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# Pesticide Residues in Bee Products

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## 1. Introduction

"The man has no divine right over the food. He must compete for this with weeds, diseases, insects and other organisms" (Grodner, 1996).

With the above quote, Grodner clearly reflects the situation in the area production and processing of food. More than 10.000 species of insects and mites, 1.500 species of fungi and 600 plant species have been identified as harmful to agriculture (Grodner, 1996; Pimentel et al., 2000). The production of plant and animal products requires the use of large quantities of chemicals (plant protection agents, veterinary drugs, fertilizers, etc.), which could lead to increased production and improved quality, as a consequence. The quality of the final product is usually reflected to particular visual parameters like color, size and general appearance. In the case of food, however, many questions are raising about their safety. The reason is the possible presence of chemical residues detected in the final product. Nowadays, consumer safety is a major priority for governments of developed countries and food safety is a criterion for the trading and prices on the market. Reports in media about alimentary scandals cause anxiety to consumers and turning a part of the market to organic products, which are considered free, or at least less contaminated by hazardous substances. In recent years, reports in media have grown at an alarming rate and any references to the consumer aimed at creating impressions, achieved by overemphasizing the disadvantages of chemical use and mainly problems related to environmental pollution and its impact on human health. In contrast, reports in media referring to the advantages of using chemicals are minimal to nonexistent. For example, the absence of appropriate chemical agents to combat rodents lead to the first epidemic of the bubonic plague disease and the death of 65.000.000 people. Moreover, the starvation in Ireland began due to the fungal disease *Phytophthora infestans*, which destroyed potatoes causing 1.000.000 deaths from 1845 to 1851 (Knutson et al., 1990). Besides health problems that preoccupied humans in the past, the economic impact on different groups of consumers will be significant in case of pesticides withdrawal. Specifically, the weekly expenses for food are expected to rise by 44% for consumers of low average income. The economies of countries with intensive agriculture will be stroke because of the decline in exports of grains and products like cotton. Finally, undesirable environmental effects are expected due to the increasing of cultivated land and the erosion problems that could be observed because of the limited growth of the root

system of plants (Knutson et al., 1990). In contrast to the above, there are few cases of pesticides proved dangerous to public health. A typical example is the chlorinated hydrocarbon DDT, which was previously used against mosquitoes. The active substance DDT contributed greatly to reduce the spread of diseases like malaria, but was withdrawn in 1970 as it was considered dangerous for human and environment safety.

Active substances are classified into five groups according to their toxicity. The first group (Ia) includes the extremely toxic agricultural plant protection agents, while the four other groups of substances are listed in order of decreasing toxicity (Ib, II, III). The fifth group (U) includes substances that are unlikely to become toxic to humans (International Programme on Chemical Safety, 2004). The adverse effects of these compounds may be observed in a short period (acute toxicity) or after a long time (chronic toxicity). In any case, it should be noted that, although the annual reported number of deaths by poisoning is 355.000, only a part of these poisonings, which is not specified in the World Health Report, are due to pesticides (WHO The World Health Report, 2003). Moreover, all cases of poisoning are due to accidents, like accidental ingestion or inhalation of chemicals, and not by food intake. However, the possibility of indication of various effects in consumer health through chronic toxicity cannot be ignored. Toxicity of a plant protection product depends on various factors, including chemical structure, temperature and humidity conditions, dose, duration of exposure, mode of action and the kind of exposure, like ingestion, inhalation, dermal etc. Different groups of pesticides and veterinary drugs are likely to be responsible for causing malaise, sore eyes, abnormalities to skin and respiratory system. Moreover, some pesticides and veterinary drugs are suspected of causing certain types of cancer, teratogenicity, chromosomal abnormalities and the weakening of the immune system of humans (Banerjee, 1999). The toxicity of various active substances, which can be detected in bee products, varies according to their chemical synthesis. In any case, poisoning or deaths due to the presence of toxic substances exclusively to bee products have not been reported. An exception is the death of infants, which was caused by *Clostridium botulinum* (Arnon, 1980). Even in these cases, however, the responsibility of honey has not been proven clearly, as this clostridium appears in the environment widely (Midura, 1996). In addition, 65% of infants that became ill had not eaten honey at all (Arnon et al., 1979). In any case, appropriate infant feeding prohibits the consumption of honey until the age of one year to eliminate possible poisoning from toxins of this micro-organism.

Another way of classification of active substances, relates to the subject of this investigation, and is based on bee toxicity. In general, active substances are classified as very toxic, moderately toxic and non-toxic to bees. The most significant impact on bee colonies has been observed after treatments with plant protection products during the blooming. Most deaths occurred during the stage of forager worker bee that collects nectar and pollen. Moreover, larvae and domestic bees die because of pesticide residues detected in pollen. Although in many cases the concentration of pesticide found in pollen is not lethal, it is however likely to cause paralysis of bees, irritability, killing and replacing of the queen bee and generally abnormal behavior. This behavior can also be caused by substances that do not kill bees directly (e.g. carbaryl as active ingredient of Sevin®) but are transferred into the hive by foragers and affect the entire population (Sanford, 1993). The long-term persistence of many pesticides in stored pollen has also serious impact of bees' survival. Arsenic from paris green and calcium arsenate was present in pollen stored in comb analyzed six months after application. Methomyl residues persisted in honeybee combs for eight months. Methyl parathion from Penncap M, persisted in combs samples of stored pollen for 7 to 14 months after use and carbaryl similarly persisted over winter for 7-9 months (Erickson et al., 1983)

Neonicotinoids like imidacloprid were also detected in stored pollen (Gregorc & Bozic, 2004; Chauzat et al., 2006). Also, the type of formulation and application of the pesticide in relation to toxicity caused to the bees proved particularly important. For example, the standardization of the active ingredient methyl parathion in microcapsules kills 13 times more bees than the formulation of the same substance as an emulsified solution. Furthermore, wettable powder or dust proved less dangerous than microcapsules, but also caused more deaths than aqueous or emulsified solutions (Sanford, 1993).

Under the development of framework concerning consumer safety, the European Union created a warning system called RASFF (Rapid Alert System for Food and Feed), which reports hazardous foods and feeds, identified in the markets of the Member States. Bee products like honey and royal jelly have been reported occasionally. The main reason why they have been reported is the detection of residues of antibiotics that have been used by beekeepers to fight various diseases of bees.

## **2. Contamination of bee products**

There are two ways of contamination of bee products with various chemicals; the indirect and the direct contamination. The indirect way reflects the transporting of toxic substances by foragers bees during the collection of nectar, honeydew, water, pollen and propolis. Many studies concern the contamination of hive products by agrochemicals and heavy metals, while few concern the presence of nitrofurans, toxins and PCBs in beehive products. The direct way, which is the most important, regards the contamination of bee products by acaricides, antibiotics and volatile pesticides caused by beekeeping practices.

### **2.1 Indirect contamination of bee products**

Many researchers supported the theory that the transferring of pesticides from fields to beehive is prevented in various ways. The bees' death at the field, the lost of orientation of the foragers, the reluctance of guard bees to permit the entrance to foragers with contaminated nectar, the retaining of contaminated food in bees' stomach, the stopping of further elaboration of contaminated nectar by hive bees and the removal of affected bees from the hive are natural provisions against general contamination of honey (Johansen & Mayer, 1990; Atkins, 1992). Contrary to the above-mentioned cases, older studies reported that worker bees may carry high concentrations of pesticides into their beehive. In some cases the concentration of pesticides in the load was 25 times greater than the lethal dose of the bee (Jaycox, 1964).

#### **2.1.1 Pesticides**

Pesticides used on various crops are classified into groups based on their chemical structure (organophosphates, pyrethroids, organochlorines, carbamates, neonicotinoids etc.), mode of action (systemic, contact), target (insecticides, acaricides, herbicides, fungicides, bactericides, nematocides) and synthesis (synthetic or natural). The residues of pesticides detected in beehive products are classified in the groups of insecticides (organochlorines, organophosphates, carbamates and neonicotinoids), acaricides, fungicides and herbicides.

