CHAPTER 2

REVIEW OF LITERATURE

2.1 Agricultural development on honey bees in Thailand

Several honey bees species are raised in Thailand. Amongst them, *Apis cerana, Apis flora, Apis dorsata and Apis andreniformis* are native while *Apis mellifera is* an introduced species because it is kept commercially all over the world. It is very gentle, not very likely to swarm, and produce a large surplus of honey. Modern beekeeping in Thailand started in the early 1940s. European honey bees in movable-frame hives were introduced at Chulalongkorn University for research but they did not survive. The second introduction of *Apis mellifera ligustica* was made by Saman Watanakit in 1953 (Wongsiri, 1995) at Kasetsart University and subsequent introductions did not succeed commercially until the early 1970s. There was a boom in beekeeping after 1980 due to low honey production from native bees and the high price of Thai honey in local markets. Since then, the beekeeping industry with the European honey bee has been expanding very fast. Thailand produces 10,000 tons of honey per year of which 3,202 tons are designated for export in 2008 (Department of Agriculture Extention, 2009). In northern part of Thailand, *Apis mellifera* is preferred by large commercial beekeepers, mainly for producing honey and royal jelly, and it is also used for pollination of longan flowers (*Euphoria longan*). The farmers of longan orchards do not need to pay anything for pollination to beekeepers but some beekeepers have to pay the farmers for land use. At present, The National Committee on Agricultural Commodity and Food Standards notifies the establishment of Thai Agricultural Commodity and Food Standards entitled Good Agricultural Practice (GAP) for bee farms to use as a voluntary standard.

2.2 Contamination in honey and bee health management

Honey and bee products have the image of being natural, healthy and clean. However, today bee products are produced in a environment, polluted by different sources of contamination. Environmental contaminants are pesticides, heavy metals, bacteria and radioactivity, contaminants from beekeeping practice includes acaricides used for parasitic mites (mainly varroa) control, bee repellents used at honey harvest, pesticides for wax moth and small hive beetle control and antibiotics used against foul brood disease. The most common insecticides that have been examined in European honeys include organochlorines, organophosphorous pesicides and carbamates. In a recent study using 50 honey samples from Spain and Portugal, residues of 42 different pesticides were examined (Blasco *et al*., 2003). There are several other European studies with no measurable residues of insecticides in honey found above the detection limit, which varied between 0,005 and 0,050 mg/kg. (Bogdanov, 2006). Acaricides used for varroa control within the hives are in the EU and USA mainly cymiazol, fluvalinate, amitraz, flumethrin, amitraz and coumaphos. In Thailand is widely used amitraz, flumethrin, nepthalene and sulfur (Bureau of Livestock Standard Certification, 2006). Residues of this control agent are detectable also in honey. Bees, more than bee products, have been used as biological monitors for pesticide contamination of geographic regions. Beekeepers can also avoid residues by placing their hives more than 3 kilomates from agricultural plants treated with pesticides (Chlebo, 2006).

Honey has two sources of contamination with microorganisms and residues (pesticides, heavy metals, acaricides and antibiotics): primary sources include pollen, the digestive tracts of honey bees, dust, air, soil and nectar; secondary sources are those arising from honey manipulation by people, they include air, food handlers, cross-contamination, equipment and buildings. Primary sources of honey contamination are very difficult to control. Conversely, secondary sources of honey contamination can be controlled by good manufacturing practices. Bee farms must have effective prevention, control, treatment, monitoring and pathogen elimination measures for bee diseases and pests. The farms must also have effective methods to prevent the accumulation of

