±0.03	3.53±0.04	3.57±0.05
B	5.64±0.03	5.25±0.03	5.04±0.01	4.75±0.01
C	6.67±0.02	6.38±0.05	6.16±0.01	6.23±0.00
D	7.44±0.01	6.77±0.07	6.84±0.00	6.62±0.01
E	6.96±0.01	6.58±0.06	6.45±0.01	6.44±0.02
F	7.83±0.00	7.35±0.03	7.00±0.00	6.93±0.03

Effect of temperature and concentration of honey on a negative reaction with normal test kit. Test kit was found to have complete yellow colour after incubation for 150 minutes. Total honey diluted at 10-30% concentrations at 30, 65, 85 and 95°C for 5 minutes were found to have a complete clear yellow colour. At 40 and 50% concentration at 30, 65, 85 and 95°C for 5 minutes, they were found to have incomplete yellow colour after incubating for 150 and 180 minutes because of had high osmotic pressure (Table 4.5). An osmotic effect draws water from bacterial cells and dehydrates them.

A good negative reaction to the test shows propagation of bacteria which changes pH value in total culture medium, leading from purple to complete yellow colour. The optimal control for a good negative reaction was 10-30% concentration of honey that was incubated at 30°C for 5 min for preparation of sample. At 10-30% concentration of honey, irrespective of temperature and time, it showed the same complete yellow colour. It means that dilution at 10-30% concentrations of honey that was a good technique to eliminate natural inhibines of honey.

Table 4.5 Colour of negative reaction at various temperatures and dilutions of honey

Temperature / Time	Concentration of honey				
	10%	20%	30%	40%	50%
30°C,5 min	yellow	yellow	yellow	yellow with purple	yellow with purple
65°C,5 min	yellow	yellow	yellow	yellow with purple	yellow with purple
85°C,5 min	yellow	yellow	yellow	yellow with purple	yellow with purple
95°C,5 min	yellow	yellow	yellow	yellow with purple	yellow with purple

4.3.3 Result of positive control

Table 4.6 Shows height (mm) of yellow colour formation on positive reaction at various temperatures and dilutions of honey

Concentration/	oxytetracycline			tetracycline			chlortetracycline		
Temperature and Time	1000 µg/kg	100 µg/kg	10 µg/kg	1000 µg/kg	100 µg/kg	10 µg/kg	1000 µg/kg	100 µg/kg	10 µg/kg
10%/30°C,5 min	4.50	8.70	18.5	4.70	8.00	18.50	7.00	10.00	18.50
10%/65°C,5 min	4.30	8.50	18.5	5.00	8.00	18.50	6.30	10.00	18.50
10%/85°C,5 min	5.00	8.50	18.5	5.10	8.00	18.50	7.00	10.00	18.50
10%/95°C,5 min	5.50	8.50	18.5	5.00	8.00	18.50	7.50	10.00	18.50
20%/30°C,5 min	4.00	8.50	16.00	4.50	7.5 0	15.50	5.60	9.50	16.50
20%/65°C,5 min	4.00	8.00	15.50	4.50	7.00	15.00	4.50	9.00	16.00
20%/85°C,5 min	5.00	8.00	15.50	4.50	7.00	15.00	5.00	9.00	16.00
20%/95°C,5 min	5.00	8.00	15.00	4.00	7.00	15.00	4.70	8.50	15.00
30%/30°C,5 min	4.50	8.00	13.00	4.00	7.00	13.00	4.50	10.00	13.00
30%/65°C,5 min	5.00	8.20	12.00	4.00	7.50	12.50	5.10	9.00	13.50
30%/85°C,5 min	4.50	8.50	12.00	4.00	7.00	12.50	5.00	9.00	13.00
30%/95°C,5 min	4.10	8.00	13.50	3.60	7.00	12.00	4.50	8.80	13.00
40%/30°C,5 min	4.00	8.00	12.50	3.50	6.70	11.50	4.00	8.00	12.50
40%/65°C,5 min	4.00	8.00	12.00	3.30	6.50	11.00	4.50	8.00	12.00
40%/85°C,5 min	4.00	8.00	12.00	3.00	6.50	11.00	3.70	8.00	12.00
40%/95°C,5 min	4.00	8.00	12.50	3.00	6.50	10.00	4.00	8.00	12.00