##### **2.1.1.1 Organochlorine pesticides (OCPs)**

This specific group of insecticides is considered particularly hazardous because of its ability to bioaccumulate into the food chain, to remain stable for many years and to move into the

environment in every potential way (air, water, soil, biota). The case of bioaccumulation of DDT in the environment is the most characteristic, while the concentration detected in the higher levels of the food chain is 10,000,000 times greater than that detected into the water. In recent decades there had been many efforts worldwide to prevent the use of substances belonging to the group of persistent organic pollutants (POP), which includes many organochlorine compounds. The continuous transfer of semi-volatile compounds from tropical regions of the world to the colder poles is suspected for long-term effects on living beings (Carson R., 1962). Chlorinated hydrocarbons are detected in high concentrations in various products, because of their low rate of degradation. Wax is the beehive product more likely to be contaminated by organochlorine insecticides because of its strong lipophilic character. Moreover, OCPs were proved to remain stable during the conversion of old combs into new (Jimenez et al., 2005). The problem is magnified by the import of wax from continents where the use of chlorinated hydrocarbons is still permitted like Asia and Africa. The encouraging news is that the percentage of honey contaminated with chlorinated hydrocarbons dropped from 96.1% to 52.3% during the decades 1980 and 1990.

#### **2.1.1.2 Organophosphorus pesticides (OPPs)**

This specific class of pesticides is of relatively high toxicity for humans and was first studied and used as an asphyxiating gas during the Second World War. Organophosphorus compounds are not stable in the environment and are not bio-concentrated and this is probably the main reason why they were detected rarely and at lower concentrations into beehive products. Most of published information concerns the compound methyl parathion, which has been used in agricultural crops as preparation in the slow-release form of microcapsules. Residues of this chemical were detected in honey and pollen (Atkins & Kellum, 1984). The microcapsules stick on the dense coat of bees, transferred into their hive and stored along with pollen. Pollen is the main component of the diet of larvae. The presence of polluted pollen might cause poisoning and eventually death to brood of bees. In a survey conducted on pollen from France, residues of parathion and methyl parathion were found in 1.2% and 4.9% of the samples, respectively. The average concentration for parathion was 0.019 mg kg<sup>-1</sup> and for parathion methyl was 0.025 mg kg<sup>-1</sup> (Chauzat et al., 2006). Blasco et al. (2003) detected only heptenophos in 4% of honey samples that were analyzed, out of 23 organophosphorus pesticides that they researched. Heptenophos concentrations in this survey ranged from 0.08 mg kg<sup>-1</sup> to 0.23 mg kg<sup>-1</sup>. Finally, Balayiannis and Balayiannis (2008) detected the organophosphorus compounds chlorfenvinphos, chlorpyrifos and phorate in honey originated from Greece, in concentrations ranged from 0.7 µg kg<sup>-1</sup> to 0.89 µg kg<sup>-1</sup>.

#### **2.1.1.3 Carbamate pesticides**

Carbamate insecticides have a similar mode of action with organophosphates but their insecticidal activity is more selective and depends to a certain extent on the insect species. Some fungicides and herbicides belong to this family. These substances are highly volatile in the environment and in some cases they were detected in beehive products. Concentration of carbamate residues detected in pollen ranged from 0.126 mg kg<sup>-1</sup> to 0.265 mg kg<sup>-1</sup> for the active ingredient carbaryl, while the maximum concentration of carbofuran was 0.14 mg kg<sup>-1</sup> (Chauzat et al., 2006). Concentration of carbaryl, carbofuran, pirimicarb and methiocarb residues, in most cases is considered low and does not exceed 0.071 mg kg<sup>-1</sup>. In only one Spanish honey the concentration of carbofuran was 0.645 mg kg<sup>-1</sup> (Blasco et al., 2003).

Nevertheless, the concentration of carbamate residues is low in pollen and honey, while no residues have been reported in other beehive products.

#### 2.1.1.4 Neonicotinoid pesticides

Most studies reported on neonicotinoid insecticides, refer to the active ingredient imidacloprid. This substance was proved toxic to bees, but the concentrations of residues detected in honey were very low ( $0.001 \text{ mg kg}^{-1}$  to  $0.005 \text{ mg kg}^{-1}$ ) (Bonmatin et al., 2003; Maus et al., 2003; Schmuck et al., 2001). In many cases residues did not exceed the limit of quantification ( $<0.002 \text{ mg kg}^{-1}$ ) (Rogers & Kemp, 2003; Schöning & Schmuck, 2003; Stadler et al., 2003; Faucon et al., 2004). Detected residues of imidacloprid in pollen were  $0.005 \text{ mg kg}^{-1}$ , while detection rate was 49.4% (Bonmatin et al., 2003). The hazard quotient (application rate in grams per hectare/ $\text{LD}_{50}$ ) of neonicotinoids is far below the trigger value of 50, but the most important is the chronic toxicity that they cause to bees. The long-term exposure to neonicotinoid after the behaviour of bees, reduce their reproduction capacity and lead of population decline. The low detectable concentrations in combination with the low toxicity of imidacloprid in humans are reassuring for consumer safety. On the contrary, the effects of imidacloprid residues on bees should be further explored. The high toxicity for bees makes neonicotinoid residues suspicious about the death of many forager bees collecting nectar from sunflower, corn and cotton crops. The implementation of neonicotinoid active substances in seed of plants like cotton, corn and sunflower led to a theory that this specific class of pesticides is responsible for the "colony collapse disorder" syndrome (CCD). The CCD is defined as the sudden depopulation of a beehive and the rapid collapse of the colony. The causes of this phenomenon are not clear yet. The suspicion is directed at the mite *Varroa destructor* Anderson & Trueman, while others blame the protozoan *Nosema ceranae*. Additionally, suspicion directed at poisoning of bees by neonicotinoid insecticides and at various forms of radiation (telephony, wireless networks etc.) as well. In fact, research on the toxicity of neonicotinoids to the bee, proved tolerance of the insect body at normal concentrations identified in honey, pollen and nectar (Schmuck et al., 2001; Faucon et al., 2004). More recent research is directed at the effect of imidacloprid to the orientation of bees (Bortolotti et al.; 2003).

#### 2.1.1.5 Fungicides

Fungicides are toxic substances that are used to kill or inhibit the growth of fungi that cause economic damage to crops and endanger the health of domestic animals or humans. Most fungicides are toxic to humans and can cause both acute and chronic problems if absorbed into food. Kubik et al. (1999; 2000) studied the possibility of contamination of beehive products with residues of chemicals used on apple and cherry trees. Vinclozolin, iprodione and thiophanate methyl residues were detected in honey and pollen collected from cherry flowers, while captan and difeniconazole were detected in beehive products collected from apple trees. Specifically, the average concentration levels of vinclozolin in honey were determined at  $0.107 \text{ mg kg}^{-1}$ . A recent review of vinclozolin by the US Environmental Protection Agency has concluded that the chemical or its breakdown products are associated with the development of testicular tumors in rats. The mean concentrations of residues of other active compounds were  $0.0006 \text{ mg kg}^{-1}$ ,  $0.009 \text{ mg kg}^{-1}$ ,  $0.023 \text{ mg kg}^{-1}$  and  $0.059 \text{ mg kg}^{-1}$  for captan, difeniconazole, iprodione and methyl thiophanate respectively. In all cases, the concentration was lower in honey, than in stored pollen (Kubic, 2000).

### 2.1.2 Antibiotic residues due to agricultural use

Antibiotics can find their ways to bee products not only from beekeepers but also from the environment. Bees collect and transfer readily in their hive bactericides that are used against *Erwinia amylovora*. Out of 166 Greek citrus honeys that had been analyzed the 146 of them were found having antibiotic residues of sulphonomides and streptomycine originating from the therapeutical products that had been used in citrus plants (Karampournioti, 2004). Similarly in South Germany 40 samples out of 183 (21%) were found having residues of that source (Wallner, 1998). Moreover, Brasse (2001) identified the antibiotic streptomycin in 27 out of 128 honey analyzed samples. Bees may also transfer antibiotics through water since sulphanimide and tetracyclines are used in drinking water from poultry farms, rabbit cages and other animals. The manure of pigs and cows treated with sulphonomides or sulphamcompounds could also be the vector. Some herbicides products, like Asulan may be degraded to sulphanimide and bees with nectar can transfer it into the hive (Bogdanov & Edder, 2004; Kaufmann & Kaenzig, 2004). Finally bees may rob honey from colonies of other apiary that had been treated by antibiotics and by this way can contaminate their product in detectable levels.

