APPENDICES

Appendix A Results of study

Appendix A.1 Data of HPLC technique

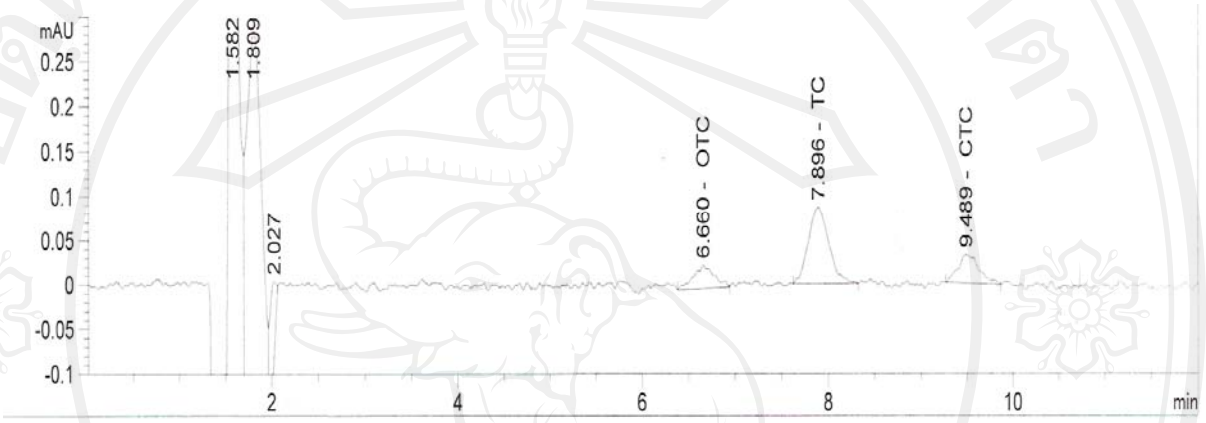
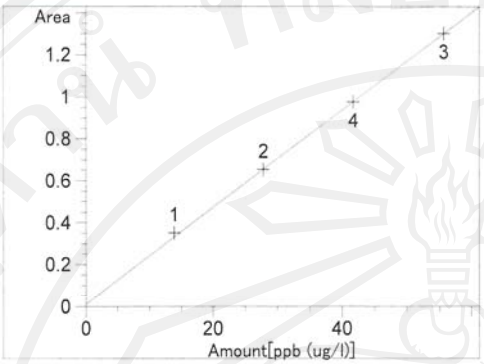


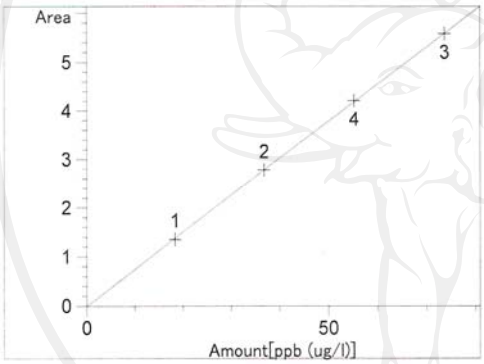
Figure A.1 Chromatograms of standard solution of Tetracycline group

RetTime [min]	Sig	Lvl	Amount ppb (ug/l)	Area	Amt/Area	Ref	Grp	Name
6.739	1	1	13.91600	3.49810e-1	39.78160			OTC
		2	27.83200	6.53010e-1	42.62109			
		4	41.74800	9.76350e-1	42.75926			
		3	55.66400	1.30153	42.76817			
7.943	1	1	18.40000	1.35489	13.58049			TC
		2	36.80000	2.78000	13.23741			
		4	55.20000	4.20700	13.12099			
		3	73.60000	5.57594	13.19956			
9.556	1	1	13.65000	6.83000e-1	19.98536			CTC
		2	27.30000	1.30435	20.92990			
		4	40.95000	2.07282	19.75569			
		3	54.60000	2.77400	19.68277			

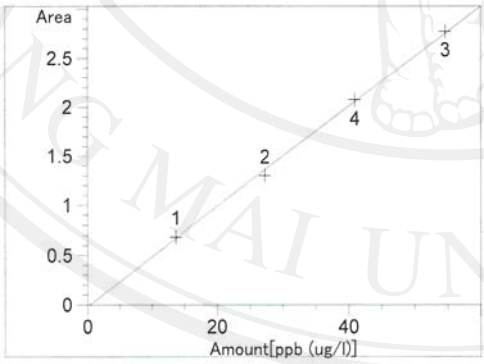
Figure A.2 Calibration report Tetracycline group



OTC at exp. RT: 6.739
DAD1 D, Sig=356,16 Ref=360,100
Correlation: 0.99981
Residual Std. Dev.: 0.01153
Formula: y = mx + b
m: 2.32078e-2
b: 1.02203e-2
x: Amount
y: Area



TC at exp. RT: 7.943
DAD1 D, Sig=356,16 Ref=360,100
Correlation: 0.99996
Residual Std. Dev.: 0.02388
Formula: y = mx + b
m: 7.61087e-2
b: -1.72346e-2
x: Amount
y: Area



CTC at exp. RT: 9.556
DAD1 D, Sig=356,16 Ref=360,100
Correlation: 0.99948
Residual Std. Dev.: 0.04068
Formula: y = mx + b
m: 5.08265e-2
b: -2.07292e-2
x: Amount
y: Area

Figure A.3 Calibration curve and Linearity of Tetracycline group

Appendix A.2 Details of commercial honey

Table A.1 Physico-chemical properties of 120 commercial honey

NO	Water activity	pH	Total Soluble solid	Collect/source	Type	Colour quality		
						L*	a*	b*
1	0.599	3.66	80.00	Farm/Thai	Longan	38.13	8.44	30.81
2	0.604	3.59	79.50	Farm/Thai	Longan	37.10	9.42	30.38
3	0.613	4.13	79.50	Farm/Thai	Longan	50.60	1.68	34.94
4	0.599	3.42	80.00	Farm/Thai	Longan	53.72	0.85	28.87
5	0.605	3.81	80.00	Farm/Thai	Longan	51.57	1.99	31.82
6	0.560	3.84	80.50	Farm/Thai	Longan	49.95	2.60	34.84
7	0.590	3.78	81.00	Farm/Thai	Longan	47.21	4.06	35.08
8	0.615	3.66	79.50	Farm/Thai	Longan	44.79	8.24	39.24
9	0.601	4.15	80.25	Farm/Thai	Longan	49.34	0.63	26.85
10	0.658	3.61	76.50	Farm/Thai	Longan	52.48	0.04	0.05
11	0.572	3.87	81.25	Farm/Thai	Longan	51.68	1.16	32.76
12	0.559	3.91	81.25	Farm/Thai	Longan	51.86	1.69	34.83
13	0.596	3.41	79.50	Farm/Thai	Forest flower	57.47	4.02	29.25
14	0.576	3.59	81.00	Farm/Thai	Bitter bush	51.57	2.11	29.89
15	0.574	3.63	81.25	Farm/Thai	Bitter bush	47.53	2.05	23.09
16	0.577	3.58	81.00	Farm/Thai	Bitter bush	51.99	1.88	29.67
17	0.578	3.59	81.00	Farm/Thai	Bitter bush	52.92	1.03	36.90
18	0.589	3.96	81.00	Farm/Thai	Longan	53.46	1.22	30.67
19	0.589	3.73	80.50	Farm/Thai	Longan	47.65	0.91	20.72
20	0.59	3.84	81.00	Farm/Thai	Longan	46.61	4.64	36.43
21	0.596	3.69	80.00	Farm/Thai	Longan	54.52	0.10	32.63
22	0.599	4.07	80.50	Farm/Thai	Longan	47.47	3.11	32.33
23	0.558	3.36	81.50	Farm/Thai	Para rubber	45.39	8.21	38.8
24	0.589	3.94	79.75	Farm/Thai	Longan	44.76	4.30	32.07
25	0.570	3.84	81.00	Farm/Thai	Longan	52.54	1.21	33.08
26	0.574	3.93	81.50	Farm/Thai	Longan	52.09	1.41	33.50
27	0.572	3.81	81.75	Farm/Thai	Bitter bush	49.79	3.15	37.67
28	0.577	3.82	81.25	Farm/Thai	Longan	50.55	2.32	36.39
29	0.606	3.62	79.75	Farm/Thai	Longan	53.39	0.52	28.66
30	0.570	3.48	81.25	Farm/Thai	Bitter bush	49.64	3.02	37.67
31	0.592	3.65	80.00	Farm/Thai	Longan	52.07	1.10	33.02
32	0.596	4.02	80.00	Farm/Thai	Longan	49.70	3.00	35.25
33	0.576	3.95	81.00	Farm/Thai	Longan	50.16	2.69	37.04
34	0.582	3.60	80.50	Farm/Thai	Bitter bush	42.78	9.69	37.57
35	0.584	3.75	80.50	Farm/Thai	Bitter bush	45.06	9.50	37.36
36	0.580	3.68	81.00	Farm/Thai	Bitter bush	43.80	10.27	38.52

Table A.1 Physico-chemical properties of 120 commercial honey (Continue)