pathogens and rapidly keep bee diseases under control. Bee diseases treatment must be done by following the recommendations of the Department of Agricultural Extension or Department of Livestock Development, Thailand. Drug application on bees must follow the regulations of the Thai Industrial Standards entitled Code of Practice for Control of the Use of Veterinary Drugs, the Standard No. TIS 7001-2540 (National Bureau of Agricultural Commodity and Food Standard, 2003). The microbes of concern in honey are primarily yeasts and spore-forming bacteria. Total plate counts from honey samples can vary from zero to tens of thousands per gram for no apparent reason. Most samples of honey contain detectable levels of yeasts. Although yeast counts in many honey samples are below 100 colony forming units per gram (cfu/g), yeasts can grow in honey to very high numbers. Standard industry practices control yeast growth. Bacterial spores, particularly those in the Bacillus genus, are regularly found in honey. Typically, honey can be expected to contain low numbers and a limited variety of microbes. A routine microbiological examination of honey might include several different assays. A standard plate count provides general information. Specialized tests, such as a count of yeasts and an assay for bacterial spore-formers, may also be useful (Snowdon and Cliver, 1996). Microorganisms in honey may influence quality or safety. Due to the natural properties of honey and control measures in the honey industry, the good honey is a product with minimal types and levels of microbes.

2.3 The quality of honey

Honey is produced by honey bees from nectar of plants. Some of the components (carbohydrates, water, traces of organic acids, enzymes, amino acids, vitamins, pigments, pollen and wax) are due to maturation of the honey, some are added by the bees and some of them are derived from the plants. Composition of the same floral source can vary due to seasonal climatic variations or to a different geographical origin (Anklam, 1998).

Honey consists mostly of the monosaccharides (glucose and fructose). The actual proportion of glucose to fructose in any particular honey depends largely on the source of the nectar. This content is dependent on the type of soil in which the original nectar-bearing plant was located. The average ratio of fructose to glucose is 1.2:1 (White, 1978; 1980). Saccharose (sucrose) is present in honey (approximately 1% of its dry weight). However, this level can be increased if the beekeeper has over-fed the bees with sugar during the spring. The protein content of honey is normally less than 0.5%. A small fraction of the proteins are enzymes, including: invertase, diastase, glucose oxidase and catalase (Anklam, 1998).

The quality of honey is mainly determined by its sensorial, chemical, physical and microbiological characteristics. Internationally, honey quality criteria are specified in Regulatory Standards, compiled in a Codex Alimentarius Standard. The Codex Alimentarius Standard (2001) for honey quality includes several chemical and physical parameters, comprising moisture content not more than 21% by weight, mineral content not more than 0.6% by weight, acidity not more than 50 milli-equivalent of acid per 1 kg, hydroxymethylfurfural (HMF) content not more than 60 mg per 1 kg, diastase activity after processing and blending not less than 8 Gothe Scale, sugar content not more than 5% by weight, and water insoluble solids content not more than 0.1% by weight. Devillers *et al*. (2004) suggest that the above quality parameters may be used in association with multivariate analyses to assign floral origin.

Thai honey shall be of the following qualities or standards: (1) Colour, odour and taste inherent of that specific characteristic of honey; (2) Reducing sugars content not less than 65% by weight; (3) Moisture content not more than 21% by weight; (4) Sucrose content not more than 5% by weight; (5) Insoluble materials content not more than 0.1 % by weight; (6) Ash content not more than 0.6% by weight; (7) Acid value not more than 40 milli-equivalent of acid per 1 kg; (8) Diastase activity value not less than 3 Gothe Scale; (9) Hydroxymethylfurfural not more than 80 mg per 1 kg; (10) Free of food additives; (11) Food colours are not to be used; (12) Free of pathogenic microorganisms; (13) Free of toxic substances released by microorganisms in quantity

which may be hazardous to health; (14) Yeast and mold shall not be more than 10 colonies per 1 g of honey; (15) Free of contaminants, except the following; arsenic not more than 0.2 mg/kg of honey and lead not more than 0.5 mg/kg of honey (Ministry of Public Health, 2000).

2.4 Antibacterial properties of honey

More intensive studies did not commence until the year 1955 when the word 'inhibine' for the antibacterial activity of honey was introduced, a term which has been widely used since the beginning of literature on honey (White *et al.,* 1963). Since then, there have been many reports on the antimicrobial activities of honey. Some reports used simple tests to demonstrate antibacterial activities of honey (Molan, 1992a; 1992b). Several species of microorganisms were used for sensitivity testing against honey. The antibacterial substances present in honey were investigated by many investigators.