### 2.2 Direct contamination of bee products

Active substances used by beekeepers themselves are likely to contaminate bee products with undesirable residues. Acaricide and antibiotic preparations are used in order to control the mite *Varroa destructor*, American foulbrood, Nosemosis and other diseases. Moreover, several volatile insecticides were used in the warehouse, in order to fend lepidopteron *Galleria mellonella* Linnaeus, which is responsible for considerable damages to stored combs.

#### 2.2.1 Acaricide residues

The use of synthesized substances for crop protection and livestock is the easiest and most effective way for beekeepers to control mites. Acaricides like amitraz, cymiazole, bromopropylate, tau-fluvalinate, flumethrin, coumaphos and malathion have been used by beekeepers all over the world. Many preparations like Apistan (a.i. tau-fluvalinate), Perizin (a.i. coumaphos), CheckMite+ (a.i.coumaphos), Bayvarol (a.i. flumethrin) and Apiguard (a.i. thymol) gain approval in most European countries. There are substances like amitraz that got approval only in certain countries and others like malathion that have not been approved at all.

##### 2.2.1.1 Amitraz

Structure: It belongs to the group of formamidines

Action: Non-systemic insecticide and acaricide, which causes stimulation of neuronal activity killing the target.

Preparation: The main commercial formulation is the Taktik, used in livestock and particularly horses and sheep. Other preparation used: Mitak and Bye Bye.

Ways to use in beekeeping: Fumigation, Spray.

Acceptable Daily Intake (ADI): 0.003 mg kg<sup>-1</sup> body weight per day or 0.18 mg per person per day (EMEA, 1999).

MRL for honey: EU established maximum residue levels (MRL) for amitraz residues in honey. The MRL of amitraz established to 0.2 mg kg<sup>-1</sup> including the parent compound and its metabolites containing 2,4-dimethylaniline moiety. It should be noted that despite the establishment of the MRL, amitraz residues in honey are not acceptable in some countries

because of the lack of approval for beekeeping use. Therefore, in this case the limit corresponds to the Limit of Quantification, which is 0.01 mg kg<sup>-1</sup>.

The use of active substance amitraz is widespread in several European countries and the United States. Moreover, the effectiveness of this substance against varroa is satisfactory. The residues of the active substance is not often detected because of the rapid degradation of amitraz, which takes place within three weeks in blossom honey and four weeks in honeydew honey. The difference in degradation interval was attributed to the lower pH of blossom honey, which accelerates the chemical reactions of decomposition (Corta et al., 1999). The active ingredient amitraz is usually detected in cases where the preharvest interval is very short. A study reports as final degradation product of amitraz in honey, the 2,4-dimethyl-aniline, which is classified as hazardous to public health (Taccheo et al., 1988a). Recently, several samples of pears found to contain significant concentrations of amitraz and its metabolites. This fact, as well as indications about carcinogenic effects of the substance, led to a series of inspections and repeated alerts reported on RASFF of EU (Rapid Alert System of Food And Feed). To date, no published RASFF on residues of amitraz in honey have been reported. Finally, a reference work published in USA, observed the development of resistance of varroa to amitraz (Eljen et al., 2000).

### 2.2.1.2 Coumaphos

Structure: It belongs to the group of organophosphorus insecticides-acaricides

Action: Substance with systemic action that causes death in insects and mites by affecting cholinergic synapses of the central nervous system.

Preparations: The active ingredient coumaphos prepared by Bayer as three different formulations; Perizin, CheckMite+ and Asuntol. The last is the only one without authorization for beekeeping use.

Ways to use in beekeeping: The active substance used as an aqueous solution or a controlled release film. Perizin is used as an aqueous solution applied as drops between the frames. Spraying or adding to food can also be used for the application of this preparation. Special mention should be made to the use of coumaphos in the form of controlled release strips (CheckMite+). Primarily, application of CheckMite+ took place in the U.S., by providing a limited number of films in beekeepers of every State (Sanford & Flottum, 1999). In Europe, CheckMite+ was granted authorization in 2006. The major advantage of this preparation is that it also controls the small hive beetle *Aethina tumida* Marey.

ADI: 0.25 mg kg<sup>-1</sup> body weight per day or 15 mg per person per day (EMEA, 2001).

MRL for honey: the established MRL for coumaphos in the EU is 0.1 mg kg<sup>-1</sup>.

Coumaphos does not control mites exclusively through contact, like most acaricides used in beekeeping, but it has a systemic action as well. The advantage of this way of action is the greater efficacy, and the rapid dispersion throughout the whole area of the hive. However, the disadvantage of substances with systemic action like coumaphos is the great persistence. According to a study, bees produce wax with residues of coumaphos, even six months after the application of the substance into the hive (Wilhelmina, 1992).

The persistence and dispersion of coumaphos in the hive after the application of CheckMite+ was studied by Karazafiris et al. (2008). According to that study, concentration of coumaphos residues was great in honey frames, which were in contact with strips. In some cases, residues exceeded the value of the established MRL. Moreover, it was observed that the concentration of acaricide in honey was at the level of MRL even 103 days after the removal of the strips. Therefore, the exclusion of frames that are in contact with the strips



could lead to a drastic reduction of residual coumaphos concentrations in the final product. On the contrary, it was observed that the concentration of coumaphos residues in honey chamber was significantly lower and in no case exceeded the MRL. Finally, the time between application of the preparation and the collection of honey affected the amount of residues. Gajduskova et al. (1990), studied the contamination of bee products under different methods of application, found that higher concentrations of coumaphos levels were recorded when the substance was added to the syrup rather than the usual method of dripping the chemical into the hive. Coumaphos proved very stable in honey, moved quickly to the wax because of its strong lipophilic character and remained there in significant concentrations even after melting of the wax (Krieger, 1991). Reports that mites became resistant to coumaphos have already been published (Maggi et al., 2009; Petis, 2004).

### 2.2.1.3 Flumethrin

Structure: It belongs to the group of pyrethroids.

Action: Non-systemic insecticide-acaricide which acts in contact through the stomach. As the majority of pyrethroid pesticides, flumethrin is characterized as a broad range pesticide presenting low toxicity to mammals. Through its action, flumethrin disrupts the functioning of the Na<sup>+</sup> pump and therefore the equilibrium of Na<sup>+</sup>/K<sup>+</sup> across the membrane.

Preparation: Bayvarol is one of the approved preparations for use in beekeeping. Production Company is the Bayer CropScience.

Beekeeping use: Flumethrin applied in the form of controlled release strips.

ADI: 1.8 mg kg<sup>-1</sup> body weight per day or 108 mg per person per day (EMEA, 1998).

MRL for honey: The very low concentration required per hive and the low water solubility, are the main reasons why no detectable residues were detected in honey after the recommended use. That is the reason why no MRL has been established for this substance (EMEA, 1998). According to recent studies, mites became resistant to pyrethroids (Milani, 1995; Thompson, 2003).

### 2.2.1.4 Tau fluvalinate

Structure: It belongs to the group of pyrethroids.

Action: This is a broad range non-systemic insecticide-acaricide that acts by contact through stomach. The way of action of tau fluvalinate is similar to that of the flumethrin.

Preparation: There are three preparations used by beekeepers containing tau fluvalinate: Apistan, Mavrik and Klartan. Out of the three, only Apistan has an approval for beekeeping use.

Beekeeping use: The use of the authorized preparation is in the form of controlled release strips (Apistan).

ADI: 0.5 mg kg<sup>-1</sup> body weight per day or 30 mg per person per day (EMEA, 1998).

MRL for honey: EU established no MRL for tau fluvalinate residues in honey, as the concentrations of detected residues were extremely low, based on the experimental results included in the file submitted (<0.01 mg kg<sup>-1</sup>) (EMEA, 1998).

The use of Apistan strips is likely to lead to accumulation of residues, if they have been left in the hive for more than 6 weeks. Balayiannis and Santas (1989) reported an increased persistence of residues in stored honey, compared with the honey in combs. This is apparently owed to the non-transfer of tau fluvalinate in wax. Tau fluvalinate is the most lipophilic of all compounds used in beekeeping. This property combined with the high stability of the substance in the wax contributes to the drastic increase in the concentration

of residues in the honeycombs (Tsigouri et al., 2004). Tau fluvalinate has been used by beekeepers for many decades. Nowadays, the beekeepers have stopped using it because the mites developed resistance towards this chemical. (Elzen et al., 2000; Milani et al., 1995; Thompson et al., 2003).