NO	Water activity	pH	Total Soluble solid	Collect/source	Type	Colour quality		
						L*	a*	b*
37	0.567	3.75	81.00	Farm/Thai	Bitter bush	43.70	7.18	29.51
38	0.570	3.73	80.50	Farm/Thai	Bitter bush	40.94	13.01	37.68
39	0.589	3.91	81.00	Farm/Thai	Longan	52.09	1.48	32.32
40	0.594	3.97	80.50	Farm/Thai	Longan	48.74	1.03	28.20
41	0.589	4.06	80.75	Farm/Thai	Longan	49.13	1.90	30.70
42	0.608	4.08	79.50	Farm/Thai	Longan	47.94	0.72	22.40
43	0.586	3.89	81.50	Farm/Thai	Longan	53.32	0.51	31.60
44	0.587	3.07	80.00	Farm/Thai	Sunflower	51.51	1.99	45.50
45	0.583	3.08	80.50	Farm/Thai	Sunflower	49.22	2.10	38.40
46	0.581	3.19	80.50	Farm/Thai	Sunflower	44.35	4.05	38.60
47	0.580	3.23	80.50	Farm/Thai	Sunflower	47.30	2.97	38.00
48	0.595	3.16	80.50	Farm/Thai	Sunflower	48.26	1.53	33.10
49	0.600	3.98	80.25	Farm/Thai	Longan	46.80	0.11	20.70
50	0.609	3.59	79.50	Farm/Thai	Lychee	44.53	8.46	39.70
51	0.601	3.89	80.00	Farm/Thai	Longan	46.70	2.86	29.10
52	0.614	3.92	79.50	Farm/Thai	Longan	43.46	0.14	29.90
53	0.616	3.81	79.00	Farm/Thai	Longan	46.26	6.96	38.10
54	0.589	3.46	80.50	Farm/Thai	Longan	46.24	7.02	41.70
55	0.604	3.82	79.50	Farm/Thai	Longan	47.29	3.14	33.20
56	0.604	3.83	80.00	Farm/Thai	Longan	48.56	4.99	38.20
57	0.614	3.90	79.50	Farm/Thai	Longan	51.72	1.41	34.60
58	0.623	3.93	79.00	Farm/Thai	Longan	48.02	4.96	37.10
59	0.608	4.06	80.00	Farm/Thai	Longan	48.94	2.97	34.20
60	0.637	3.54	78.75	Farm/Thai	Longan	43.31	4.83	30.70
61	0.626	3.83	79.00	Farm/Thai	Longan	50.13	2.97	36.60
62	0.623	3.82	79.00	Farm/Thai	Longan	52.44	1.57	35.50
63	0.637	3.86	77.50	Farm/Thai	Longan	49.24	0.54	27.20
64	0.614	3.85	78.50	Farm/Thai	Longan	48.32	3.71	34.70
65	0.607	3.8	79.50	Farm/Thai	Longan	49.09	2.29	33.00
66	0.582	3.89	81.00	Farm/Thai	Longan	47.26	5.35	41.40
67	0.598	3.19	80.25	Market/Thai	Sesame	49.18	4.67	38.80
68	0.618	3.85	80.00	Market/Thai	Rambutan	44.76	8.81	41.60
69	0.583	4.11	81.50	Market/Thai	Longan	47.42	0.85	25.50
70	0.567	4.17	81.00	Market/Thai	Longan	43.43	4.40	29.70
71	0.619	4.29	78.50	Market/Thai	Forest flower	48.17	1.62	33.00
72	0.609	3.95	80.25	Market/Thai	Longan	46.56	4.37	34.70
73	0.535	3.62	83.00	Market/Abroad	Unknown	49.67	3.37	37.40
74	0.530	3.38	83.50	Market/Abroad	Unknown	51.12	1.02	33.40
75	0.576	3.31	80.50	Market/Abroad	Unknown	53.50	0.88	35.90
76	0.573	4.01	81.50	Market/Thai	Unknown	51.94	1.36	33.80
77	0.565	4.16	82.00	Market/Thai	Unknown	44.28	2.55	23.80
78	0.593	3.70	81.00	Market/Abroad	Unknown	44.77	6.08	34.50

Table A.1 Physico-chemical properties of 120 commercial honey (Continue)

NO	Water activity	pH	Total Soluble solid	Collect/source	Type	Colour quality		
						L*	a*	b*
79	0.568	3.48	81.25	Market/Thai	Unknown	53.94	1.22	30.50
80	0.609	3.97	81.00	Market/Thai	Unknown	48.86	3.42	38.90
81	0.586	4.00	80.50	Market/Thai	Unknown	51.74	0.68	34.72
82	0.620	3.96	80.00	Market/Thai	Unknown	48.71	5.02	39.97
83	0.743	3.72	78.00	Market/Thai	Forest flower	49.22	0.18	25.82
84	0.603	4.03	80.50	Market/Thai	Forest flower	43.23	3.83	25.77
85	0.609	3.86	81.00	Market/Thai	Longan	46.09	6.44	38.05
86	0.616	4.14	79.00	Market/Thai	Forest flower	46.08	1.43	25.89
87	0.581	3.32	81.50	Market/Thai	Unknown	44.94	8.91	39.74
88	0.579	4.00	81.00	Market/Thai	Forest flower	47.91	5.91	33.65
89	0.561	2.90	81.00	Market/Thai	Sunflower	52.67	0.38	44.21
90	0.584	3.35	81.50	Market/Abroad	acacia	52.78	0.57	35.06
91	0.562	3.00	81.75	Market/Abroad	Orange	52.37	0.15	33.65
92	0.594	4.03	80.50	Market/Thai	Longan	42.60	11.01	40.66
93	0.586	3.08	80.50	Market/Thai	Sunflower	51.55	0.04	49.08
94	0.582	4.10	81.50	Market/Thai	Unknown	52.49	0.54	32.57
95	0.579	3.42	81.50	Market/Abroad	Eucalyptus	43.23	4.09	28.89
96	0.738	4.02	75.00	Market/Thai	Forest flower	52.68	0.90	26.44
97	0.537	4.06	83.00	Market/Thai	Unknown	50.29	1.92	36.09
98	0.590	3.68	81.00	Market/Thai	Forest flower	45.59	0.89	24.95
99	0.654	3.89	82.50	Market/Thai	Longan	52.58	0.14	29.73
100	0.604	3.88	80.00	Market/Thai	Longan	52.25	0.10	31.18
101	0.619	4.24	79.25	Farm/Thai	Longan	46.19	0.61	23.40
102	0.569	3.72	81.50	Farm/Thai	Longan	46.36	3.83	33.66
103	0.591	3.80	80.25	Farm/Thai	Longan	40.42	12.43	36.15
104	0.595	4.25	80.50	Farm/Thai	Longan	52.19	0.91	34.56
105	0.588	3.90	81.00	Farm/Thai	Longan	49.43	2.98	35.53
106	0.572	3.91	81.50	Farm/Thai	Longan	52.10	1.55	35.14
107	0.584	4.22	81.00	Farm/Thai	Longan	51.81	0.76	32.52
108	0.616	4.24	79.50	Farm/Thai	Longan	52.20	0.65	33.72
109	0.616	4.27	79.50	Farm/Thai	Longan	48.40	0.29	23.76
110	0.606	3.77	80.00	Farm/Thai	Longan	39.37	10.59	33.56
111	0.626	3.95	82.00	Market/Thai	Unknown	48.95	1.15	25.95
112	0.602	3.84	80.00	Market/Thai	Macadamia	52.65	0.73	28.62
113	0.586	3.66	80.50	Market/Thai	Forest flower	42.87	3.00	23.96
114	0.560	3.72	81.00	Market/Thai	Unknown	47.56	1.01	23.41
115	0.589	3.52	80.50	Market/Thai	Forest flower	43.30	3.31	25.09

Table A.1 Physico-chemical properties of 120 commercial honey (Continue)

NO	Water activity	pH	Total Soluble solid	Collect/source	Type	Colour quality		
						L*	a*	b*
116	0.625	3.42	78.50	Market/Thai	Lychee	50.19	1.30	22.44
117	0.581	3.46	80.50	Market/Thai	Forest flower	43.60	8.92	34.84
118	0.570	3.93	81.25	Market/Thai	Longan	52.17	0.12	33.24
119	0.600	3.67	80.50	Market/Thai	Forest flower	39.41	13.83	33.79
120	0.554	4.51	81.00	Market/Abroad	Unknown	34.44	7.04	20.10

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

Appendix A.3 Experimental figures

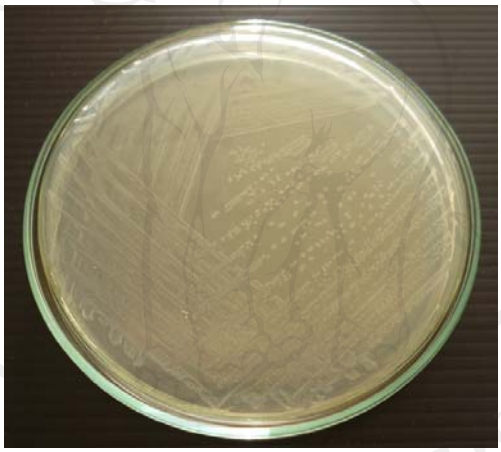


Figure A.4 *G. stearothermophilus* on nutrient agar

Figure A.5 10, 100 and 1000 µg/kg antibiotics and negative control of normal test kits

Figure A.6 Test pH with negative control by pH paper

Figure A.7 Test pH with positive control (high concentration) by pH paper

Figure A.8 Test pH with positive control (low concentration) by pH paper

Appendix B The measurement of the assay

Appendix B.1 Color analysis by a colorimeter[1]

CIE L*, a*, b* values of honey samples were measured by a colorimeter (Minolta CR-300, Japan). Samples of honey were prepared by pouring 25 ml of honey into a white plastic cup. The colorimeter probe was then dipped into the honey samples and the L*, a* and b* values that were shown by the colorimeter were recorded. The colorimeter was calibrated against a standard white tile prior to the honey measurement at 25± 2 ^{o}C.