Positive control: Test kit was found to have purple colour at upper part and yellow colour at lower part of test kit after 150 minutes incubation. Honey sample mixed with 10 µg/kg oxytetracycline, tetracycline and chlortetracycline in normal test kit clearly showed a complete yellow colour with 10% concentration honey and almost a complete yellow colour with 20% concentration honey. They propogated bacteria more than the

sample at 30-40% concentration and results were negative (Table 4.6). The good results could be read from the bottom three-fourth parts of the microvial because of media in test kit height was 18.5 millimetres. There was no significant difference for the height of yellow colour at each concentration of honey and concentration of antibiotic incubated at 30, 65, 85 and 95°C for 5 minutes ($p > 0.05$). The measurement of height of yellow colour formation was in test kit because of easier than visual observation (Figure 4.6).

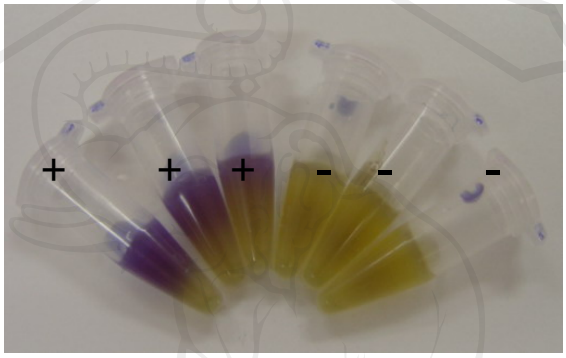

Figure 4.6 Coloury formation in test kit with positive and negative control

In positive control, 10-1000 µg/kg antibiotic in honey, the solid medium turned partly yellow at 30% honey concentration incubated at 30, 65, 85 and 95°C for 5 minutes, and bacteria growth was not different ($p > 0.05$). Use of diluted honey at 30% concentration was the optimum concentration for preparation of negative sample and positive sample. But 40% concentration of honey cannot be used for negative control because of high osmotic pressure which can inhibit the growth of bacteria (Table 4.3).

4.4 Result of freeze-dried test kit

Screening test kit of tube diffusion method in microvial polypropylene tube with cap from freeze-drying contained bacteria at about 10^8-10^9 cfu/ml, after that 0.1 ml of inoculum was added into 0.4 ml of medium and mixed together by a vortex mixer. Test kits were separated into two groups. The first group was stored at -20°C for 18 hours and the second group was dropped in liquid nitrogen (-196°C) for 5-10 minutes, after

that they were taken in a lyophilizer (Christ, alpha 1-4, Germany) for 24 hours. Freeze-dried test kits were stored at 4-8^{o}C in darkness until testing. All two studies appeared to have same colour results. All the samples were analyzed in triplicates.

Table 4.7 Colour of positive control in freeze-dried test kit

Concentration of antibiotic	F2 test kit		
	concentration of honey		
	10%	20%	30%
Oxytetracycline 100 µg/kg	-	+	+
Oxytetracycline 25 µg/kg	-	-	-
Oxytetracycline 10 µg/kg	-	-	-
Tetracycline 100 µg/kg	-	+	+
Tetracycline 25 µg/kg	-	-	+
Tetracycline 10 µg/kg	-	-	-
Chlortetracycline 100 µg/kg	-	-	+
Chlortetracycline 25 µg/kg	-	-	-
Chlortetracycline 10 µg/kg	-	-	-

+ = Purple colour mixed yellow colour

- = Yellow colour

Kirsop (1985) explained that freeze-drying techniques have become standard methods for the long-term maintenance of bacterial culture. Many methods of preservation provide varying degrees of success with different species of bacteria, and neither technique results in 100% recovery of preserved.