#### 2.2.1.5 Bromopropylate

Bromopropylate is one of the oldest compounds that had been used against *Varroa* under the commercial product FOLBEX-VA. Its use was totally abandoned in Switzerland in 1991. An analysis made 19 years later showed that bromopropylate was still in beeswax in high concentrations and scientists believe that more than 20 years will pass before it is expected to fully disappear from beeswax.

Structure: It belongs to the group of chlorinated derivatives of benzene.

Action: bromopropylate is a broad spectrum, non-systemic insecticide-acaricide with high residual activity, which acts by contact and inhibits the synthesis of ATP.

Preparations: The name of the commercial preparation is Folbex VA, manufactured by Giba-Geigy.

Beekeeping use: The use of this preparation is in the form of fumigant.

ADI: 0.03 mg kg<sup>-1</sup> body weight per day or 1.8 mg per person per day.

MRL for honey: no MRL exists for this chemical at E.U. Bromopropylate was used in crops such as pome fruits, stone fruits and plants of Solanacea family. The agricultural use has led to an establishment of MRL under the provisions of EU Regulation 396/2005. This new MRL corresponds to a concentration of 0.1 mg kg<sup>-1</sup> and concerns the contamination of bee products through the use of agricultural pesticides.

Bromopropylate is not toxic to bees, while its metabolite 4,4-dibromobenzolic acid is likely to be detected. The use of bromopropylate was particularly widespread in Central Europe. The observation that bromopropylate degradation is slow and, therefore very stable in honey and wax, forced Europeans beekeepers to start using acaricides that are more environment and consumer friendly. According to a survey conducted by Taccheo et al. (1988b), concentration of bromopropylate residues was greater in honey from an uncapped comb than from a capped one. Moreover, the burning of fumigant strips in an empty floor above the hive reduced the concentration of residues in honey (Taccheo, 1988b). The use of bromopropylate has stopped in many countries and no samples with residues of bromopropylate were found in Greek honey (Karazafiris et al., 2007).

#### 2.2.1.6 Malathion

Structure: It belongs to the group of organophosphorus compounds.

Action: malathion is broad spectrum, non-systemic insecticide-acaricide which acts through stomach. The way of action is similar to that of the organophosphate acaricide coumaphos. In addition, malathion oxidized to malaoxon, which is a substance with high toxicity to insects and mites. This chemical reaction does not occur in the body of mammals, limiting the toxicity of the acaricide for humans.

Preparations: Malathion

Beekeeping use: Malathion has never had approval for beekeeping use. Despite this, many beekeepers use it as spraying material or as powdered sugar. Due to the high bee toxicity, malathion requires special attention during application. A slightly increased dose can be fatal for the colony, especially when used as a solution.

ADI: 0.03 mg kg<sup>-1</sup> body weight per day or 1.8 mg per person per day.

MRL for honey: No MRLs have been established in honey, and therefore the threshold corresponding to the LOQ is 0.01 mg kg<sup>-1</sup>.

Two different studies were conducted by Thrasyvoulou et al. (1988) and Balayiannis et al. (1989) concerning the time of degradation of malathion in honey. Both two studies proved that the time of degradation of malathion is three months. In a survey conducted in 50 samples of Greek honey, 4% were found contaminated in concentrations that did not exceed 0.005 mg kg<sup>-1</sup> (Thrasyvoulou et al., 1988). Furthermore, malathion was detected in 23 out of 593 honey samples analyzed in the laboratory of Apiculture-Sericulture, Aristotle University of Thessaloniki during the years 2003-2006 (Karazafiris et al., 2005). In Cuba, Pelayo et al. (1987) detected malathion in 12 out of 110 samples. The concentration of the active substance did not exceed 0.02 mg kg<sup>-1</sup> in any case.

### 2.2.1.7 Cymiazole

Structure: It belongs to the group of iminophenyl thiazolidine.

Action: Cymiazole is another substance, like coumaphos, that used in beekeeping and has systemic action.

Preparation: The name of the commercial preparation is Apitol and is manufactured by Giba-Geigy.

Beekeeping use: Cymiazole can be applied in different ways.

ADI: 1 mg kg<sup>-1</sup> body weight per day or 60 mg per person per day (EMEA, 1996).

MRL for honey: MRL that was established for cymiazole was 1 mg kg<sup>-1</sup>, but latest E.U. regulations established new MRL that corresponds to LOQ (0.01 mg kg<sup>-1</sup>).

### 2.2.2 Antibiotic residues

The term antibiotic originally refers to any agent with biological activity against living organisms; however, "antibiotic" nowadays refers to substances with antibacterial, anti-fungal, or anti-parasitical activity. There are currently about 250 different substances registered for use in medicine and veterinary medicine (Künmerer & Henninger, 2003).

Antibiotics such as tetracycline, chloramphenicol, sulfathiazole, streptomycin, tylosin, erythromycin etc, are commonly used by beekeepers, in order to control European Foulbrood Disease (EFB), American Foulbrood Disease (AFB) and Nosemosis caused by *Paenibacillus larvae larvae*, *Streptococcus pluton* bacteria and fungus of the genus *Nosema*, respectively. The use of antibiotics is not allowed in beekeeping since no MRLs have been set for honey. Some countries, like Switzerland, UK and Belgium, have established action limits for antibiotics in honey, which generally lie between 0.01 to 0.05 mg kg<sup>-1</sup> for each antibiotic group. An action limit is the concentration of antibiotics in honey, above which the sample is considered non-compliant. The presence of antibiotic residues in honey and other hive products is not accepted in Europe for products imported from third countries. In case a product is found contaminated with antibiotics then it should be destroyed and the producer should be penalized. In the U.S.A., Canada and Argentina, preventive treatments with antibiotics are considered a routine procedure to control AFB. As a result, various strains of *P. larvae* have developed resistance to antibiotics, such as oxytetracycline (OTC). Such strains have been isolated in Argentina (Alippi, 2007) as well as in many areas of the U.S.A. (Miyagi et al., 2000). Generally, the presence of antibiotics in the environment

especially in foods may lead to the rapid emerge of resistant bacterial strains and consequently to the demand of new substances to replace the old. Moreover, the emergence of resistant bacteria involves the use of powerful antibiotics leading to serious consequences in the normal flora of the human body.

### 2.2.2.1 Chloramphenicol

Chloramphenicol is a potent antibiotic that has limited uses; it has been declared carcinogenic and causing fatal aplastic anemia, which makes it an unacceptable substance for use in production of food products where any residue may be found. Several reports document human fatalities resulting from ophthalmic preparations containing chloramphenicol, with exposure doses that could be found in residues in food (Settepani, 1984). Chloramphenicol was detected in bee products, honey and royal jelly, imported from China and India. In 2002, alerts appeared from the U.S., Canada, and Europe that honey samples from China often contained traces of the antibiotic chloramphenicol with a range of 0.3 to 34  $\mu\text{g kg}^{-1}$  (LOD=  $\mu\text{g kg}^{-1}$ ). Since China did not have stringent controls on veterinary use of various antibiotics, this drug had been used (along with streptomycin) by the Chinese beekeepers to control a bacterial epidemic that affected bee hives (Dharmananda, 2003). The EU, in an effort to protect consumers, banned the import of Chinese products of animal origin since 2004. Also in Switzerland, chloramphenicol residues detected in thirteen out of 75 (17%) of commercially obtained honey samples, ranged between 0.4 and 6.0  $\mu\text{g kg}^{-1}$  (Ortelli et al., 2004).

### 2.2.2.2 Tetracyclines

Tetracycline is used by beekeepers in order to control AFB and EFB. Normally, it degrades in 6-10 weeks (Matsuka & Nakamura, 1990; Gilliam et al., 1979). In some cases tetracycline was detected in honey, even after three years, because of the high dose used by beekeepers (Shakaryan & Akopyan, 1973). Acidity, viscosity and organic acids of honey contribute to the stability of antibiotics (Gilliam et al., 1979). The treatment of the hive with antibiotics results in tetracycline residues in honey and wax (Gilliam et al., 1979; Corner & Gochnauer, 1971). In two studies of Shakaryan & Akopyan (1972 & 1973), 1.2% of the initial concentration of the antibiotic residues remained stable even after the heating of honey for three successive times in 90°C (30 minutes). Tetracyclines (tetracycline, oxytetracycline, chlortetracycline, doxycycline) have been found in honey in various countries. In a study conducted in Greece, tetracycline residues were found in 23% of the spring floral honey samples tested (Karazafiris et al., 2007). In another study, out of 251 greek honey samples, 29% were found contaminated with tetracycline residues ranged from 0.018 to 0.055  $\text{mg kg}^{-1}$  (Saridaki-Papakonstadinou et al., 2006).