The *L** (luminosity), *a** (redness) and *b** (yellowness) colour measurements were determined according to the CIELab colour space system, where *L** corresponds to light/dark chromaticity (changing from 0% dark to 100% light), *a** to green/red chromaticity (changing from −60% green to 60% red) and *b** to blue/yellow chromaticity (changing from −60% blue to 60% yellow). The instrument was calibrated with a white reference tile (*L** = 97.10, *a** = −4.88, *b** = 7.04) before the measurements.

[1] Instruction manual of Minolta Chroma Meters (CR-300, Japan) by Minolta Co.,Ltd

Appendix B.2 pH values by pH meter*****

A volume of 15 ml honey samples was transferred in to 25 ml beaker. Into this sample, a pH meter probe was immersed for 5-8 minutes until the pH meter **(**Cyberscan, model 310, Singapore**)** showed a constant pH value. The pH value shown by the pH meter was recorded. Prior to the measurement of honey samples, the pH meter was calibrated using 3 standard buffer solutions, which were pH 4, 7 and 10 at room temperature.

* Instruction manual of pH meter **(**Cyberscan, model 310, Singapore**)**

Appendix B.3 Total soluble solid by a refractometer*

Total soluble solid of honey samples were measured with a hand refractometer (ATAGO, model N-3E, Japan) at room temperature and reported as ^{o}Brix.

* Instruction manual of refractometer **(**ATAGO, model N-3E, Japan**)**

Appendix B.4 Water activity measurement*

Water activity measurements were performed with an Aqualab apparatus (Model Series 3TE; Decagon Devices Inc., Pullman, WA, USA). Pure water (1.000 ± 0.003%) was used as standard for equipment calibration at 25 ^{o}C temperature.

* Instruction manual of Aqualab apparatus **(**Model Series 3TE, USA**)** by Decagon Devices Inc.

Appendix B.5

Viable counts of *Geobacillus stearothermophilus*

Geobacillus stearothermophilus count from each preparation of test kit was mixed thoroughly and a sample amount of 1 ml was 10-fold serially diluted (10^5 to 10^8) in 0.85% sterilized normal saline. Enumeration was carried out using a drop plate technique on Nutrient agar. Plates were incubated aerobically at 65 ^oC for 24 hours. Drops of sample containing 5 to 50 colonies were enumerated, calculated as colony forming units (CFU/g) per ml by ISO 4833 (1991). All microbial tests were performed in duplicate.

Appendix B.6

Viable counts of total bacteria, yeasts and mold

Ten grams of each honey sample were homogenized into 90 ml of peptone water solvent. Decimal dilutions were made into the same solvent. Aerobic mesophilic bacteria followed the protocol ISO 4833 (1991) were counted onto standard plate count agar (PCA) after incubated at 30^{o}C for 72 hours. Moulds and yeasts counts followed the protocol of ISO 21527-2:2008. Microbial counts were expressed as colony-forming units per gram of honey (CFU/g). All microbial tests were performed in duplicate.

Appendix B.7

QUANTOFIX ®Peroxide 25*

Method of application:

Remove only as many test sticks as are required, and reseal the container immediately after use. Do not touch the test paper zone. Dip test stick briefly (approximate 1 second) into the test solution. Shake off excess liquid and after 15 seconds, compare the paper zone with the colour scale. In the presence of hydrogen peroxide the test paper turns blue. QUANTOFIX ®Peroxide 25 can also be used for determination of peracetic acid, other organic and inorganic hydroperoxides. To test for hydroperoxides in organic solvents, the test zone is wetted with one drop of water after evaporation of the solvent.

Test sticks of QUANTOFIX ®Peroxide 25 for semiquantitative determination of peroxide (0.5-25 mg/ l H_2O_2). In the pH range of 2-12, the accuracy of the determination is independent of the pH of the test solution. Avoid exposing the sticks to sunlight and moisture. Store in a cool and dry place.

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* Instruction manual of QUANTOFIX ®Peroxide 25 of Germany by MACHEREY-NAGEL Gmbh & Co.

Appendix C

Details of commercial test kits for honey

C.1 Primi® test

Testing samples: honey, meat, egg, fish

Packing: 100 ampoules/ box

Place of Origin: Geleen, Netherlands of DSM

Type: Microbial inhibition assay

Limit of detection : 50-80 µg/kg for Tetracyclines group

Assay time: 3 hours

Protocol for honey without extraction

-Heat the honey at 45^{o}C for 30 minutes.

-Dilute the honey with water (1:1).

-Honey may have a very low pH (around 3), which influences the result of the Premi®Test. So adjust the pH to 5.5-6.0.

-Adjustment can be done with NaOH or with an acetate buffer

-Transfer 100 µl of this mixture onto the agar in the ampoule.

-Depending on the origin of the honey, incubation can be done directly at 64^{o}C or with the 2-Step incubator (first 10 minutes at 80^{o}C)

- Be aware of the dilution: detection level of the Primi®Test increases.

Protocol for honey with the extraction method

-Take 2 gram of honey.

-Add 5 ml of acetonitrile/acetone (70:30 v/v)

-Homogenize for 30-40 seconds

-Sonicate for 5 minutes, than vortex mix for 30-40 seconds.

-Centrifuge at 4500 rpm for 10 minutes at 4°C.

-Remove supernatant and evaporate under nitrogen at 40-45°C until approximately 100 µl remains.

-Resuspend residue into 250 µl 8 gram/liter Lab Lemco broth (Oxoid: Cat No CM0015) and mix well.

-Apply 100 µl of this mixture onto the Primi®Test.

The Primi®Test validated for honey using the acetone extraction method. The detection limits obtained were as follow:

Penicillin G:	< 12.5 µg/kg	Sulphadiazine	75 µg/kg
Amoxicillin:	< 12.5 µg/kg	Sulphamethizole	75 µg/kg
Ampicillin:	25 µg/kg	Oxytetracycline	75 µg/kg
Oxacillin:	75 µg/kg	Chlortetracycline	80 µg/kg
Cloxacillin:	100 µg/kg	Tetracycline	50 µg/kg
Dicloxacillin:	< 75 µg/kg	Doxycycline	50 µg/kg

Reading the test results

-When the negative control changes color from purple to yellow (approximate 3 hours), the results can be read.

-Read the results from the bottom 2/3 part of the ampoule.

- A clear colour change purple to yellow indicates that the antimicrobial compounds are below the Premi®Test detection limits.

-A purple colour indicates the presence of antibiotics at or above the detection limits of the Premi®Test .

C.2 Quicking Tetracycline Rapid Test (Honey)

Testing samples: milk, honey, meat, aquatic product

Packing: 10 tests/box

Shelf Life: 18 months at room temperature

Place of Origin: Shanghai China (Mainland) of Quicking Biotech Co., Ltd.

Type: Immunoassay System

For price: US $1 - 10 / Piece

Limit of detection : 20 µg/kg

Assay time: 10-15 minutes

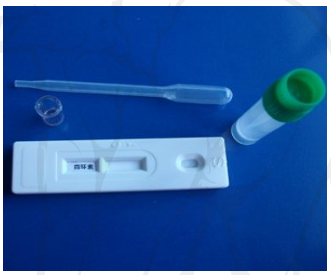

Figure C.1 Quicking Tetracycline Rapid Test (Honey)

1. Intended use

Quicking Tetracycline Rapid Test is a competitive immunoassay for the semi-quantitative detection of the presence of Tetracycline residue in honey sample.

2. Principle of the assay

Quicking Tetracycline Rapid Test is based on competitive lateral flow immunochromatographic assay. The Tetracycline conjugate in the test zone will capture the immuno-gold (colloid gold- Tetracycline antibody conjugate), when there is very little dissociative Tetracycline in the samples. A visible red test band indicates a negative result when the control line (C zone) shows that the card is valid. The test band (T zone)

will be not visible if Tetracycline is present in concentration of 20 ppb and above which explains a positive result.