The fact that honey has antibacterial properties has been known for more than a century . It is well established that honey inhibits a broad spectrum of bacterial species. There are many reports of bactericidal as well as bacteriostatic activity. The major variations seen in overall antibacterial activity are due to variation in the level of hydrogen peroxide that arises in honey, and in some cases to the level of non-peroxide factors or phytochemical nature, i.e. its content of tetracycline derivatives, amylase, fatty acids, phenols, ascorbic acid, flavonoides, trepens, benzyl alcohol and benzoic acids (Bogdanov, 1984; Molan, 1992a; 1992b) However the production and type of honey produced by honeybees is depend on the natural flowers blooming in different seasons.The hydrogen peroxide amount in honey is very small and it can be produced only after aerobic incubation of diluted honey solutions. Hydrogen peroxide was found to rapidly disappear when added to dilute honey, and the level of hydrogen peroxide accumulated from enzymatic action was seen to decline with time (White *et al*., 1963). Molan (1992b) reported the loss of antibacterial activity on exposure of honey to heat by varying temperature, time and pH. It was found that the activity was more rapidly lost at low pH and exposure to light. However, a certain antibacterial test might be sensitive

only to certain types of antibacterial substances. Mundo *et al*. (2004) studied the broad spectrum inhibition assay and found that each pathogen or food spoilage organism showed different sensitivity to the honey samples, ranging from highly sensitive (*Geobacillus stearothermophilus*) to unaffected microorganisms (*Staphylococcus aureus*, *Pencillum expansium, Aspergillus niger, Geotrichum candidum*). Other researchers have also found differences in susceptibility of microorganisms to the antimicrobial activity of honey (Dustmann, 1979; Radwan *et al*., 1984; Allen *et al.*, 1991; Willix *et al.*, 1992; Bogdanov, 1997; Nzeako and Hamdi, 2000; Ceyhan and Ugur, 2001; Taormina *et al.*, 2001). This variation could neither be attributed to the floral source or geographic region from which the honey was produced nor by the phylogenetic relatedness of the various different species and growth rate of bacteria, nutritional requirements, temperature, inoculum's size, inoculum's volume and the test method itself (Galli and Washington, 1995). In this study, reduction of inhibines would be studied, using an agar well diffusion method with *Geobacillus stearothermophilus*.

Geobacillus stearothermophilus is a new name of *Bacillus stearothermophilus* that is phylogenetically closely related to the members of the *Bacillus* rRNA Group 5 (Nazina *et al*., 2001a; 2001b). It is a rod-shaped, Gram-positive bacterium and a member of the division Firmicutes. The bacteria is a thermophile. It will grow within a temperature range of 30-75ºC. Its optimum functional temperature is between 60 and 65°C. The optimum pH for growth is 7.0. The spores will not develop if the product is stored at temperature below 43°C. It can cause food spoilage. The organism ferments carbohydrates to produce acids without gas but with some off-flavor and cloudiness (Tucker and Featherstone, 2010).

2.5 Main chemicals used in honey bees of Thailand

Chemical usage by commercial apiarists and government apiary officers in Thailand is currently limited to the following: (i) use of the antibiotics for controlling of bee brood diseases (ii) use of the acaricides against Varroa and Tropilaelaps species of

mites. Both of them are exotic pest of honey bees which are spreading in various third countries, thereby creating serious problems for the apiculture industry. Several commercial acaricide products which are used by beekeepers, such as amitraz, pyrethrins, nephthalene and sulfur, are not registered for bees. There is no report of chemical residues and Maximum residue limit (MRL) for honey and wax of Thailand. MRL is the maximum concentration of a residue that is legally permitted or acceptable in or on a food. It is expressed in µg/kg of that food. Thailand would like to propose priority list of active ingredients used for honey bee treatment for further consideration and proposing MRL in honey as follows (Suijidta, 2008): oxytetracyclin, fluvalinate, tau-fluvalinate, flumethrinne, coumaphos and sulphur (Codex alimentarius commission, 2010). On the other hand, usage of chemicals for beekeeping has become a more sensitive subject for consumers. So many countries have set MRL values of acaricide residues in honey. The degrees of contamination of honey by the different contaminants are shown in this review