### 2.2.2.3 Sulfonamides

The sulfonamides are analogues of para-aminobenzoic acid, which include sulfapyridine, sulfadimidine, sulfadiazine, sulfamethoxazole, sulfadimethoxin, sulfamethopyridazine, sulfadoxine, sulfamethoxypridazine, sulfadoxine and sulfamethopyrazine. They are suspected to cause aplastic anemia, like chloramphenicol. It is the most stable antibiotic in honey (Bonvehi & Pajuelo, 1983). In the past, sulfathiazole was detected regularly in honey produced in the European countries. Beekeepers used sulfa-drugs in order to control AFB and EFB and in some cases Nosemosis.

In 2002, sulfa drugs were detected in 3 out of 91 samples of honey collected from the Belgian market. Moreover, 12 out of 203 honey samples collected in 2003 were contaminated by residues of sulfonamides (Reybroeck et al., 2004).

#### **2.2.2.4 Streptomycin**

The problem with streptomycin is that it may cause ototoxicity and nephrotoxicity. It is considered more dangerous than oxytetracycline and less hazardous than sulfathiazole and chloramphenicol regarding side effects. According to the Food Standards Agency of UK, an Indian honey was found to be contaminated by streptomycin in 2003 (Mayande, 2007).

#### **2.2.2.5 Fumagillin**

This is the active ingredient of the preparation Fumidil used by beekeepers to treat nosemosis. It could cause teratogenesis and have genotoxic effects (Stanimirovic et al., 2007). Nowadays, it is not permitted to use fumagillin in Europe and no MRLs have been established, neither for honey nor for any other products of animal origin.

#### **2.2.2.6 Monitoring of antibiotics in bee products**

Many other antibiotics have been used worldwide. One of these is tylosine, which got an approval for use in the U.S.A. in the form of preparation Tylan. Moreover, beta-lactams are suggested to be the ideal antibiotic group in terms of efficiency and lack of residues to the final product.

Fifty chestnut, pine, linden and multifloral honey samples from Southern Marmara region of Turkey were analysed for erythromycin residues by Liquid Chromatography-Mass Spectrometry. Four of the honey samples were contaminated with erythromycin residues at concentrations ranging from 50 to 1776  $\mu\text{g kg}^{-1}$  (Gunes et al., 2008).

A percentage of 1.7% out of 3855 honey samples of European market, which was analyzed for antibiotic residues, were non compliant with the EU standards. Antibiotics were detected in the honey samples in a range of 3–10.820  $\mu\text{g kg}^{-1}$ , 5–4.592  $\mu\text{g kg}^{-1}$ , 5–2.076  $\mu\text{g kg}^{-1}$ , 0.1–169  $\mu\text{g kg}^{-1}$ , 0.3–24.7  $\mu\text{g kg}^{-1}$ , 2–18  $\mu\text{g kg}^{-1}$ , 1–504  $\mu\text{g kg}^{-1}$  for streptomycin, sulfonamides, tetracyclines, chloramphenicol, nitrofurans, tylosine and quinolones respectively (Diserens, 2007).

In the period 2000–2001, samples of honey of Belgian market were monitored for the presence of residues of antibiotics. Streptomycin was detected in 4 out of 248 (1.6%) samples that, tetracycline in 2 (2.8%) and sulfonamides in 3 (4.2%) out of 72 samples analyzed. No residues of  $\beta$ -lactams and chloramphenicol were detected. In imported honey samples, streptomycin was detected in 51 out of 108 samples (47.2%), tetracyclines in 29 out of 98 samples (29.6%), sulfonamides in 31 out of 98 samples (31.6%) and chloramphenicol in 40 out of 85 samples (47.1%). Residues of  $\beta$ -lactams were not detected in any sample (Reybroeck, 2003).

A total of 57 samples of royal jelly were collected from beekeepers and the Chinese market. The royal jelly was analyzed for seven fluoroquinolones used in beekeeping (ciprofloxacin, norfloxacin, ofloxacin, pefloxacin, danofloxacin, enrofloxacin, and difloxacin). Ofloxacin, ciprofloxacin and norfloxacin residues were detected in concentrations ranging from 0.012 to 0.056  $\text{mg kg}^{-1}$ . Difloxacin was found at a concentration of 0.047  $\text{mg kg}^{-1}$  in one sample (Zhou et al., 2009).

### 2.2.3 Residues of volatile insecticides in bee products

The greater wax moth *Galleria mellonella* is a serious pest of stored combs and weak colonies. Adult female wax moths enter hives and lay their eggs on wax combs or in small crevices between wooden parts of the hives not easily accessible to honey bees. After few days the larvae hatch and begin feeding on bees-wax, pollen, cast larval skins and other remains in cells. This devastating activity of wax moths leads to great financial losses every year in the field of beekeeping.

Strong colonies are the best control against the wax moth in the field. In comb storage chests, technical, physical, biological and chemical methods have been used to control the pest. The most effective method to avoid the destruction of combs from wax moth is their continuous maintenance in temperatures of the refrigerator, or their passing from the freezer for a short time. Cantwell and Smith (1970) confirmed that temperature lower than -18 °C destroys all stages of the wax moth insect (egg, immature forms and adult). Although this treatment requires expensive facilities, it is successfully applied nowadays protecting the honeycombs from the wax moth without contaminating the beehive products.

In addition, biological and environment-friendly control method were developed such as the male sterile technique with gamma-rays (Jafari et al., 2010), the trapping of moths by using pheromone (Flint & Merkle, 1983) and the use of the bacterium *Bacillus thuringiensis* that kills the wax moth larvae when it ingests the spores (Burges & Bailey, 1968; Burges 1997; Charriere & Imdorf, 2004).

Chemical methods, includes substances that are considered friendly to environment like methyl salicylate, clove oil, formic acid, sulphur, acetic acid, basil oil and other have been used (Wilson, 1965; Williams, 1980; Owayss & Abd-Elgayed, 2007). Most of these compounds are dangerous for bee brood and human health, while they require repeated application and may react and destroy the metal parts of the combs. Besides these, 1,2-dibromo-ethane (DBE), 1,4-dichloro-benzene (p-DCB), naphthalene had been used for many years in different countries even though their use causes significant contamination of bee products.

DBE is a manufactured chemical. In nature, it is produced in small amounts in the sea water, where it is formed, probably by algae and kelp. It is dissolved in water and by this way it can stay in groundwater and in soil for a long time. In air it breaks down quickly. This substance has been used as a pesticide in soil, and on citrus, vegetables, and grain crops. EPA has banned most of these uses since 1984. The same organization has also set a limit of 0.05 µg.cm<sup>-3</sup> of 1,2-dibromo-ethane in drinking water (ATSDR, 1992).

The compound p-DCB is one of the three di-chloro-benzene isomers (1,2-DCB, 1,3-DCB and 1,4-DCB), which is commonly used as a space deodorant in toilets and for moth control. It is a volatile colorless to white crystalline material with a mothball-like, penetrating odor and it is commercially, the most important isomer (ATSDR, 2006).

Naphthalene is a white solid substance that evaporates easily. Its major use is in the manufacture of polyvinyl chloride (PVC) plastics and it is also used in moth repellents and toilet deodorant blocks. That use of naphthalene accounted for 73% and 60% of commercial demand for naphthalene in Japan and the United States, respectively in 1999, (ATSDR, 2005).