3. Kit component

- 10×foil pouches each containing one cassette and a desiccant
- 10×assay buffer (0.75 mL each)
- 20×pipettes
- 1×plastic canister contained 10 microwells and a desiccant
- Products Manual

4. Test procedure

- Add 0.25 mL of honey sample into an assay buffer tube and mix well. If there is crystal, thaw the sample in a water bath (60-80°C) beforehand.
- Take out the microwells strip from the plastic canister. Take one well and tear off the film.
- Suck 0.2 mL of the extract into the well with another pipette (the reticle level is 0.2 mL content). Repeatedly suck and extrude the sample until all red reagents are completely dissolved. Wait for 1 min.
- Take out the cassette from the foil pouch and place it horizontally.
- Suck the mixture in the well and gradually drop 3 drops into the assay sample hole "S".
- Interpret the result in 10-15 min. Result after 15 min is considered as invalid.

5. Interpretation of results

Positive: Only one clear band in C zone C indicates a positive result. If a vague T band can be seen but apparently weaker than C band, we also consider it as a positive result. Positive shows that the concentration of Tetracycline is above 20 ppb (ng/mL) in the sample.

Negative: The presence of both clear bands in C zone and T zone. (T band is close to or stronger than C band.)

Invalid: No colored band appears in C zone.

6. Specificity

The results are negative when the test card is applied to detecting 100 ppm (μg/mL) of Chloramphenicol, Aminoglycosides, Beta-lactams, Sulfonamides, and Macrolides.

7. Storage

The kit can be stored at room temperature (2-30°C). The test kit is stable through the expiration date (18 months) marked on the package label. **DO NOT FREEZE**. Do not store the test kit in direct sunlight.

8. Precautions

- For best results, please strictly adhere to these instructions.
- All reagents must be at room temperature before running the assay.
- Do not remove test cassette from its pouch until immediately before use.
- Do not reuse the test kit.
- Do not use the test beyond its expiration date marked on the foil pouch.
- The components in this kit have been quality control tested as standard batch unit. Do not mix components from different lot numbers.

9. Limitation

Quicking Tetracycline Rapid Test is a useful tool offering a rapid and accurate testing in field screening, exceeding with its convenience. It provides a semi-quantitative method to detect the Tetracycline above 20 ppb in honey samples. If you want a quantitative result, please adopt other method such as ELISA/ HPLC in practice.

C.3 Tetrasensor Honey Kits

Place of Origin: Vienna, Austria of Noack group companies
Type: A competitive immunoassay
Packaging Detail: 25 or 100 assays/kit
Detection range: 10 μg/kg
Assay Time Requirement: 30 minutes

Introduction

Tetrasensor honey is a receptor-based assay for rapid determination of the amount of every tetracyclines. The sensitivityis set at 10 μg/kg**.** The means that 100% of honey having 10 μg/kg of tetracycline gives a positive answer.

Reaction mechanism

Tetrasensor is a competitive test that exploitis the activity of a receptor for the recognition of tetracycline molecules present in the honey. The test requires the use of two elements provided in the kit. The first element is a reagent containing a certain amount of labeled receptor and the second is a dipstick consisting of a set of membrane where two capture lines are printed in green. The diluted honey sample is first added together with the receptor where incubation takes place at room temperature for 15 minutes. After 15 minutes the dipstick is dipped into the vial and a second incubation take place for 15 minutes. When the liquid passes through the green capyure lines, red colour appear. The first line caplures the remaining active receptor and the second line takes a certain amount of the excess of reagent that has passed through the first line. This second upper line serves as a control line and becomes visible in all cases.

Test Protocol and Interpretation of the Results

For liquid and semisolid honey, there is no sample preparation requested. Solid honey can be made liquid by heating in a glass test tube in a water bath at 37°C. The lid of the plastic vial is filled with honey so that a correct amount of honey (around 600 mg)

is diluted with the buffer content of the vial (1.8 mL). A total of 200 µL of diluted honey sample is added to the lyophilized receptor present in a glass vial and incubated at room temperature (20 ± 5 °C) for 15 min. During this first incubation period, tetracyclines possibly present in the honey bind with the specific receptor. After 15 min, the dipstick is dipped into the vial, and a second incubation at room temperature takes place for 15 min. When the liquid passes through the green capture lines, a red color appears. The first line captures the remaining active receptor, and the second line takes a certain amount of the excess reagent that passed through the first line. The second line serves as a control line and always has to become visible; otherwise, the test is invalid (figure I.2). Results were read both visually and using the Quantisensor, comparing the color intensity of both capture lines. The visual interpretation is as follows: when the color of the test line is more intensive than the color of the control line, the honey sample is negative ("vis neg"). In all other cases, the honey is contaminated with tetracyclines ("vis pos"). The visual interpretation is always done before the instrumental reading in order to prevent an influence on the judging by the technician.

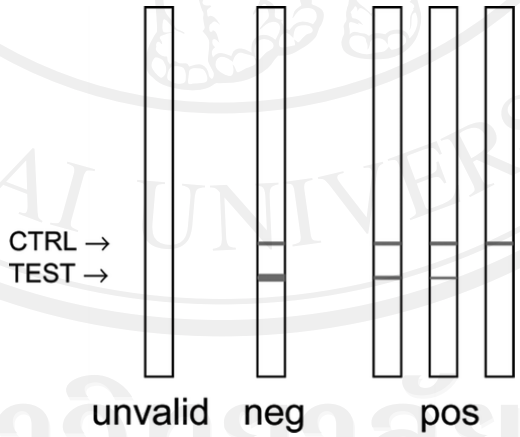

Figure C.2 Visual interpretation of Tetrasensor Honey dipsticks

C.4 Charm II Test Antibiotic

Testing samples: meat, milk, animal urine, fish, shrimp, egg, feed and honey.

Packing: 10 tests/box

Shelf Life: 18 months at room temperature

Place of Origin: Cologne, Germany of Charm Sciences, Inc.

Type: A indirect method competitive enzyme-linked immunoassay

Detection limit: 10-50 µg/kg

Assay Time Requirement: Less than 30 minutes.

Principle

Charm II uses H3 and C14 tagged drug tracers with broadly specific binding agents in a receptor assay format. Samples with high count (CPM) results are considered negative while samples with low count are considered positive. There are separate reagents for each antibiotic drug class. Honey is diluted 1 part to 3 parts supplied MSU buffer and ph adjusted to 7.5 with M2 buffer. This honey extract has active reagents added in sequential and competitive assay formats at various incubation temperatures optimized for drug detection.

This honey extract has active reagents added in sequential and competitive assay formats at various incubation temperature optimized for drug detection.

The detection reaction is stopped with a centrifugation step where unbound tracer is separated from bound tracer-binder complex. The pellet (tracer-binder complex) is analyzed in a scintillation counter for 1 minute to give a resulting count. The higher the count, the less drug contamination in the sample. The lower the count the more drug contamination in the sample. The result is simplified to a presen/absent result using a control point.

Appendix D Regulation for Chemical residues in Honey

Table D.1 Comparision of different regulation for Antibiotics in Honey

Antibiotic	Codex Alimentarius[1]*	EU[2]*	USA[3]*	Australia[4]*	Canada [5]*	India-EIC[6]*	Thailand[12]
Oxytetracycline	No MRL	Provisional MRL -25 µg/kg	No MRL	300 µg/kg MRL[7]	300 µg/kg AMRL[8]	10 µg/kg	No MRL
Chloramphenicol	No MRL	No MRL RPA[9] -0.3 µg/kg	No MRL	No MRL	No MRL	0.3 µg/kg	No MRL
Erythromycin	No MRL	No MRL	No MRL	No MRL	100 µg/kg - AMRL 30 µg/kg WRL[10]	No LOA[11]	No MRL
Ampicillin	No MRL	No MRL	No MRL	No MRL	No MRL	No LOA	No MRL
Enrofloxacin	No MRL	No MRL	No MRL	No MRL	No MRL	No LOA	No MRL
Ciprofloxacin	No MRL	No MRL	No MRL	No MRL	No MRL	No LOA	No MRL

*Data from Johnson, S., Jadon, N (2010)

Notes:

1. FAO/WHO, 2008 Codex Alimentarius: Veterinary Drugs Residues in Food Maximum Residue Limits. http://www.codexalimentarius.net/mrls/vetdrugs/jsp/vetd-q-e.jsp

2. EU- Http://www.emea.europa.eu/index/indexv1.htm. Veterinary medicines and Information technology Units Committee for Veterinary medicinal products.

3. USA- Tolerances for residues of new animal drugs in food in Title 21, Part 556 (21 CFR 556). http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=556

4. Australia /New Zealand Food Standards Code http://www.foodstandards.govt.nz

5. Canada HC 2008a Health Canada WRLs in honey. http://hc-sc-gc.ca/dhp-mps/legislation/vet/pol/cfia-acia_amrram_ table_e.htmll HC 2008 b Health Canada. Drugs& Health Products, Veterinary Drugs, Administrative Maximum Residue Limits (AMRLs)(MRLs) set by Canada and Maximum Residue limits available at http://www.hc-sc.gc.ca/dhp-mps/vet/mrllmr/ mrl

6. According To Export Inspection Council of India's Residue Monitoring Plan (RMP) Honey 2010-2011

7. Maximum residue level (ppb or part per billion)

8. AMRL – is administrative MRL means that the scientific evaluation and decisions are complete and that regulatory process to publish this information is in progress. Once the regulatory process is complete the AMRL becomes an MRL

9. RPA – Reference point for action set by EU

10. WRL – Working Residue Levels. There are no MRLS for antibiotics in honey therefore WRLs are set. WRLs are recommended levels for drug residues in honey below which there is considered to be no undue risk to human health. The WRLs for honey have been derived by extrapolating from AMRL/MRLs for antimicrobials that are approved for use in other food-producing animals such as chickens, swine and cattle.