The results of negative control using the freeze-dried test kit showed the yellow colour and white colour. The results of positive control showed the purple colour and white colour (Figure 4.7). From the results of colour on negative control and positive control of freeze-dried test kit, it was difficult to read colour than the normal test kits and the freeze-dried test kit could effectively test using 30% concentration of honey with 25 µg/kg

tetracycline residue and 100 µg/kg oxytetracycline and chlortetracycline residues (Table 4.8). The next study did not use test kit from lyophilization because minimum detection limit of tetracyclines residues was higher than normal test kit, besides difficulty to read the result of colour. Disadvantages from production of test kit from freeze-drying were that addition 0.5 ml deionized water in test kit was required (Figure 4.8) before dropping the honey sample in the test kit, and cost of production of the test kit is high.

Figure 4.7 Coloured results of freeze-dried test kit

Figure 4.8 Before and after to add 0.5 ml deionized water in freeze-dried test kit

Mean values for the height (mm) of yellow colour of F2 test kit using 30 % concentration honey sample with antibiotic are shown in Table 4.8. The mean values for height (mm) of F2 test kit with 10, 20, 50 and 100 µg/kg antibiotics was

significantly different ($p \leq 0.05$). For each group of antibiotic, the mean height significantly decreased with increasing antibiotic concentration ($p \leq 0.05$). This means that higher concentration of antibiotic could reduce the microbial growth, hence reducing pH change leading to lesser colour formation in the media of the test kit

Table 4.8 Height (mm) of yellow colour formation on positive control of normal F2 test kit

Concentration of antibiotic	F2 test kit
Tetracycline 10 µg/kg	12.36 [a] ±0.84
Tetracycline 20 µg/kg	12.00 [b] ±0.00
Tetracycline 50 µg/kg	10.00 [c] ±0.50
Tetracycline 100 µg/kg	8.00 [d] ±0.20
Oxytetracycline 10 µg/kg	13.53 [a] ±0.32
Oxytetracycline 20 µg/kg	12.85 [b] ±0.37
Oxytetracycline 50 µg/kg	10.00 [c] ±0.50
Oxytetracycline 100 µg/kg	8.70 [d] ±0.30
Chlortetracycline 10 µg/kg	13.32 [a] ±0.63
Chlortetracycline 20 µg/kg	12.00 [b] ±0.00
Chlortetracycline 50 µg/kg	11.00 [c] ±0.54
Chlortetracycline 100 µg/kg	10.10 [d] ±0.50

- = not available

Values were the means of three replications ± Standard deviation

*Means in the same column with different small letters are significantly different ($P < 0.05$) into same antibiotic

Now, there is Premi®test (Screening test kit) which is based on the inhibition of the growth of *Geobacillus stearothermophilus,* same as developed test kit of this research (Stead *et al*., 2005). Premi ®test has Detection limit of Tetracycline (50 µg/kg), Oxytetracycline (75 µg/kg) and Chlortetracycline (80 µg/kg) in honey. The negative control changes color from purple to yellow (approximate 3 hours). Determination of the

colour change for positive sample can be done at the lower two-thirds of the contents of the ampoule. The prepared honey sample was heated at 45^{o}C for 30 minutes or using the acetone extraction method and the 2-step incubator (first; 10 minutes at 80^{o}C , second; 3 hours at 64^{o}C). The developed test kit of this research read the result from the bottom three-fourths part of the contents of the ampoule for the positive control, requires only dilution of honey sample without heating and the one-step incubation (2-3 hours at 65±1^{o}C). The reading of the positive reaction by the presence of antibiotic was shown purple or partly yellow colour (from the bottom three-fourths of the medium).

The test will be considered positive when, after completing optimal incubation, there was no change or only a little change of colour. While in negative reaction, the bacteria grow and completely change the colour of the medium.