Amitraz has a chemical formula of $C_{19}H_{23}N_3$. From the study of, 70 samples honey and beeswax from different beehives (Iran), markets and store shelves of (Iran) all samples were collected during (2006-2007) and analysis for detection of amitraz residues, it could be conformed to EU standard (MRL = 200µg/kg) and were declared as appropriate for human consumption (Hejazy *et al.,* 2010). Amitraz residue was found in 230-5,350 µg/kg in 6 out of 32 Turkey honey samples by HPLC technique (Cobanoglu *et al*., 2008). Maximum residue levels (MRLs) for amitraz in honey were 10 µg/kg in Italy, Germany and Switzerland, 100 µg/kg in New Zealand and 1 mg/kg in the USA. No MRL has been established for amitraz in beeswax, since the substance has never been found as a residue in beeswax (Caldow *et al.,* 2007).

Flumethrin is a synethetic pyrethroid ectoparasiticide for the diagnosis and treatment of mite in honey bees. Official maximum residue levels for flumethrin in honey range from 5 µg/kg in Switzerland to 10 µg/kg in Italy and Germany. Neither the USA nor the EU have an MRL for flumethrin in honey (Wallner,1999).

Naphthalene is an organic compound with formula $C_{10}H_8$. It is a white crystalline solid with a characteristic odor that is detectable at concentrations as low as 80 µg/kg by mass (Amoore and Hautala, 1983). As an aromatic hydrocarbon, naphthalene's structure consists of a fused pair of benzene rings. MRL of naphthalene, was revised to 10 µg/kg by The Ministry of Agriculture and Rural Affairs of Turkey (Alpat and Sunay, 2008). Research between 2004-2006 found only 17%, 1% and 2% of the 3199 honey samples which were over the set MRL (10 µg/kg) of naphthalene, respectively by a SPME (Solid Phase Micro Extraction) method followed by Gas Chromatograph-Flame Ionization Detector). The result indicates that the problem is solved for Turkish beekeeping (Alpat and Sunay, 2008).

Naphthalene was found in 78.9% of the 90 unifloral Greek honey samples by solid-phase microextraction coupled to gas-chromatographic/mass spectrometry but only 5.6% of them contained concentrations above the MRL. Maximum concentrations was 193.74 µg/kg honey for naphthalene (Harizanis *et al.,* 2008).

Naphthalene residues were detected in 2 of 10 honey samples tested. These were at concentrations of 88 and 120 µg/kg (The Veterinary Residues Committee, 2007).

Sulfur or sulphur is the chemical element with atomic number 16, represented by the symbol S. Sulphur, no maximal limits of residues are fixed for the honey even when it is used for pharmaceutical purposes. It appears that it is not necessary for the protection of public health to establish a maximum residue limit, because the amount of sulphur is small and the contribution of honey to the recommended daily intake. 100 g honey contains 0.7-26 mg/kg sulphur. Sulfur is an essential element for all life (Bogdanov, *et al.,* 2008).

2.6 Residues of antibiotics in honey

Antibiotics are used for veterinary purpose. They have been an extremely important aid during the development of intensive methods of animal husbandry (Corry and Sharma, 1983). In general, Thai beekeepers usually buy drugs themselves

and some veterinary drugs are used unlicensed. Many beekeepers do not register to the competent authority and some beekeepers get good agricultural practice for bee farms. Therefore, it can not traceable in food chain.

The tetracyclines that were discovered first in 1948 were the broad-spectrum antibiotics introduced into clinical practice. Their spectrum of activity includes gram-positive and gram-negative bacteria and bacteriostatic. The members of the tetracycline group of antibiotics which occur naturally are tetracycline, chlortetracycline, oxytetracycline and demeclocycline and from semi-synthetic are doxycycline, lymecycline, meclocycline, methacycline, minocycline and rolitetracycline. They bind primarily to the 30S ribosomal subunit, where they inhibit protein synthesis by blocking the binding of aminoacylated tRNA to the A site of the ribosomes (Mascaretti, 2003).