11. LOA- Level of Action- is the concentration of a drug residue in a sample at which it is deemed non-compliant.

12. Suijidta, P. 2008. Honey and drug residue standard section 1. Part of analysis drug residues and hormones. Group of Authenticated Quality Meat and Animal Products, Office of Authenticated Quality Domestic Animals, Department of Livestock Development. (In Thai).

Table D.2 Comparision of different regulation for Acaricides in Honey

Acaricides	EU[1]	Switzerland[2]	Thailand[3]
Amitraz	200 µg/kg	1000 µg/kg	No MRL
Flumetrin	No MRL	5 µg/kg	No MRL
Naphthalene	10 µg/kg	No MRL	No MRL
Sulphur	No MRL	No MRL	No MRL

Notes:

1.Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC Official J Eur Union L70: 1–16.

2. Bogdanov, S., Kilchenmann, V., Imdorf, A. 1998. Acaricide residues in some bee products. *Journal of Apicultural Research*, 37, 57–67.

3. Suijidta, P. 2008. Honey and drug residue standard section 1. Part of analysis drug residues and hormones. Group of Authenticated Quality Meat and Animal Products, Office of Authenticated Quality Domestic Animals, Department of Livestock Development. (In Thai).

Appendix E

Presentations and publications

E-1 Suitable condition to eliminate natural antibacterial properties in Longan honey to develop screening test kit for detection of antibiotic residues

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Abstract

Study of suitable condition to eliminate natural antibacterial properties in Longan honey was developed screening test kit for detection of antibiotic residues in Longan honey by study 3 diluted solution, pH 2-5, concentrated honey (10-20%) and heated honey (30°C, 85°C, 95°C) at 5 minutes and 65°C 3 hours in antibacterial properties of Longan honey and antibiotic residues with Agar well diffusion test had *Geobacillus sterothermophilus* on Nutrient agar that applied to reservoirs (holes cut) and put sample 100 microlites then incubated at 65°C at 18 hours. The results the concentrated honey 10-20% and temperature 85°C and 95°C at 5 minutes by diluted solution A were suitable condition to eliminate natural antibacterial properties to against *Geobacillus sterothermophilus* in Longan honey had not or had 100 ppb Tetracycline, Oxytracycline and Chlortetracycline.

Keywords: Suitable condition, Eliminate, Natural antibacterial properties, Longan honey

E-2 Development of Test Kit for Detection of Antibiotic Residues in Longan Honey

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Keywords: Test kit, antibiotic residues, longan honey

Introduction

Honey bees, like other insects and livestock, can be infected by many bacterial pathogens. Beekeepers prophylactically administer antibiotics to prevent outbreaks and to treat bacterial pathogens. This extensive use of antibiotics in honey bee colonies can cause an accumulation of residues in honey and can become a public health hazard. Tetracyclines have been used since 1967 and are still being used for the treatment and control of a wide variety of bacterial infections (1). At present, the most commonly applied antibiotic against *Mellissococcus pluton* and *Paenibacillus larvae* (causes of the European foulbrood and American foulbrood, respectively) in bees is oxytetracycline, followed by tetracycline, chlortetracycline and doxycycline (2). This extensive use of antibiotics in honey bee colonies can cause an accumulation of residues in honey and can become a public health hazard. In Thailand, there is still no recommended data of maximum residue limits (MRL) for veterinary medicine products in honey. In practice, it implies to prohibit the use of drug or no drug residues in honey (zero tolerance) (3). This research only investigated on the use of tetracycline, chlortetracycline and oxytetracycline because these drugs are cheap and easy to obtain from drug stores or chemical shops. At present, Thai beekeepers generally use antibiotics to control microbial infections in bees because of convenience and quick results. This problem leads to significant antibiotic residues in honey and other bee products. Screening test for

antibiotic residues in honey is required to protect consumers. However the cost of this test is generally too high for the beekeepers and bee product processors. The objective of this research was to develop a cheap screening test kit for detection of teteacycline group residues in longan honey by microbial inhibition assay.

Materials and methods

Preparation of honey: The honey samples to be analysed in this study was longan honey that was produced by *Apis mellifera* in standard bee farm which practiced a good beekeeping system. The honey was collected during February-April 2009 from northern part of Thailand. The honey was prepared to eliminate its osmotic or inhibine effect on microbial assay using different dilutions (10-40 %) with sterile solutions. Before test, the prepared sample (honey) will be keep at 4-8 ^{o}C in refrigerator and examined the microbiological, chemical and physical properties.

Preparation of bacteria: *Geobacillus stearothermophilus* (DMST 8041) was acquired from the Microbiological Resources Center of Thailand, Institute of Scientific and Technological Research, Thailand. Prior to the use, the cells were streaked on nutrient agar plates and incubated at 65^{o}C for 24 hour under aerobic condition. After incubation, they were propagated in nutrient broth at 65^{o}C for 72 hour. After that, the cells were harvested by cooling centrifugation at 2192 *g* for 20 minutes at 25^{o}C and washed three times with sterile normal saline and then incubated at 95^{o}C for 20 minutes. Then, the harvested cells were mixed with normal saline in order to prepare cell suspension. The bacteria were prepared at 10^{8}-10^{9} cfu/ml.

Production of test kit with negative and positive control:

-The media was mixed with bromocresol purple.

-The bacteria were prepared at 10^{8}-10^{9} cfu/ml, after that 0.1 ml of inoculum was added into 0.4 ml of media.

-Negative control was prepared by adding 0.1 ml of 10-40% concentration of honey at 30, 65, 85 and 95^{o}C for 5 minutes. Positive control was prepared by adding 10-1000

µg/kg antibiotics in 10-40% concentration of honey at 30, 65, 85 and 95°C for 5 minutes into prepared media.

-Negative and positive controls were incubated at 65°C for 120-180 minutes. The test was read negative or positive by the change of colour. In the absence of antimicrobial substances, the whole solid medium turned into total yellow colour. The medium remained purple in the presence of sufficient concentrations of antibiotics. At intermediate concentrations of antibiotic, the solid medium turned partly yellow. The experiment was carried out using a complete randomized design (CRD) with 5 replications. Comparison of means was conducted using analysis of variance (ANOVA) and least significant difference (LSD) at 95% confidential level.

Validation and result comparison with HPLC technique: The result obtained from the best antibiotic residues test kit using optimal formula and test condition was validated and compare against HPLC *technique* (4). Analysis of tests was validated based on sensitivity, specificity and accuracy of the test (5).

Study on the effect of storage time on effectiveness of test kit: The test kit with optimal components for the microbial assay was stored at 4-8 °C. Effectiveness of the test kit was evaluated during the storage from 1 to 270 days. The assay was conducted using diluted honey without antibiotic and the diluted honey with antibiotic 10-1000 µg/kg (tetracycline, chlortetracycline and oxytetracycline) in optimal diluted solution. The experiment was conducted using a complete randomized design (CRD) with 5 replications. ANOVA and least LSD analysis were conducted with the data at 95% confidential level in order to compare mean values.

Result

Quality in 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. It had pH, total soluble solids and water activity (a_w) of 3.97, 80.6 °Brix and 0.55, respectively. Yeast and mold, total bacteria count were lower than 10 cfu/g and 100 cfu/g, respectively.

Negative control: Test kit was found to have complete yellow colour after incubation for 150 minutes. Honey was dilute 10-30% concentrations and was found to have a complete clear yellow colour. At 40% concentration, it was found to have incomplete yellow colour after incubate for 150 and 180 minutes.

Positive control: Test kit was found to have purple colour at upper part and yellow colour at lower part of test kit at 150 minutes. Honey sample with 10 % concentration mixed with 10 ppb oxytetracycline, tetracycline and chlortetracycline in media showed a complete yellow colour very clearly. It propogated bacteria more than the sample at 20-40 % concentration. The results could be read from the bottom three-fourth part of the ampoule. There was no significant difference for the height of yellow colour in each concentration of honey and concentration of antibiotic at 30, 65, 85 and 95^{o}C for 5 minutes ($p \leq 0.05$).

Table 1 Validity of 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)

Validity of 100 test kit with honey has antibiotic (oxytetracycline, tetracycline and chlortetracycline) and 100 test kit with honey has no antibiotic showing**:**

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%

Table 2 Detection limits by the new test kit and HPLC technique

Antibiotic	Detection limit by the test kit	Detection limit by HPLC
Tetracycline	10 µg/kg	2.72 µg/kg
Oxytetracycline	10 µg/kg	7.44 µg/kg
Chlortetracycline	10 µg/kg	3.40 µg/kg

From the study of the effect of storage time on effectiveness of the test kit, it was found to have complete yellow colour at 150 minutes (negative control) at 1 day shelf life at 5 ^{o}C. At 270 days at 4-8 ^{o}C storage the sample had complete yellow colour at 180 minutes incubation (negative control). From observation of yellow colour in test kit, it was found that 10-20% concentration of honey, that was mixed with 10 µg/kg oxytetracycline and chlortetracycline could not inhibit the growth of bacteria, same as 10 % concentration of honey that was mixed with 10 µg/kg tetracycline. There was no significant difference between the mean values for the height of yellow colour at 30-40 % concentration of honey ($p \leq 0.05$).