4.5 Validation of normal test kit with HPLC technique

The false positive and false negative rate in the field laboratories were occasionally high, mainly at the beginning of the test. there was not enough evidence for such definite conclusions to be justified using in field laboratories or honey collection centers and bee farms because the data were from small numbers of sample honey, and must be validated with other techniques. (Parish and Davidson,1993)

Table 4.9 Detection limits by the screening test kit and HPLC technique

Antibiotic	Detection limit by the test kit	Detection limit by HPLC
Tetracycline	10 µg/kg	1.048 µg/kg
Oxytetracycline	10 µg/kg	4.462 µg/kg
Chlortetracycline	10 µg/kg	2.811 µg/kg

The tetracycline residue separation was carried out on a HPLC system. Data of Limit of Detection (LOD) by HPLC technique in test Tetracycline, Chlortetracycline and Oxytetracycline in honey are lower than minimum detection limit in screening test kit (Table 4.9).

Table 4.10 Retention time and regression results of HPLC technique

Tetracyclines	Retention time (minutes)	Regression equation	Correlation coefficient (r^2)
Oxytetracycline	6.660	y = 0.232078x + 0.102203	0.99981
Tetetracycline	7.896	y = 0.761087x- 0.172346	0.99996
Chlortetracycline	9.489	y = 0.508265x- 0.207292	0.99948

* Based on two replicates of four concentrations in the range 13-74 µg/kg.

Under HPLC conditions, three tetracycline standards were separated within 10 minutes and the chromatogram is illustrated (Appendix A.1). Tetetracyclines were found to have good linearities between their concentration and peak area responses ranging from 13-74 µg/kg with correlation coefficient (r^2) more than 0.999 (Table 4.10). Method validation results were satisfactory with linear regression equations show in Appendix A.1.

Table 4.11 Values of spiked honey samples with antibiotic by HPLC technique

Concentratios of antibiotics	Oxytetracycline		Tetracycline		Chlortetracycline	
	100 µg/kg	10 µg/kg	100 µg/kg	10 µg/kg	100 µg/kg	10 µg/kg
HPLC technique	90.29±1.25	9.54±1.15	97.18±0.15	8.87±0.24	89.27±1.05	8.11±0.92

Values were the means of three replications ± Standard deviation

A study of honey spiked with 100 and 10 µg/kg antibiotics by HPLC technique shows that the detected concentration of antibiotics was lower than the spiked concentration (Table 4.11). It has been reported that tetracycline group is more stable in acidic conditions (approximately pH 4) than at normal to alkaline. In acidic conditions of honey (pH range 3.0 to 4.51), the greater number of hydrogen atoms may prevent epimerization of the drug, thus maintaining antimicrobial ability (Moreno-Cerezo et al., 2001; Wu and Fassihi, 2005). Antibiotic standard solution and spiked honey samples were prepared fresh daily and kept below -20 ^{o}C until analysis. They were aseptically collected in sterile screwed caps. So the decrease of the concentration of antibiotics under the

storing conditions and effect of honey were scarcely resulted. Recoveries of the antibiotics were different depending on the compound and substrate of HPLC technique. This research obtained the mean recoveies at 10 and 100 µg/kg antibiotic concentrations of 88.79% and 97.18% for tetracycline, 95.46% and 90.29% for oxytetracycline and 81.17% and 89.27% for chlortetracycline, respectively.

Table 4.12 Validity of normal F2 test kit

	Honey with antibiotic	Honey without antibiotic	
Result of positive	a (100)	b (2)	a+b (100+2)
Result of negative	c (0)	d (98)	c+d (0+98)
	a+c (100+0)	b+d (2+98)	n (200)

The clinical sensitivity, specificity and accuracy of the assays were determined by using two-by-two contingency tables (Table 4.12). Validity of 100 test kit with honey containing antibiotic (oxytetracycline, tetracycline and chlortetracycline) and 100 test kit without antibiotic is as follows:

Sensitivity = (a/a+c) X 100% = (100/100+0)X 100% = 100%

Specificity = (d/ b+d) X 100% = (98/2+98)X 100% = 98%

Accuracy of the test = (a+d/n) X 100% = (100+98/200)X 100% = 99%

Analytical techniques are validated and have a false compliant rate < 5% at the level of interest shall be used for screeing methods. In the case of a suspected noncompliant result, this result shall be confirmed by a confirmatory method (Annex of Commission Decision 2002/657/EC). So accuracy is important value because accuracy means the closeness of agreement between a test result and the accepted reference value. It is determined by determining trueness and precision. Accuracy of the F2 test kit was 99%. Because the test kits had accuracy more than 95%, it was a good screening test kit.

4.6 Storage time on effectiveness of normal test kit

From the study of the effect of storage time on effectiveness of the F2 test kit, it was found to have complete yellow colour at 150 minutes incubation of negative control at 1 day shelf-life at 4-8^{o}C. At 270 days at 4-8^{o}C storage, the sample had complete yellow colour at 180 minutes incubation (negative control). From 1 day and 270 days observation of complete yellow colour in test kit at 10 and 20 % concentration of honey respectivety, that were mixed with 10 µg/kg oxytetracycline, tetracycline and chlortetracycline could not inhibit the growth of bacteria (Table 4.13). It was found that 30% concentration of honey was optimal dilution. After 270 days storage of F2 test kit the reaction showed less propagation of bacteria which changed the pH value of total culture medium compared to the new test kit.

Table 4.13 Effect of storage time on yellow colour formation in normal F2 test kit

	concentration in honey							
	40%		30%		20%		10%	
Concentration of drug	1 day	270 day	1 day	270 day	1 day	270 day	1 day	270 day
TC 10 µg/kg	11.50	7.16^{a,A}	13.00	8.00^{b,A}	18.50	18.50^{c,A}	18.50	18.50^{c,A}
TC 100 µg/kg	6.70	7.00^{a,A}	7.00	7.20^{a,A}	7.00	7.50^{a,B}	8.00	8.83^{b,B}
TC 1000 µg/kg	3.50	5.16^{a,B}	4.00	5.33^{a,B}	4.50	5.50^{a,C}	4.70	6.33^{b,C}
OTC 10 µg/kg	12.50	8.00^{a,A}	13.00	8.50^{b,A}	18.50	18.50^{c,A}	18.50	18.50^{c,A}
OTC 100 µg/kg	8.00	8.10^{a,A}	8.00	8.05^{a,B}	9.00	9.10^{b,B}	8.70	18.50^{c,A}
OTC 1000 µg/kg	4.50	6.33^{a,B}	4.00	6.16^{a,C}	4.00	6.16^{a,C}	4.00	7.33^{b,B}
CTC 10 µg/kg	12.50	8.50^{a,A}	13.00	8.00^{a,A}	18.50	18.50^{b,A}	18.50	18.50^{b,A}
CTC 100 µg/kg	8.00	7.23^{a,B}	9.00	8.00^{a,A}	9.50	8.33^{a,B}	10.00	10.33^{b,B}
CTC 1000 µg/kg	4.00	6.33^{a,B}	4.50	7.00^{a,B}	5.60	7.50^{b,B}	7.00	7.50^{b,C}

*Means in the same row with different small letters are significantly different ($p \leq 0.05$) after 270 days storage.

*Means in the same column with different capital letters are significantly different ($p \leq 0.05$) after 270 days storage.

4.7 Analytical acaricide residues by normal test kit

The residues most likely to be present in honey are due to the use of medicines to treat honeybee diseases, introduced during some form of honeybee management, or from environmental pollution. Amitraz, flumetrin, naphthalene and sulphur are acaricides used to control and treat the infestions produced by the mite in the beehives. Usage of this acaricide can cause the presence of it's residue in honey and aparian products, same as beeswax. In many years, acaricides were used more frequently in Thailand (Bureau of Livestock Standard Certification, 2006). With regard to increasingly public concern about health risk from acaricide residues in the honey and bee products, the analysis of acaricide residues in honey and bee products has received special attention. Under preparation of acaricide solutions for spiked positive honey samples was diluted to the required 3 concentrations (10^{-1}, 10^{0} and 10^{1} dilutions of the acaricides at MRL). The MRLs for four acaricides: amitraz, flumetrin, naphthalene. were 200, 5 and 10 µg/kg, respectively. Sulphur has not maximal limits of residue.