The presence of residues of antibiotics in products from bee becomes a problem. The residues can cause enormous economic losses. There is ample incentive, therefore, it is important for screening methods to be developed to exclude contaminated products from bee. Many countries recommended measures to restrict antibiotic. Further recommendations include monitoring other animal products. There are many hazards that could be posed by antibiotic residues in foods (Corry and Sharma, 1983).

1. Allergic reactions have occasionally been attributed to the consumption of food containing antibiotics, especially penicillin. Other antibiotics which have been implicated include tetracyclines. Fortunately, symptoms are not usually severe. Hypersensitivity reactions including urticaria, angioneurotic edema, anaphylaxis, anaphylactoid purpura, pericarditis and exacerbation of systemic lupus erythematosus may occur. Side effects from tetracyclines are not always common, but of particular note is possible photosensitive allergic reaction which increases the risk of sunburn under exposure to UV light from the sun or other sources.

2. Direct toxic effects due to antibiotic residues are very unlikely because of the low levels involved. There have been reports, however, of toxic effects from ingesting. Tetracyclines are teratogens due to the likelihood of causing teeth discolouration, enamel hypoplasia and fluorescence in the fetus as they develop in infancy. For this same

enamel hypoplasia and fluorescence in the fetus as they develop in infancy. For this same reason, tetracyclines are contraindicated for use in children under 8 years of age. They are, however, safe to use in the first 18 weeks of pregnancy. Some patients taking tetracyclines require medical supervision because they can cause steatosis and hepatotoxicity (Deboyser *et al*., 1989; Amacher and Martin, 1997) and tetracyclines may cause side effects (upset stomach, diarrhea, itching of the rectum or vagina, sore mouth, redness of the skin) (Jawetz *et al*., 1984).

3. Selection of antibiotic-resistant organisms resulting directly from the consumption of antibiotic residues in food seems rather unlikely because the levels of antibiotics would be low and the food would be diluted by other foods, gastric secretion, etc. However, very low levels of antibiotics are present in food, the mechanism by which this occurs is still not clear, but alteration of intestinal flora appears to be involved. In humans, any antibiotics cannot be denatured or absorbed in the upper part of the intestinal tract due to water absorption, and might affect the intestinal flora and hence, resulting in resistant strains. A more important reason for monitoring antibiotic residues in foods form part of a general policy to prevent unnecessary and indiscriminate use of therapeutic or prophylactic doses of antibiotics as a substitute for good animal husbandry. In particular, this contributes to the selection of multiple-resistant strains of bacteria, which can be acquired via food and cause serious infections in both man and animals. It is known that the resistance genes can easily be passed onto other unrelated bacteria and small doses stimulate the bioresistance of bees too. Consequently, life-saving antibiotics become less effective against bacteria and there will be fewer alternatives available for successful treatment on infection.

Bacterial diseases of the honey bee may severely decrease the honey bee population and honey production, causing significant damage to the beekeeping industry. The pathogens have been treated in bee colonies by using antibiotics for many years. Due to their low cost and large availability, veterinary drugs are most commonly used in several countries (Martel *et al*., 2006). Because recommendations for drug withdrawal are not respected or veterinary drugs are used unlicensed, there is a significant risk of

detecting tetracyclines antibiotic residues in honey.

Antibiotic residues in honey have recently become a major consumer concern, however there are very few reports of antibiotics in honey. Heering *et al*. (1998) reported that 100 honey samples (various countries from Eurasia, Oceania and the Americas) were analysed by enzyme immunoassays (EIA). They found tetracyclines in 12 samples exceeding a level of 50 µg/kg. In France, Faucon *et al.* (2001) found tetracyclines residues in 34 of 75 French lavender honeys, and 14 of them had residues above 0.02 µg/kg. In 2002, residues of tetracyclines (ranging from 0.019-2.152 µg/kg) were detected in 19 out of 113 French honey samples collected according to Directive 96/23/EC (European Union, 2002). In Belgium, Wim (2003) reported that residues of veterinary drugs were found in a very limited number of locally-produced honey and imported honey on the Belgian market. Tetracyclines 2/72 (2.8%) and 29/98 (29.6%) were determined with ELISA test with detection limit of 10 µg/kg.