No MRL's in honey for the above three compounds were defined until 2005 when the European regulation 396/2005 EC set the limit at 10 µg kg<sup>-1</sup> for substances for which no MRL had been established. This limit for p-DCB was also the Swiss Tolerance Limit (STL)

and was already used as action level in Greece. ADI values for DBE, p-DCB and naphthalene, range according to Table 3. Besides killing the moth those chemical are absorbed by the wax and when bees store honey into combs, they are transferred into the product. Laboratory comb-melting experiment showed that p-DCB is not removed from wax during the comb recycling (Bogdanov et al., 2004). Residues up to 0.002 mg kg<sup>-1</sup> may be detected in honey due to the use of precontaminated wax. Residues of p-DCB exceeding 0.01 mg kg<sup>-1</sup> indicate contamination of bee product by beekeeping practices. Countries that have reported problems with residues from the above volatile insecticides are Germany, Switzerland, Greece and Turkey (Wallner, 1992; Bogdanov et al., 2004; Tananaki et al., 2005; Beyoğlu & Omurtag 2007).

Wallner (1992), stated in his paper that the problem of p-DCB residues in Germany is serious, since 50% of the analyzed honey samples had been found contaminated from 3 to 50 µg kg<sup>-1</sup>. He noticed that p-DCB is very stable in honey and it cannot evaporate from the sealed glass containers. Finally, he stated that beeswax works like a sponge as it has large capacity for fat-soluble active compounds. The more the p-DCB crystals are added to combs the higher is the substance stored in the wax. The evaporation of p-DCB from wax is impossible even after prolonged ventilation.

Compound	ADI (mg kg <sup>-1</sup> bw day <sup>-1</sup> )	
	US EPA	Canadian health
1,2-dibromoethane	0.009	0.009
1,4-dichlorobenzene	0.03	0.11
naphthalene	0.02	0.02

Table 1. ADI of three compounds that have been used against wax moths

Bogdanov et al. (2004) analyzed Swiss commercial honey samples during five years period for p-DCB residues and they found that the contaminated samples ranged from 14% to 46% (fig. 1). The percentage of the imported samples was lower, on average 7%. Although there is no MRL for p-DCB, Switzerland has established a "Swiss tolerance value" (STV) for honey at 10 µg kg<sup>-1</sup>. From the total 173 Swiss and 287 imported honey samples, 13% and 0.8% exceeded the STV respectively.

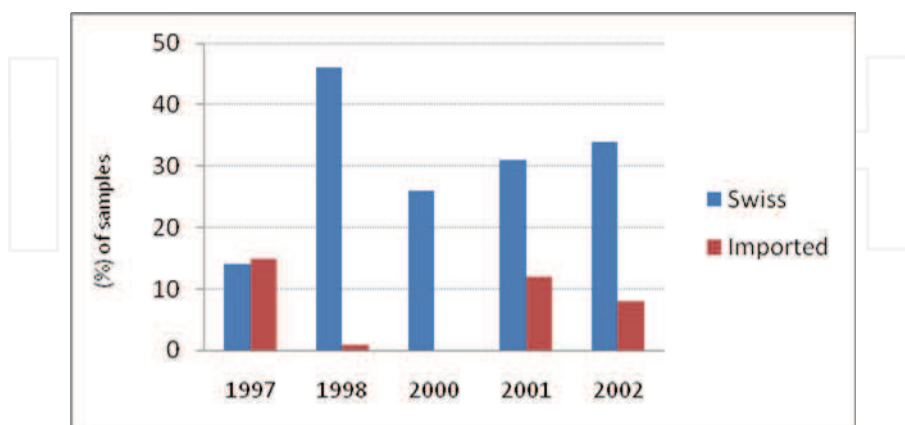


Fig. 1. Residues of p-DCB in honey samples in Switzerland (Bogdanov et al., 2004)

Contamination of bee products by chemicals that are used against wax moths was also noted in Greece by Tananaki et al. (2005). Initially a multi-method had been developed for the determination of DBE, p-DCB and naphthalene and then this method had been applied in twenty five honey samples produced in different areas of Greece. The 8% of the samples had detectable amounts of DBE, 92% had p-DCB and 88% had naphthalene residues. Concentrations of naphthalene, p-DCB and DBE that exceeded  $10 \mu\text{g kg}^{-1}$  were measured in 6.7%, 32% and 8% of tested samples, respectively.

After confirming the mass contamination, beekeepers had been informed to stop the treatment with those chemicals and to destroy all the combs that had been treated before. Meanwhile a monitoring program for the residues of volatile insecticides in Greek honey was initiated by laboratory of Apiculture - Sericulture of Aristotle University. A total of 1,519 samples were analyzed during the period 2004 - 2010 (Tananaki et al., 2006). From those, 209 samples were bought from Greek supermarkets (commercial) while 1,310 were collected from beekeepers or from their associations (bulk honey). Results of this research are indicated in Fig. 2.

Comparing the results of eight years' monitoring of p-DCB, a considerable reduction of residues is observed both in commercial and bulk honey samples. During the first year the 82.9% of commercial samples had residues more than  $10 \text{ mg kg}^{-1}$  which is the established action limit in Greece since 2005. In the following three years this percentage decreased gradually and finally p-DCB wasn't detected at concentrations more than  $10 \text{ mg kg}^{-1}$  in 2010. Similar behavior was observed for the samples collected from beekeepers. These results demonstrate that the Greek beekeepers' efforts to restrict the problem and to find alternative solutions for the control of the wax-moth (*Galleria mellonella*) have been accomplished.

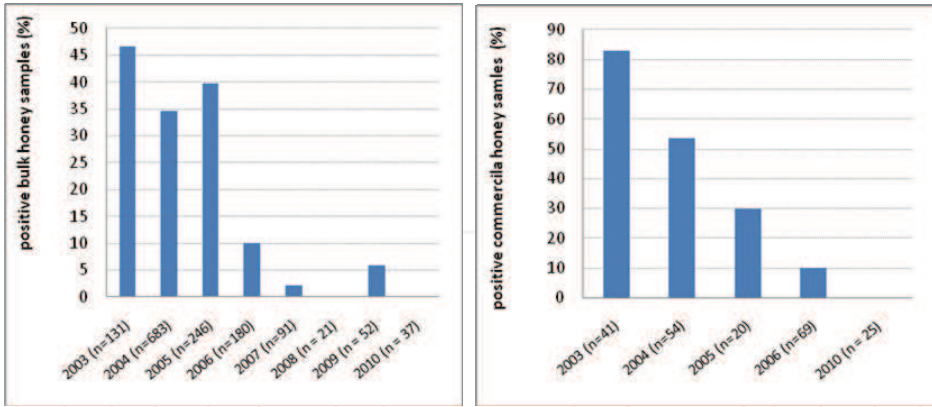
The great percentage of commercial samples in all years of study have either no detectable amounts or below  $10 \mu\text{g kg}^{-1}$  DBE. Only one sample was found exceeding  $40 \mu\text{g kg}^{-1}$  in year 2003. This sample had  $60.5 \mu\text{g kg}^{-1}$  DBE, which is the maximum concentration found in samples bought from stores. Samples that had been collected from beekeepers had higher concentration of DBE than the commercial ones. This is because commercial samples usually are mixtures from different producers. During year 2003, a percentage of 9,9% of the samples exceeded the level of  $10 \mu\text{g kg}^{-1}$  and a maximum value of  $132.5 \mu\text{g kg}^{-1}$  was found in one of them. During the following two years this percentage decreases to 1.9% and 2.8% respectively, but still some beekeepers continue to use the chemical as indicated by the high concentration of  $331.2 \mu\text{g kg}^{-1}$  detected in one sample in 2004.

Figure 2 summarizes the results of naphthalene residues in honey from the Greek market and from beekeepers as well. Contrary to p-DCB and DBE, naphthalene was found in more commercial samples than in samples from beekeepers during the first year of monitoring program. This could be attributed to blending of Greek commercial honeys with imported honey originating from countries where naphthalene is still used to control wax-moth. During the following years the residues in commercial samples dropped below  $10 \mu\text{g.kg}^{-1}$  and very few beekeepers' samples were contaminated at higher levels. The highest concentration of naphthalene found in one sample was  $523.6 \mu\text{g.kg}^{-1}$  in 2004.