From the study of the effect of analytical acaricides levels in spiked positive honey by the screening test kits, all tests were found to have complete yellow colour after 150 minutes incubation, except for the screening test kits with 50 µg/kg flumetrin which showed complete purple colour after 150 minutes incubation. The screening test kit detected flumetrin at 50 µg/kg (Table 4.14), which was above the standard allowable concentration. Its quantity has to be confirmed by certain instruments such as HPLC, SPME (Solid Phase Micro Extraction) method followed by GC-FID (Gas Chromatograph-Flame Ionization Detector) (Alpat and Sunay, 2008).

The lack of detection acaricide residues by screening test kit in honey was related to the fat-soluble compound of amitraz, naphthalene and sulphur. Amitraz, naphthalene and sulpher were absorbed easily by the comb and leaves residues in honey (Alpat and Sunay, 2008; Jimenez *et al*., 1997; Bogdanov *et al*., 1998; Caldow *et al*., 2007; Wallner, 1999; Wikipedia, 2010; Lodesani *et al.,* 1992). Test kits appeared yellow colour completely on all test because amitraz, naphthalene and sulphur could not be absorbed by the test kit media but bacteria could propagate in media. Most of these

acaricides are easy to apply, economically convenient. Furthermore, as lipophilic substances they are mainly absorbed by the bee' wax and the less the sugar feed and the honey (Rosenkranz *et al.,* 2010). Test kit appeared purple colour on positive control because flumetrin is soluble in water and 50 µg/kg flumetrin can absorb in test kit to rest growing bacteria. At low concentration of flumetrin at 10 µg/kg appeared complete very clear yellow colour on positive control because bacteria grew completely.

Table 4.14 Analytical acaricide residues in honey by normal test kit

Concentration of acaricides (µg/kg)											
Amitraz			Flumetrin			Nephthalene			Sulphur		
20	200	2000	0.5	5	50	1	10	100	1	10	100
–	–	–	–	–	+	–	–	–	–	–	–

+ = Purple colour

- = Yellow colour

4.8 Physico-chemical properties of commercial honey

From data (Table 4.15) of investigated honey, 120 honey samples collected from bee farms, honey factories and markets during June-August 2010 had mean values of L* (luminosity), a* (redness) and b* (yellowness) values of 48.30±0.23, 3.31±0.45 and 32.67±0.23, respectively. It had mean values of pH, total soluble solids and water activity (a_w) of 3.77±0.30, 80.41±1.15^{o}Brix and 0.59±0.02, respectively (Appendix A.2).

The pH of the samples of honey were found to be in the normal range for fresh honeys. The average pH of honey is 3.89 (range of 3.0 to 4.51), same as reported by National Honey Board, 1999 that the average pH of honey is 3.9 (with a typical range of 3.4 to 6.1).

The luminosity (L*), redness (a*), yellowness (b*) level were in the ranges of 34.44-57.47, 0.04-13.83 and 0.05-44.21, respectively (Appendix A.2). There were many

types of honey varying in colors (from pale yellow to dark amber), depending on the type of blossoms visited by the honeybee. Different flowers have different kinds of nectar. The properties of different honey batches will depend upon the nectar and pollen sources. For example, different honey sources can differ in terms of their taste, smell and color (from light yellow to darkish brown) (National Honey Board, 2010). The water activity level was in the ranges of 0.53-0.743 (Appendix A.2). The total soluble solids was in the range of 75.0-83.5% (Appendix A.2). From results of high water activity, it appeared to low have total soluble solids. Two wildflower honey had values of water activity and the total soluble solids of 0.743; 78% and 0.738; 75%, respectively. Besides, fermentation occurred in some bottles of honey. Moisture conditions influencing growth of microorganisms in honey has long been used to control the spoilage of honey. Microbes of concern in post-harvest handling are those that are commonly found in honey (i.e., yeasts and spore-forming bacteria), those that indicate the sanitary or commercial quality of honey (Snowdon and Cliver, 1996).