Research in Thailand, by Thai Nestle Company in 2004 found 1 chloramphenicol sample from 229 honey samples and tetracyclines 30/229 (13.1%) (Maneetup, 2004). Liawruangrath *et al*. (2006) found chloramphenicol in 10 out of 14 samples of honey (0.29-3.26 mg/g) and no tetracyclines were found in honey from Chiang Mai Province that were analyzed by High Performance Liquid Chromatography (HPLC). Pathomchai and Sujaritpun (2007) found chloramphenicol in 3 out of 6 samples of honey (2.49-10.69 µg/kg) and no tetracyclines were found in honey from Chiang Mai Province as analyzed by HPLC. Pochalearn (2007), found tetracycline group in 2 out of 267 samples of honey from Chiang Mai Province as measured by LC-MS/MS technique. Taokaenchan and Sangsrichan (2010) found oxytetracycline residue (60.61 mg/kg) in a commercial longan honey that was purchased from a market in Chiang Mai Province during 2008.

2.7 Techniques for antibiotic residue test in honey

An antibiotic was originally defined as a substance produced by one micro-organism that inhibits the growth of other micro-organism. This definition is no longer strictly accurate because some antibiotics have now been produced synthetically or semi-synthetically. Thus, the term antibiotic is now considered as referring to a substance produced by a micro-organism or to a similar substance that, in low concentrations, inhibits the growth of micro-organisms (Quesnel and Russell, 1983). Now there are many techniques for detecting antibiotic in honey.

2.7.1 Agar diffusion test

Early technique or a classical method of assaying antibiotics which relied on the inhibition of growth of a test organisms is the agar zone diffusion technique. The reference standard and unknown were applied to reservoirs (holes cut by a cork-borer) in a layer of agar. The principles of measurement involved the formation of inhibition zones within 18 hours. This technique is still widely used. The advantages are that this technique is cheap, relatively sensitive, easy to perform and highly productive (Dinkov *et al*., 2005). However, this method has a great disadvantage of being a slow technique which is mostly undesirable when results are needed rapidly. It can be sped up by the use of heavy inoculums. Moreover, as the presence of antibiotics is measured by inhibition zones, under well-controlled standard conditions, the zone size is directly related to the sensitivity of the test organism for discs of specified potency. Accurate measurement of zone diameter, using calipers, can be used as a definitive assessment of sensitivity on a comparable basis (Linton, 1983) and to smooth inequalities connected with precise estimate, breadth zones in three directions situated at angle of 120^0 to each other were measured to calculate average quantity. The sensitivity was determined on the test microorganisms such as *Micrococcus luteus*, *Geobacillus stearothermophilus* and *Bacillus subtilis* to pure substances of oxytetracyclin, tetracycline, chlortetracycline, doxacyclin and methacyclin added to honey specimens in concentrations of 0.1 and 1.0 mg/kg. The most sensitive microorganism to honey artificially contaminated

with the antibiotics is *Bacillus subtilis* at residual of 1.0 mg/kg (Petkov, 2000). According to the information taken from different sources, when using bacteria *Bacillus cereus* species as a test specimen, the concentration of the antibiotic in honey detected by this method should not be less than 0.04–0.09 mg/kg (Gordon, 1989). When a test specimen *Bacillus subtilis* culture was used, the minimum determined concentration of tetracycline in honey was 0.3 mg/kg.. There was no clear zone at 0.15 mg/kg antibiotic content (Khismatullin *et al*., 2003).