Discussion

The longan honey had quality following of Thai standards (6). In positive control, 10-1000 ppb antibiotic in honey, the solid medium turned partly yellow at 30 % honey concentration incubated at 30, 65 85 and 95^{o}C for 5 minutes, and bacteria growth was not different. Use of diluted honey at 30% concentration was the optimum concentration for preparation of negative sample and 10 µg/kg positive sample. But 40% concentration of honey cannot be used because of high osmotic pressure which can inhibit the growth of bacteria.

Sensitivity of test kit was 100% and accuracy of the test was 99% from the preparation with or without antibiotic sample within the concentration range that antibiotic could be detected (≤10 µg/kg). Specificity of test kit was only 98% because concentration of bacteria in some test kit was lower than in other test kit.

Data of Limit of detection (LOD) by HPLC technique in test Tetracycline and Oxytetracycline in honey is lower than minimum detection limit of the test kit but minimum detection limit for chlortetracycline was lower than HPLC technique.

Conclusions

This research had developed a cheap screening test kit in microvial polypropylene tube for antibiotic residue detection in longan honey. The test was easy to detect negative and

positive reaction. A microbial inhibition assay was carried out using spores of *Geobacillus stearothermophilus* (DMST 8041) in optimal medium. 0.1 ml of 30% honey solution incubated at 65^{o}C for 2-3 hours in waterbath. The shelf life of the test kit kept at 4-8^{o}C was 9 months. The test kit could not detect at lower than 10 μg/kg, therefore, it has to be confirmed by certain instruments such as HPLC and LCMS.

Acknowledgement

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E-3 Development and Evaluation of a Screening Test Kit for Detection of Tetracycline Group Residues in Honey

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Extensive use of antibiotics in bee colonies can cause an accumulation of residues in honey and become a public health hazard. Analysis of antibiotic residues, therefore, is essential for consumer protection purposes. This study developed and evaluated a simple, rapid, and inexpensive screening test kit for detecting tetracycline group residues in honey. A microbial inhibition assay was carried out using spores of Geobacillus stearothermophilus (DMST 8041) in optimal medium with bromocresol purple as an indicator. Prepared 0.1 ml of 30% honey solution was incubated at 65 ± 1°C for 2-3 hours in a water bath. A positive reaction was detected by negligible change of the purple medium, indicating the presence of a substance(s) that can prevent the growth of the test organism. A negative reaction, indicating an absence of antibiotic residue in sufficient quantity to inhibit bacterial growth, showed a complete change of the medium's color, due to bacterial propagation, from purple to yellow.

Using negative and positive controls (honey free of any residues and honey spiked with varying concentrations of residue, respectively), the screening test kit was 97.5% accurate. The test kit had a detection limit for tetracycline group residues in honey of 10 µg/kg. The shelf life of the test kit kept refrigerated at 4-8°C was 9 months.

The test kit was then used to test 120 commercially available honey samples from across northern Thailand for tetracycline group residues. All 120 samples tested free of antibiotic residues using the new test kit. Of these samples, 30 were randomly selected and subjected to antibiotic residue test using HPLC technique to validate the results from the new screening test kit. Only one of the samples tested positive (8.85 µg/kg of chlortetracycline) at a concentration below the 10 µg/kg detection limit of the test kit.

Keywords: Test kit, Tetracycline, Residues, Honey

Introduction

Honeybees play a vital role in the environment by pollinating both wildflowers and many agricultural crops as they forage for nectar and pollen, in addition to producing honey and other products. The essential and valuable activities of bees depend upon beekeepers to maintain a healthy population of honeybees because, like other insects and livestock, many microorganisms can infect honeybees. Beekeepers prophylactically administer antibiotics to prevent outbreaks and to treat bacterial pathogens. Tetracyclines, in use since 1967, are still used for the treatment and control of a wide variety of bacterial infections (Dinkov et al., 2005). Currently, the most commonly applied antibiotic against *Mellissococcus pluton* and *Paenibacillus larvae* (causes of the European foulbrood and American foulbrood, respectively) in bees is oxytetracycline; followed by tetracycline, chlortetracycline, and doxycycline (Lehnert and Shimanuki, 1980). This extensive use of antibiotics in honeybee colonies can cause an accumulation of antibiotic residues in honey and can become a public health hazard.

Thailand does not specify recommended maximum residue limits (MRL) for veterinary medicine products in honey, as it has a zero tolerance limit similar to European legislation (Passantino and Russo, 2008). In practice, it effectively prohibits the use of drugs or does not allow for any drug residues in honey products (Suijidta, 2008). This research only investigated the use of tetracycline, chlortetracycline, and oxytetracycline as these drugs are inexpensive and readily available from drugstores or chemical shops for use by beekeepers.

In 2004, the Thai Nestle Company tested honey in Thailand and found chloramphenicol in one of 229 samples and tetracyclines in 30 of 229 samples (Maneetup, 2004). Liawruangrath et al. (2006) found chloramphenicol in 10 of 14 samples of honey tested (0.29-3.26 mg/g) from Chiang Mai Province but found no tetracyclines using High Performance Liquid Chromatography (HPLC). Pathomchai and Sujaritpun (2007) found chloramphenicol in three out of six samples of honey from Chiang Mai Province (2.49-10.69 μg/kg) but again no tetracyclines as analyzed by HPLC. Pochalearn (2007) found tetracycline group residues in 2 out of 267 samples of honey from Chiang Mai Province using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) technique. (Note: Chloramphenicol is no longer available for purchase/use in beekeeping in Thailand.)

In northern Thailand, the majority of orchards is occupied by plantations such as longan, forest flower, and bitter bush. Northern Thailand has many beekeepers and honey factories and is an important source of honey exports. Thai beekeepers generally use antibiotics to control microbial infections in bees because of its convenience and quick results. This may lead to significant antibiotic residues in honey and other bee products. A screening test for antibiotic residues in honey is, therefore, required to protect consumers.[1]

To address this issue, this study has aimed to develop an effective and commercially applicable microbial inhibition assay screening test kit for detecting

[1] Currently, Premi®Test (DSM Nutritional Products Company, 2010) is the only commercially available microbial inhibition assay test kit for testing antibiotic residues in honey, and is it unavailable in Thailand.

tetracycline group residues. To do so, this study will seek to develop a test kit based on using a microbial inhibition assay as it has several advantages–it is inexpensive, easy to use, adaptable to screening large numbers of samples, and can detect a wide variety of antibiotics (and/or chemicals toxic to bacteria). However, such a test kit cannot be used to indentify the specific antibiotic residue present, produces qualitative (presence of residuals or not) rather than quantitative result (specific concentration levels), and requires several hours (2-3) before results are available. For positive results, a validated HPLC or LC-MS/MS method must subsequently be used to confirm and quantify the specific antibiotic presence. The test kit developed here will then be evaluated for its effectiveness in detecting tetracycline group residues and compared against and validated using the HPLC technique.

Materials and Methods

A simple screening test kit was developed at the Central Laboratory of the Veterinary Medicine Faculty, Chiang Mai University based on the microbial inhibition assay method. To evaluate this test kit, three studies were subsequently carried out. The first study determined the detection limit of the screening kit and validated results using the HPLC method. The second study investigated the shelf life of the screening kit. The final study used the screening kit to test for tetracycline group residues in commercially available honey samples from private beekeepers and markets in northern Thailand.

Test Kit Development

The screening test kit developed here is an ampoule, containing an agar medium, imbedded spores of *Geobacillus stearothermophilus* (DMST 8041)[2], and a color indicator. The screening test combines the principle of an agar diffusion test with a color change indicator. Bromocresol purple is a pH indicator that detects changes in acidic conditions, changing color over a pH range of 5.2-6.8. In the case of active metabolism of the included microorganism, acid is produced and the bromocresol changes color from purple to yellow in response to the changing pH levels. The optimum functional temperature of the included bacteria is 60-65°C and the optimum pH for growth is 7.0. The spores will not develop if the test kit is stored at temperatures below 43°C. The test kit should be stored at 4-8°C, not frozen, and away from direct sunlight.

When using the test kit at optimal growth conditions, if microorganism growth is inhibited by the presence of an antibiotic at or above the detection limit, the test will remain purple. The color of all test sample ampoules is read at the moment that the negative control sample changes color from purple to yellow. The following steps are required to prepare the microbial inhibition assay screening test kit for use:

Preparation of blank honey. Longan honey was collected from a typical bee farm that practiced good beekeeping in Chiang Mai, Thailand during February-April 2010. The honey was prepared to eliminate its osmotic or inhibine effect on microbial assay by dilution at 10-40% with sterile solutions at pH 5 and heating at 30, 65, 85, and

[2] The bacteria used spoils food but are not dangerous to humans (Tucker and Featherstone, 2010).