From 120 honey samples from bee farms, honey factories and markets collected during June-August 2010, tetracyclines group residues were not found by the screening test kit of 3 batches of test kit. From randomly selected 30 samples, only one sample was found to have chlortetracycline (8.85 µg/kg) by HPLC method. Minimum detection limit of Tetracyclin group residues by screening test kit was 10 µg/kg. Screening test kit could not detect 8.85 µg/kg chlortetracycline in lychee honey. Although small drug residue was found, Thai beekeepers should be aware of the use of antibiotics in bee honey in order to control honey quality and for the safety of consumers. Most of beekeepers and businessmen in the honey industry think that consumers regard honey as a pure, natural product free from any residues at all. Honey sales would go down if consumers knew of the possibility of these residues in honey.

Table 4.15 Physico-chemical properties of commercial honey

Type of honey	Color quality			pH values	soluble solids (°Brix)	water activity
	L* values	a* values	b* values			
Longan (65)	48.61±0.22	2.92±0.61	32.12±0.20	3.89±0.18	80.20±1.00	0.59±0.02
Bitter bush (11)	47.24±0.21	3.98±0.08	28.53±0.23	3.80±0.27	79.54±1.66	0.57±0.00
Wildflower (12)	46.62±0.33	3.75±0.08	28.17±0.24	3.80±0.25	79.50±1.78	0.62±0.05
Sunflower (7)	49.27±0.13	1.86±0.03	40.98±0.32	3.10±0.00	80.50±0.26	0.58±0.00
Lychee (2)	47.36±0.45	4.88±0.12	31.07±0.46	3.50±0.08	79.00±0.50	0.61±0.00
Rambutan (1)	44.76±0.33	8.81±0.19	41.60±0.18	3.85±0.00	80.00±0.00	0.61±0.00
Sesame (1)	49.18±0.26	4.67±0.09	38.80±0.06	3.19±0.00	80.25±0.00	0.59±0.00
Para rubber (1)	45.39±0.17	8.21±0.08	38.80±1.13	3.36±0.00	81.50±0.00	0.56±0.00
Acacia (1)	52.78±0.09	0.57±0.06	35.06±0.08	3.35±0.00	81.50±0.00	0.58±0.00
Orange (1)	52.37±0.37	0.16±0.08	33.65±0.31	3.00±0.00	81.75±0.00	0.56±0.00
Eucalyptus (1)	43.23±0.21	4.09±0.08	28.89±0.19	3.42±0.00	81.50±0.00	0.57±0.00
Macadamia (1)	52.65±0.19	0.73±0.06	28.62±0.07	3.84±0.00	80.00±0.00	0.60±0.00
Unknown (16)	48.58±0.28	2.88±0.10	32.54±0.28	3.82±0.33	81.51±0.94	0.57±0.02

L*: luminosity; a*: redness; b*: yellowness;

Data were mean±standard deviation values of triplicate measurements except colour quality used ten replicates.

In the future scientists, researchers, beekeepers, etc., would need the analytical technique that has high sensitivity, accuracy, specificity and being cheap. Economy of scale is beginning to reduce capital costs of instruments and improving accessibility. Food legislation must protect consumers. Consumers perceive honey as a natural, healthy product and expect it to be pure and unadulterated. In order to meet these expectations and to prevent allergic reactions or the development of antibiotic resistance, many countries prohibit the use of antibiotics in apiculture altogether. Some countries, however, authorize the use of certain antimicrobials for the treatment of bee diseases. Accidental contamination via the pollen-nectar-bee-honey chain also presents a risk.