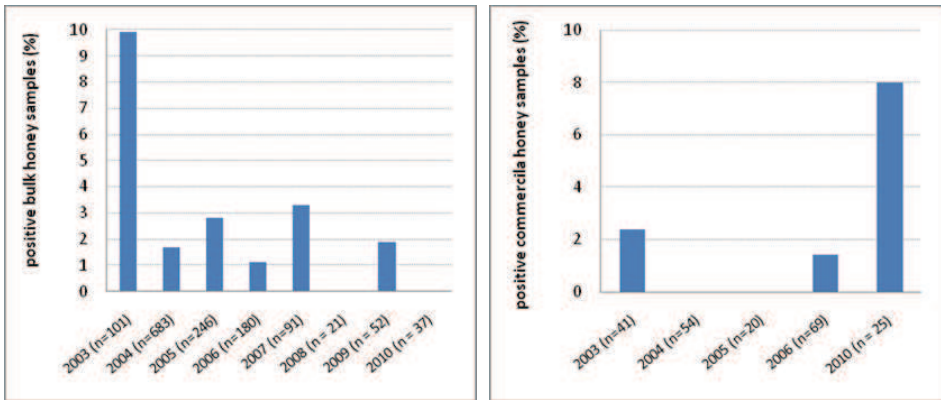
Tananaki et al. (2006) also found differences in the level and the frequency of contamination among different types of honeys. Honey produced during the spring honey flow (blossom and fir honeys) was contaminated in a higher percentage than the honey produced later in the season (thymus and pine honey). Thymus and blossom honey have higher contamination in naphthalene than other types of honey. This might happen because both thymus and blossom are the types of Greek honey that are probably mixed with imported honey. Paleologos et al., (2006), Tsimeli et al., (2008) and Harizanis et al., (2008) have also analyzed samples of Greek honey with similar results.



1,4-dichlorobenzene



1,2-dibromoethane



Naphthalene

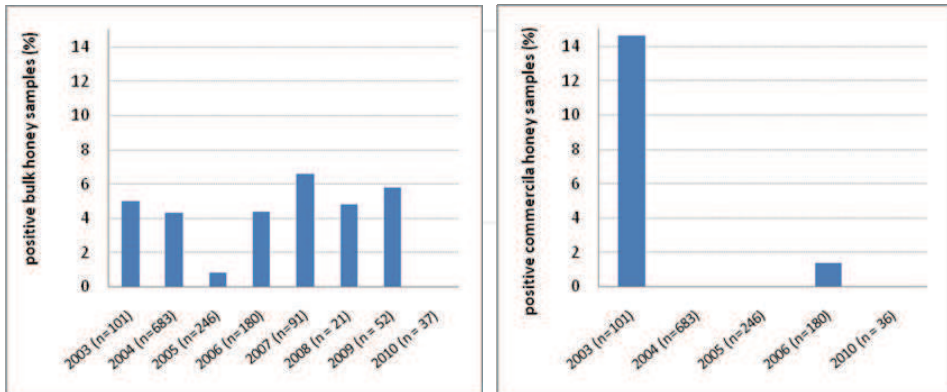


Fig. 2. Residues of volatile insecticides in Greek honeys

Residues of p-DCB were also detected in royal jelly. Tananaki et al. (2009) found that the concentrations of p-DCB in honey were significantly lower than in the royal jelly; in some cases, royal jelly had some hundred times more residues than honey from the same comb. The maximum concentration of p-DCB found in royal jelly was 1,520  $\mu\text{g kg}^{-1}$ . Bogdanov et al. (2004) checked the p-DCB residues in wax. They analyzed wax samples from manufactures during the years 1994 -1998 and 2002 and they found residues in 66% of the wax sample, in concentrations from 0.7 to 74.9  $\text{mg kg}^{-1}$ . The concentrations of p-DCB in new wax after melting of old combs were the same with those of the old combs. This indicates that p-DCB is not being removed from wax during the comb recycling process.

### **2.3 Methods of analysis of pesticide and acaricide residues detected in bee products**

The need of monitoring residues of acaricides used by the beekeeper in conjunction with the need to monitor the contamination of bee products from other sources, such as pesticides used on crops and environmental pollutants, makes the development of appropriate methods of analysis obligatory. Furthermore, it is necessary to analyze products like honey and pollen randomly in order to find any violations of existing legislation on the part of producers or sellers. The suitability of each method lies in its ability to give a reliable result of the concentration of residues. A complete method should usually includes four sub-stages, which are described as:

- Sampling
- Sample Preservation
- Sample Preparation
- Analysis

The particular physicochemical properties of each product (moisture, fat, protein content etc.) in conjunction with specific physicochemical properties of each substance (polarity, volatility, etc.) do not permit the use of one methodology for the determination of all active substances in all products. Various techniques have been reported in order to clean -up the sample and isolate the analyte.

#### **2.3.1 Sampling**

The first step of an analysis is the sampling. Specifically, the meaning of a sample is to take a part of the product, which should be as representative as possible. The way of the sampling varies, depending on the type of sample. In homogeneous samples such as water, the sampling is simple and does not require complicated procedures. On the contrary, heterogeneous samples such as fruits, vegetables and animal products require additional measures during sampling in order to reduce the uncertainty. The contribution of sampling to the total uncertainty is so great that in some cases approaches 40%. The EU issued a special directive on the sampling of food (2002/63/EK) and requires the accurate implementation of the official control laboratories.

#### **2.3.2 Sample preservation**

The second stage of the analysis is the preservation of the sample. The common practice of laboratories is the storage of collected samples for a period ranging from some hours to years. Storage conditions must ensure the preservation of the sample during the period required for the analysis. Food should normally be preserved under freezing conditions,

until the day of analysis, in order to minimize the evaporation or chemical reactivity of these compounds.

### 2.3.3 Sample preparation and analysis

The next step includes the preparation and the analysis of the sample. The analysis is performed directly, i.e. without pretreatment of the sample, where a direct measurement is possible (e.g. measurement of moisture in honey). In most cases, however, a preparation of the sample should take place before the analysis. The preparation of the sample includes the removal of interferences and the isolation of compounds of interest. This step is necessary in methods of analysis for residues of pesticides and veterinary drugs. Especially in the case of residue analysis, this stage is divided into separate stages that vary in terms of the number and type. Typically, these steps are five and consist of:

- The homogenization, which may include a stage of subsampling.
- The extraction, including the isolation of analytes in a suitable solvent.
- The removal of a significant amount of solvent in order to make the stage of purification of the sample easier and faster (optional step).
- The cleaning of the sample in order to remove interferences that prevent the proper evaluation of chromatograms.
- The final concentration of solvent, allowing qualitative identification of analytes and the minimization of the quantitative limits.

The cleaning of the sample is the most complicated stage. That is the reason why various techniques have been used. The main techniques used for the preparation and analysis of honey samples are summarized in a review of Rial-Otero et al. (2007). Techniques that have been used in order to achieve the determination of acaricide and pesticide residues in bee products are:

- Solvent Extraction (SE). This is the first technique developed in order to detect pesticide residues. In this technique, the sample is dissolved in water, or mixtures of water and alcohols. After the dilution of the sample, an extraction with suitable organic solvents takes place, in order to collect the analyte and remove a large portion of co-extractives components. Several methods of the SE used combined with acidification of the sample (Waliszewski et al., 1998; Waliszewski et al., 2003; Bernal et al., 1997) or the use of ultrasound (Jimenez et al., 2000; Rezig et al., 2005), in order to improve the efficiency. Due to the use of large quantities of organic solvents, the SE is particularly aggravating for the environment and the health of laboratory staff. Moreover, the cost is quite high due to the large quantity of supplies. Finally, many hours are required for analysis of a sample and the automation of the process is very difficult. Despite these drawbacks, the SE has been used with satisfactory results in various methods of analysis of honey (Jimenez et al., 2002; Menkissoglu-Spiroudi et al., 2000; Taccheo et al., 1988a), royal jelly (Balayannis, 2001), pollen and bees (Bernal et al., 1997) for the determination of pesticide and acaricide residues.
- Accelerated Solvent Extraction (ASE). This technique includes steps of extraction with organic solvents at predetermined conditions of pressure and temperature. In ASE, the extraction solvent is carried out in a special device and the extraction under steady environmental conditions (pressure and temperature) allows efficient and reproducible isolation and collection of analytes. The quantities of solvents used in

this technique are very small compared to the SE and the automation of the extraction procedure much easier. Disadvantage of this technique is the high cost of required equipment. However, the small amount of solvent and the possibility of automation make it possible to recover the cost within a short period of time (especially for laboratories that analyze large numbers of samples). The ASE has been used successfully in many cases of food and water analysis by EPA (Chuang et al., 2001). There is only one study on the analysis of bee products with ASE. Results indicated good efficacy in the determination of acaricides in honey by the use of High Performance Liquid Chromatography (HPLC). The recovery rates of this method ranged from 58% to 103% and limits of quantification ranged from 0.01 mg kg<sup>-1</sup> to 0.2 mg kg<sup>-1</sup> (Korta et al., 2002). ASE is likely to be referred in the literature with the names of PLE (Pressurized Liquid Extraction), PSE (Pressurized Solvent Extraction) and PFE (Pressurized Fluid Extraction).