95°C for 5 minutes. The prepared samples were kept at 4-8°C in a refrigerator. The samples were examined for their microbiological, chemical, and physical properties as well as the presence of any tetracycline group residues. If the samples contained any chemical residues, they were rejected for use with the negative and positive controls.

Preparation of bacteria. *Geobacillus stearothermophilus* (DMST 8041) was acquired from the Microbiological Resources Center of Thailand, Institute of Scientific and Technological Research, Thailand. Prior to use, the cells were streaked on nutrient agar plates and incubated at 65°C for 24 hours under aerobic conditions. After incubation, they were propagated in nutrient broth at 65°C for 72 hours. The cells were then harvested by cool centrifugation at 2,192 g for 20 minutes at 25°C and washed three times with sterile normal saline and then incubated at 95°C for 20 minutes (Holt et al., 1994). The final cells were harvested by refrigerated centrifugation at 2,192 g for 20 minutes at 4°C. The harvested bacteria cells were mixed with normal saline to prepare the cell suspension at 10^8 -10^9 cfu/ml.

Production of test kit. The medium (yeast extract 0.25%, peptone 0.5%, glucose 0.1%, polysorbate 0.1%, L-cystein 0.01%, agar 1.5%, starch 0.8%, NaCl 0.05%, and bromocresol purple 0.006%) was mixed with deionized water and brought to 100 ml volume and sterilized at 121°C and 15 psi pressure for 15 minutes. 0.4 ml of medium was dropped in a polypropylene tube with cap and 0.1 ml of the bacteria suspension was added.

Production of negative and positive controls. Negative controls were prepared by adding 0.1 ml of the prepared blank honey samples. Positive controls were prepared by adding 10-1000 µg/kg antibiotics in the prepared blank honey samples at 30, 65, 85, and 95°C for 5 minutes. Antibiotic standards of HPLC grade (Sigma Chemical Co., USA) were tetracycline (98% purity), oxytetracycline (≥ 97% purity), and chlortetracycline (≥ 78% purity).

Screening of the controls. Negative and positive controls were incubated at 65°C for 120-180 minutes. The test was read as negative or positive by the change of color. In the absence of antimicrobial substances, the entire solid medium turned yellow. The incubation time was recorded when the negative controls turned completely yellow. The medium remained purple in the presence of sufficient concentrations of antibiotics. At intermediate concentrations of antibiotic, the solid medium turned partly yellow. The medium changed color from the bottom up. Positive results were identified if the bottom three-fourths part of the ampoule turned yellow. The experiment was carried out using a complete randomized design (CRD) with five replications.

Screening Test Validation and Result Comparison with HPLC Technique

The results obtained from the antibiotic residues screening test kit using optimal formula and test conditions was validated and compared against the HPLC technique

(Pena et al., 2005). Analysis of the tests was validated based on sensitivity, specificity, and accuracy of the test (Smith, 2006).

Shelf Life of Screening Test Kit

The test kit with optimal components for the microbial assay was stored at 4-8°C. Effectiveness of the test kit was evaluated during the storage from 1 to 270 days. The assay was conducted using diluted honey without antibiotic and the diluted honey with antibiotic at 10-100 µg/kg (tetracycline, chlortetracycline and oxytetracycline concentrations) in optimal diluted solution. The experiment was conducted using a complete randomized design (CRD) with five replications.

Using the Test Kit to Screen Commercially Available Honey from Northern Thailand

Honey samples from different geographic locations in northern Thailand were obtained from private beekeepers (76 samples) and local food stores (44 samples) during June-August 2010. A total of 120 honey samples were examined for their physical properties as follows: color quality by using colorimeter (Minolta, Chroma Meter CR 300, Japan), pH by using pH meter (Cyberscan, model 310, Singapore), total soluble solid content by using hand refractometer (ATAGO, model N-3E, Japan), and water activity (a_w) by using water activity analyzer (AQUA. LAB, model Series 3TE, USA). All tests were done in triplicate except color quality, which was done in ten replicates, with appropriate controls at each step. The screening test kit was then used to test for the presence of tetracycline group residues in the 120 honey samples. For validity, thirty of the samples were randomly selected by Win Episcope (Thrusfield et al., 2001) and subjected to tetracycline group residues analysis by HPLC technique.

Sample preparation and HPLC analysis of antibiotics from commercial honey. The sample of honey (10-15 g) was weighed into a polypropylene tube and dissolved in 50 ml of 0.1 M Na_2 EDTA-McIlvaine buffer (pH 4.0). The sample solution was shaken for 5 minutes and centrifuged at 2,850 g for 15 minutes. After filtration, it was loaded on C 18 Clean-up (VertiPak^{TM}) 6 cm^3 (500 mg) cartridge previously conditioned with 5 ml of methanol and 5 ml of water. The cartridge containing the sample was then washed with 5 ml of methanol. Then, the tetracyclines were eluted with 5 ml of oxalic acid (1 M)/acetronitrile (80/20). The final eluate was filtered through a 0.45 µm cellulose acetate membrane and stirred in vortex before injecting into the HPLC system.

HPLC analyses of tetracycline group residues was applied according to Geertsen and Pedersen (2009) with a Diode Array Detector and Zorbax Eclipse Separation Column (15 cm x 4.6 mm, 5 µm) at a light wavelength of 356 nm with C 18 Guard Column. The mobile phase used acetonitrile:methanol:oxalic acid 0.01M (10:10:80) at a flow rate of 1.0 ml/minutes, injection volume of 100 µl, and column temperature of 30°C. The calibration graphs were constructed with standard solution concentrations ranging from 13-74 µg/kg. The linearities were evaluated by linear regression analysis, which was calculated using least square regression. All the solvents used (acetonitrile, methanol,

ethyl acetate, and water) were of HPLC grade. Other reagents were of analytical grade and purchased from Merck Ltd., Germany.

Statistical evaluation. All results, where possible, were statistically analyzed by analysis of variance using Microsoft Office Excel 2003. If a significant primary effect was detected, the means are followed by ± *x*, where *x* refers to the standard deviation. The predetermined acceptable level of probability was 5% ($p \leq 0.05$) for all comparisons (e.g., *t*-tests, one-way analysis of variance).

Results

Quality of Blank Longan Honey

The longan honey sample had L* (luminosity), a* (redness) and b* (yellowness) values of 37.27±0.47, 6.22±0.05, and 23.21±0.86. It had pH, total soluble solids, and water activity (a_w) of 3.97, 80.6°Brix and 0.55, respectively. Yeast and mold and total bacteria counts were lower than 10 cfu/g and 100 cfu/g, respectively. The longan honey samples did not contain any tetracycline group residues or any chemical residues**.**

Negative Control

Using the negative control, the screening test kit turned completely yellow after incubation for 150 minutes. Honey diluted at 10-30% concentrations and incubated at 30, 65, 85, and 95°C for 5 minutes all resulted in the test kits turning a completely clear yellow color. At 40% concentration and incubated at 30, 65, 85, and 95°C for 5 minutes, the test kits turned an incomplete yellow color (purple in the upper and yellow in the lower portion of the test kit) after incubating for 150 and 180 minutes because of the high osmotic pressure of honey, which can inhibit bacterial growth.

Positive Control

The various samples of positive control (10-1000 µg/kg antibiotic in honey) were tested in the screening kit for 150 minutes. Before testing, the entire 18.5 mm assay column of media was purple. The more the bacteria were able to flourish (indicating low or non-existent levels of antibiotic residue), the more the column changed to yellow from the bottom up. The measurements in Table 1 indicate the mm of yellow produced.

The honey samples mixed with 10 µg/kg oxytetracycline, tetracycline, and chlortetracycline in media turned completely yellow at a 10% honey concentration and almost completely yellow at a 20% honey concentration producing false negatives. The test kit was unable to reliably detect the presence of antibiotic residue in the positive control samples with very low residue concentrations (10 µg/kg) coupled with low honey concentrations – the combination of which created very low residue concentrations (shaded area in Table 1).

For higher residue concentrations and/or higher honey concentrations, the color purple remained in at least a portion of the column, indicating the screening test was able to reliably detect the presence of residue in the positive controls (unshaded area in Table 1).

There was no significant difference in the height of yellow color part, regardless of the antibiotic, between all tested samples at the same concentration.