2.7.2 The microbial growth inhibition assays

These are the earliest methods used for the detection of antimicrobial residues in food based on the detection of growth inhibition of various sensitive bacterial strains. Such methods, originally developed for use in clinical medicine, were based on microbial agar diffusion tests of the inhibition of acid production of coagulation by starter organism (Mitchell *et al*., 1998). The basic microbial inhibition assay format involves a standard culture of a test organism, usually *Geobacillus stearothermophilus, Bacillus subtilis, Bacillus cereus, Micrococcus luteus or Streptococcus thermophilus* seeds in an agar or liquid growth medium which is then inoculated with a milk, urine, meat and incubated for periods of up to several hours. The presence of an inhibitory substance is indicated by change in the color of the medium (Mitchell *et al*., 1998). The major disadvantages of microbial inhibition assays are that they are not very specific for antibiotic identification purposes, are qualitative, have limited detection levels to many antibiotics and require several hours before results are available (2.5 to 4 hours). Growth inhibition tests are subjected to the effects of many natural inhibitory substances found in foods of animal origin such as lysozyme, hydrogen peroxide, lactic acid. These compounds may give false positive test results. Example, Premi®Test has protocol to preparation of sample by heating the honey at 45°C for 30 minutes and dilute the honey with water (1:1). 100 µl of extract was applied to an ampoule, the ampoules were sealed using plastic film. The ampoule was then incubated at 64 ± 0.5°C in a circulating water bath until the yellow end-point was visible in the negative control (3–3.5 hours). The Premi®Test is based on

the inhibition of growth of *Geobacillus stearothermophilus*. It had detection limit of tetracycline,oxytetracycline and chlortetracycline at 50 µg/kg, 75 µg/kg and 80 µg/kg, respectively (DSM Nutritional Products Company, 2010). Advantages of these tests are that they are inexpensive, easy to perform, adaptable to the screening of large numbers of sample and have reasonably broad antimicrobial detection spectrum (Mitchell *et al*., 1998).

2.7.3 The immunological methods

The immunological method mainly consists of ELISA test kits. There are many kits commercially available. ELISA is an enzyme-linked immunosorbent assay in which antibiotics are quantitatively assayed by specific antibodies. In immunological technique, antigen and antibody reaction has been used for many years to detect a wide variety of food constituents including substances responsible for adulterations and contaminations. The interaction between antigen–antibody is very specific and useful for the detection of residues of chemical and veterinary drugs in animal foods. Immunological assay should not be affected by antibiotics from families other than that being assayed or nonspecific inhibitory sources. The basis of immunological assay for antibiotics depends upon the binding of the antibiotic with a specific antibody in a reversible reaction. Antibodies in conventional antisera are heterogeneous in nature, since they are secreted by many different clones. Some of them lack specificity, since they are directed against impurities in the antigen preparation, rather than to the antigen alone. It is virtually impossible to separate the different antibodies, a problem that has led to a major drawback in their application in biology and medicine (Quesnel and Russell, 1983). The most usual technique consists in the ELISA and the detection system is usually based on enzyme-labelled reagents. There are different formats for antigen quantification. In double antibody or sandwich ELISA tests, a primary antibody is bound to the plate well. The antigen of the sample extract added to the well complexes with the bound antibody and remains bound to the plate after washing. Then, a second antibody labelled with an enzyme such as peroxidase is added to the well followed by a new wash. The quantity of

conjugate bound to the plate is detected after incubation with a specific substrate. Colour is developed during incubation and measured with a microplate reader, which is proportional to the amount of analyte in the sample. Now, there are screening test kits on the competitive enzyme immunoassay for the detection of tetracycline in honey and this method is still reliable enough to indicate. Example, a commercially-available enzyme immunoassay, the honey samples diluted with buffer were filled into the microtiter wells coated with a tetracycline-protein-conjugate and anti-tetracycline antibodies were added. Any unbound antibodies were removed by washing steps. After adding enzyme substrate and chromogen, the absorption was measured. ELISA has been tested successfully in honey (Heering *et al.,* 1998). The detection limit is 10-50 µg/kg.

Main advantages of ELISA test kits are easy to use, available kits for a good number of specific and families compounds, large number of samples (42) per kit for a single analysis, reduced time (few hours) to obtain the results: about 2–2.5 hours for most kits, high sensitivity, high specificity, possibility to use within the food-processing facility. Disadvantages are expensive, limited storage (few months) under refrigeration, cross reactivity with other antibiotics from the same group the method can be used only for semiquantitative determination.