Table 1. Height (mm) of yellow color formation on positive reaction at various honey concentrations and incubation temperatures

Concentration and condition of honey sample (all 5 min)	Oxytetracycline			Tetracycline			Chlortetracycline		
	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	4.50	8.70	18.50	4.70	8.00	18.50	7.00	10.00	18.50
10%, 65°C	4.30	8.50	18.50	5.00	8.00	18.50	6.30	10.00	18.50
10%, 85°C	5.00	8.50	18.50	5.10	8.00	18.50	7.00	10.00	18.50
10%, 95°C	5.50	8.50	18.50	5.00	8.00	18.50	7.50	10.00	18.50
20%, 30°C	4.00	8.50	16.00	4.50	7.5 0	15.50	5.60	9.50	16.50
20%, 65°C	4.00	8.00	15.50	4.50	7.00	15.00	4.50	9.00	16.00
20%, 85°C	5.00	8.00	15.50	4.50	7.00	15.00	5.00	9.00	16.00
20%, 95°C	5.00	8.00	15.00	4.00	7.00	15.00	4.70	8.50	15.00
30%, 30°C	4.50	8.00	13.00	4.00	7.00	13.00	4.50	10.00	13.00
30%, 65°C	5.00	9.00	12.00	4.00	7.50	12.50	5.10	9.00	13.50
30%, 85°C	4.50	8.50	12.00	4.00	7.00	12.50	5.00	9.00	13.00
30%, 95°C	4.10	8.00	13.50	3.60	7.00	12.00	4.50	8.50	13.00
40%, 30°C	4.00	8.00	12.50	3.50	6.70	11.50	4.00	8.00	12.50
40%, 65°C	4.00	8.00	12.00	3.30	6.50	11.00	4.50	8.00	12.00
40%, 85°C	4.00	8.00	12.00	3.00	6.50	11.00	3.70	8.00	12.00
40%, 95°C	4.00	8.00	12.50	3.00	6.50	10.00	4.00	8.00	12.00

Note: Shaded area represents false negatives. Antibiotic residues are present at a concentration undetectable by the screening test.

When the positive control (honey spiked with 100 and 10 µg/kg antibiotics) was further analyzed by HPLC technique, the detected concentration of antibiotics was lower than the spiked concentration (Table 2). It has been reported that the tetracycline group is more stable in acidic conditions (approximately pH 4) than at normal to alkaline conditions (Moreno-Cerezo et al., 2001; Wu and Fassihi, 2005). Antibiotic standard solution and spiked honey samples were prepared fresh daily and kept below -20°C until analysis. The decrease in concentration was not attributed to the storage method as the samples were aseptically collected and stored in sterile bottles with screwed caps.

Recoveries of the antibiotics were different depending on the compound and substrate used with the HPLC technique. The mean recoveries at 10 and 100 µg/kg antibiotic concentrations were 88.8% and 97.2% for tetracycline, 95.4% and 90.3% for oxytetracycline, and 81.2% and 89.3% for chlortetracycline, respectively. 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 2. Values of spiked honey samples with antibiotic by HPLC technique

Concentrations 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

Note: Values were the means of three replications ± Standard deviation.

Screening Kit Validity

The clinical sensitivity, specificity, and accuracy of the assays were determined by using two-by-two contingency tables (Table 3). For validity, positive controls (honey with antibiotics present – oxytetracycline, tetracycline, and chlortetracycline) were tested 100 times and negative controls (honey with no antibiotics) were tested 100 times. The results showed:

- Sensitivity = (a/a+c) X 100% = (95/95+5) X 100% = 95%
- Specificity = (d/b+d) X 100% = (100/0+100) X 100% = 100%
- Accuracy of the test = (a+d/n) X 100% = (95+100/200) X 100% = 97.5%

Table 3. Validity of screening test kit

	Honey with antibiotic	Honey without antibiotic	
Result of positive	a (95)	b (0)	a+b (95+0)
Result of negative	c (5)	d (100)	c+d (5+100)
	a+c (95+0)	b+d (0+100)	n (200)

Detection Limits of Screening Kit

The Limits of Detection (LOD) by HPLC technique for tetracycline, chlortetracycline, and oxytetracycline in honey are lower than the minimum detection limits of the screening test kit (Table 4).

Table 4. Detection limits of the screening test kit compared to HPLC technique

Antibiotic	Detection limit of test kit	Detection limit of 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

Shelf Life of Screening Kit

From the study of the effect of storage time on the effectiveness of the screening test kit, after storing the kit for 1 day at 4-8°C, negative control honey turned the kit completely yellow after 150 minutes. After storage for 270 days at 4-8°C, negative control honey turned the kit completely yellow after 180 minutes incubation time. Honey samples mixed with 10 µg/kg oxytetracycline, tetracycline, and chlortetracycline showed complete, almost complete, and partial inhibition of bacteria growth in test kits with 1-day shelf life at 10, 20, and 30-40% honey concentrations, respectively. The test kit with 270-days shelf life did not show microbial growth inhibition when tested with the sample containing 10% honey concentration and 10 µg/kg oxytetracyclin but did with the 30% honey concentration. Given this, a 30% honey concentration was the optimum dilution for use with the new antibiotic residual screening test kit (Table 5).

Table 5. Mean values for the height (mm) of yellow color on shelf life of antibiotic residues test kit with effect of 10-40% concentration in honey on a positive reaction

Honey Concentration	Oxytetracycline				Tetracycline				Chlortetracycline			
	100 µg/kg		10 µg/kg		100 µg/kg		10 µg/kg		100 µg/kg		10 µg/kg	
	1 day	270 day	1 day	270 day	1 day	270 day	1 day	270 day	1 day	270 day	1 day	270 day
10%	8.7	7.2	18.5	18.5	8.0	6.5	18.5	8.7	10.0	7.5	18.5	12.5
20%	9.0	6.0	18.0	9.0	7.0	5.2	16.5	8.0	9.5	7.5	18.0	8.0
30%	8.0	6.0	13.0	8.0	7.0	5.2	13.0	8.0	9.0	7.0	13.0	8.0
40%	8.0	6.5	12.5	8.5	6.7	5.2	11.5	7.0	8.0	6.5	12.5	7.5

Using the Test Kit to Screen Commercially-Available Honey Samples

The analysis of the 120 honey samples collected from bee farms, honey factories, and markets revealed 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. The samples had mean values of pH, total soluble solids, and water activity (a_w) of 3.77±0.30, 80.41±1.15°Brix, and 0.59±0.02, respectively.

Using the new screening test kit, no tetracycline group residues were detected in any of the 120 samples. From the 120 samples, thirty samples from a variety of flower sources (longan [15], bitter bush [4], sunflower [3], forest flower [2], unknown [2], rambutan [1], sesame [1], lychee [1], acacia [1]) were randomly selected for validation by HPLC technique. Only one of the 30 (the lychee honey sample) tested positive for chlortetracycline residue (8.85 µg/kg).

Discussion

In positive controls, containing 10-1,000 µg/kg of antibiotic, the solid medium turned partly yellow when the samples with 30% honey concentration incubated at 30, 65, 85, and 95°C C for 5 minutes were tested, and bacteria growth was not different. For use in the new screening test kit, it was found that diluted honey at 30% concentration was the optimum concentration for preparation of honey samples with incubation at 65°C for 2-3 hours in a water bath to destroy natural inhibines. But a 40% concentration of honey cannot be used because of its high osmotic pressure, which can naturally inhibit the growth of bacteria.

The sensitivity of the test kit was 95% and the accuracy of the test was 97.5 from the preparation with or without antibiotic samples within the concentration range that the antibiotic could be detected. Specificity of the test kit was 100%.

The test kit had a detection limit for tetracycline group residues of 10 µg/kg. The tetracycline group residue concentrations in the honey samples detected by the screening test kit were low and not likely to cause any acute health effects although chronic effects cannot be ruled out. However, even very low levels of antibiotics could over time lead to antibiotic resistance in pathogenic bacteria making their treatment difficult.

As tested, the usable shelf life of the test kit was at least nine months, assuming optimal storage conditions, including immediate and continual storage in refrigerated

conditions. After nine months, the bacteria's viability in the nutritional medium decreases, even under optimal conditions, so it is not recommended to use the kit after this period.

Recently, a commercial screening test kit has become available (Premi® test), which is also based on the inhibition of the growth of *Geobacillus stearothermophilus*, the same microbial used in this research (Stead et al., 2004). However, Premi® test has detection limits for tetracycline (50 µg/kg), oxytetracycline (75 µg/kg), and chlortetracycline (80 µg/kg) that are higher than the screening test kit developed here. In addition, to use the Premi® test kit, the honey sample has to be heated at 45°C for 30 minutes or use the acetone extraction method, followed by a two-step incubation (first = 10 minutes at 80°C; second = 3 hours at 64°C). These steps are in contrast to the newly developed screening test kit evaluated here that only requires dilution of the honey sample and one-step incubation (2-3 hours at 65±1°C). The screening test kit developed here is easier to use and has a lower LOD.

Testing commercially available honey from northern Thailand found virtually no evidence of tetracycline group residues. Of the 120 samples tested with the new screening kit, all tested negative for the presence of antibiotic residues. Only when 30 randomly selected samples were further tested for validation by HPLC technique, did one reveal any residue (8.85 µg/kg of chlortetracycline) at a concentration below the limits of detection (10 µg/kg) of the new microbial inhibition assay screening test kit.

Conclusion

This research has developed a screening test kit in a micro vial polypropylene tube for antibiotic residue detection in honey. Even though the screening test kit developed in this research produces qualitative results only, it is useful for rapid screening of honey for antibiotic residual contamination. The test kit is able to detect antibiotic residues at very low concentrations (10 µg/kg) and has a useable shelf life of 9 months. Moreover, the test kit is inexpensive and simple to use. Beekeepers and general staff at honey collection centers can follow simple application instructions for testing for antibiotic residues. If required, the quantity and type of residue can be analysed and confirmed by commercial and more sophisticated techniques such as HPLC and LC-MS/MS technique.

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