2.7.4 Other screening test kits

The tetrasensor honey test kit is a receptor-based assay, using dipsticks for a rapid screening. The test detects tetracycline, oxytetracycline, chlortetracycline and doxycycline in honey in a specific and sensitive way. Depending on the type of tetracyclines, detection capabilities ranges from 4-12 mg/kg (Reybroeck *et al*., 2007) but Alfredsson *et al*. (2005) reported detection capabilities of 5-25 µg/kg.

Charm II Tetracyclines Honey test uses an antibody (binder) with specific receptor sites that bind all of the test antibiotics. The binder is added to a sample extract together with an exect amount of H^3 or C^{14} labled antibiotics (tracer). The antibiotics in the samples combine with the receptor sites and the radio-labeled antibiotics occupy the remaining sites. After this reaction, the concentration of either H^3 or C^{14} associated with

the binder are measured as counts per minute value using the Charm II system (Morlot and Beaune, 2003). The Charm II Tetracyclines Honey kits were from Charm Sciences Inc. (Massachusetts, USA). The Charm II Tetracyclines Honey (detection capability for tetracycline, oxytetracycline, chlortetracycline and doxycycline in honey ≤ 10 μg/kg).

2.7.5 Quantitative confirmatory methods

Commonly used procedures for the detection of veterinary drug residues include HPLP and LC-MS/MS. The use of high performance liquid chromatography (HPLC) expanded during the 1990s and the availability of automation some how facilitated its use as a screening technique. HPLC is a superlative technique and its ability to detect compounds depends on the type of detector used. It is increasingly becoming the premier choice for analyzing antibiotics and anti-infective agents, according to regular agencies. HPLC is versatile enough to permit several methods of analysis to be developed, starting with almost any type of separating column and this method is very expensive. HPLC is getting expanded use in controlled laboratories due to the possibility to analyse simultaneously multiple residues in a sample in relatively short time. Recent developments of high speed HPLC can reduce sample treatment and analysis time. In addition, this technology is fully automated (injection, elution, washing of column, detection) and computer-controlled, facilitating its use as a screening technique.

The HPLC is good for routine work where known antibiotics are analysed. There is no 100% certainty regarding the safe identification of the antibiotics. HPLC methods with fluorescence detection help increase the sensitivity and improve the specificity of the method and have been used in the detection limit of about 50 μg/kg (Bogdanov, 2003). The use of HPLC technique with fluorimetric and mass detection, together with an appropriate separation/concentration step, allows to obtain good validation parameters such as recovery, quantification limits, linearity response and repeatability with the detection limit of 21-50 μg/kg. (Gallina *et al*., 2005).

Main advantages of HPLC: advantages are short time (few min/sample) to obtain the results, sensitive, specificity depending on detector, automation leading to

leading to higher productivity, possibility to find more information from spectra when using diode array detector. Disadvantages are expertise required, need of sample preparation (extraction and filtration, addition of internal standard, etc.), high initial investment (equipment) and cost of column.

Liquid chromatography-tandem mass spectrometry (LC/MS-MS) is the most modern and promising method. The MS-MS insures an almost 100 % safe specific detection with a very low background noise. The limit of detection is 0.5-1.0 µg/kg (Bogdanov, 2003) depending on the substance. Indeed, tetracyclines are not stable molecules and the epimerisation phenomenon was evaluated in this work. Appropriate correction factors of the MS/MS responses of each epimer were studied for each of the four tetracyclines to accurately quantify them (Khong *et al*., 2005). One reason for this trend is the increasing number of analyses to perform due to demands from EU and consumers' interest in safe food. Another reason is the special criteria which has to be fulfilled for the confirmation of illegal substances.

Because of disadvantages of commercial techniques, the aim of this research is to develop antibiotic residual screening test kit. This research will use modification of microbial inhibition assay for detection of tetracycline, chlortetracycline and oxytetracycline residues in longan honey; microbiological assays measure the ability to inhibit the growth of microorganisms. The test kit will be developed to be cheap, easy to perform and reliable.