- Supercritical Fluid Extraction (SFE). The SFE is a technique similar to the ASE with similar advantages and disadvantages, while the equipment used for this technique is rather expensive (Mitra, 2003). The difference between the two techniques lies in the type of solvent, which is carbon dioxide (CO<sub>2</sub>) for the SFE. Adding a small amount (1-10%) of an organic solvent (such as methanol, ethanol, etc.) improves the efficiency of extraction of more polar compounds, which otherwise would be very small. Two types of supercritical fluid extraction techniques, called static and dynamic were developed. In the case of static SFE, the solvent enters the cell, which contains the lyophilized sample and remains an exact time at constant pressure and temperature conditions. However, in the dynamic SFE, the flow of solvent into the cell remains constant and stable for perfectly accurate time and at constant pressure and temperature conditions. The final extract is transferred to a vial containing an organic solvent. The SFE is a rapid technique that requires very small quantities of organic solvents and does not contaminate the environment significantly. Unlike ASE, there are several publications on the analysis of residues in honey using SFE. Rissatto et al. (2004) developed a method to analyze samples of honey combined SFE system and gas chromatography. The limit of quantification was 0.01 mg kg<sup>-1</sup>, while recovery rates ranged from 75% to 94%. In a second study conducted by Atienza et al. (1993) the average recovery rates ranged from 53% -94% while the RSD of the method ranged from 1.3% to 1.6%. In one case, this technique was used for the analysis of organophosphorus and carbamate insecticide residues in bees. The recovery rate exceeded 75% for all substances except omethoate (Jones & McCoy, 1997).
- Gel Permeation Chromatography (GPC). This technique allows the separation of different components based on their size (larger particles move faster). Gels of various porosity and organic solvents are used in order to achieve the separation. Usually, this technique is used to remove lipids, proteins, polymers and other macromolecules contained in the sample. Especially for the pesticide analysis, the technique is suitable for removing high boiling point compounds, which are deposited to the inlet of gas chromatography. Rossi et al., (2001) have used the GPC on the analysis of residues in bees. The recovery was satisfactory for 25 of 29 substances analyzed (percentage recovery ranged from 70.9% to 106.8%). In contrast, the recovery rate for active substances pirimicarb, ethiofencarb, methiocarb and fenoxycarb was 38.7%, 48.6%, 46.6% and 58.4% respectively.

- Stir Bar Sorptive Extraction (SBSE). This extraction technique is using an appropriate stirring bar, which adsorbs the analyte. The bar was either eluted with suitable organic solvents or placed directly to the inlet of gas chromatography systems (Baltussen et al., 1999). Particularly, the bar is made by stainless steel coated with a thin layer of glass and poly-dimethyl siloxane (PDMS), which adsorbs the analyte in the sample (Popp et al., 2001). It is very important for the efficiency of extraction to be accurate in temperature and extraction time. The greater the precision, the more improved the repeatability of the method. In the final phase, the bar is placed in a special unit, which in turn is attached to the inlet. The adsorbed substances led to the column and detector with a carrier gas flow rate increasing with temperature. There is also the option for the bar to be extracted with organic solvents (e.g. acetonitrile), which constitute the final sample for chromatographic analysis (Sanchez-Rojas et al., 2008). SBSE has been used on bee products with good results compared the SPME. Based on data given by Blasco et al. (2004), the SBSE is more efficient as a technique than SPME, while accuracy and repeatability are much better. More specifically, the limit of quantification was 0.04 mg kg<sup>-1</sup> for SBSE technique, while those for SPME technique ranged from 0.8 mg kg<sup>-1</sup> to 3.0 mg kg<sup>-1</sup>. Moreover, the recovery of SBSE ranged from 40% to 64%. Finally, the relative standard deviation of repeatability did not exceed 10% in both cases.
- Solid Phase Micro Extraction (SPME). The SPME is a relatively modern technique, developed by Pawlyszyn et al. (1997). The principle of this technique relies on the use of a fiber, which adsorbs the analyte, which then eluted to the inlet of gas chromatography systems. The SPME technique is suitable for the determination of volatile compounds in liquid or solid samples. SPME can be used in two ways. The first method involves an extraction by sinking the fiber into the sample solution directly. This is an advantage in terms of sensitivity and the number of identified substances. The second way relates to cases of extraction in the supernatant layer of sample. The advantage of this method is the higher level of purity of the final sample (Arthur & Pawlyszyn, 1990; Louch et al., 1992; Zhang & Pawlyszyn, 1993; Page & Lacroix, 1993). Both SPME and SBSE are based on the logic of the adsorption of chemicals in various absorbents, which in the first case is a fiber (SPME), and in the second a bar (SBSE). Another important parameter, which can greatly improve the results of SPME, is pH (Volante et al., 2001). The adjustment of pH by using buffers could improve efficiency or reduce the time of extraction. It should be noted that there is a variety of fibers, which differ in the type and thickness of the adsorbent material. The advantages of SPME include: (i) the lack of use of organic solvents, (ii) the purest final samples, (iii) the minimization of time, (iv) the good linearity of the method, (v) the non-requirement for full adsorption of the analyte & (vi) the relatively simple automation (Pawlyszyn, 1997). The major disadvantage of SPME is the low efficiency for the semi-volatile or non-volatile compounds and the inability to repeat the analysis of a sample (same bottle). SPME was used for the detection of pesticide and acaricide residues in honey. More specifically, the fiber of SPME was immersed in an aqueous solution of honey and remained there until equilibrium of the analyte between the fiber and the environment was achieved. After this, the fiber was removed and placed in the inlet of gas chromatography in order to desorb the

ingredients. The period of immersion of the fiber, as well as the temperature was strictly defined and determined by tests during the development of the method. The technique of solid phase microextraction has been applied for the determination of OCP, OPP, pyrethroid and acaricide residues in honey (Blasco et al., 2004; Yu et al., 2004; Jimenez et al., 1998). In a comparative study, two different types extraction fibers (PDMS 7 mm, PDMS 100 mm and PA 85mm) were tested. The fiber made of PDMS proved significantly superior in terms of reproducibility, sensitivity, linearity and time of extraction obtained (Jimenez et al., 1998).

- Matrix Solid Phase Dispersion (MSPD). The MSPD includes a stage of dilution of the sample in an organic solvent (e.g. methanol) and mixing a quantity of the solution with a sorbent, which is usually C<sub>18</sub> or Florisil. Next phase involves addition of solvents (hexane, ethyl acetate, etc.), working as means of extraction and elution. After good homogenization in an ultrasonic bath and centrifugation, the extract is collected and analyzed in chromatography systems. The advantages of this technique include the limited use of solvents and the rapid process of the sample. Although MSPD was a promising technique, it is expected to be replaced by QuEChERS, which is a new method of analysis described in next paragraph. The MSPD is rarely used in the analysis of acaricide and pesticide residues in bee products. However, there are few studies used MSPD and gas chromatography for the detection of pesticides in honey. Limits of quantification in these studies were lower than 0.015 mg kg<sup>-1</sup> for any pesticide, while the recovery ranged between 60% and 113% (Albero et al., 2001; Sanchez et al., 2002).
- QuEChERS. The name of the technique derives from the characteristics of this method, which is described as Quick, Easy, Cheap, Effective, Rugged and Safe (Schenck & Hobbs, 2004). The QuEChERS is a new technique used for the determination of pesticide residues in food analysis. This technique is based on solid-phase dispersion extraction (Matrix Solid-Phase Dispersion). QuEChERS developed and validated by Anastassiades (2005) and quickly began to be used by many laboratories. Nowadays, QuEChERS is the common sample preparation technique of official laboratories of European Union. This technique was developed primarily for the analysis of products with high water content. The addition of water to the sample makes possible the use of this technique for the analysis of products like honey. The disadvantage of this technique is the need of expensive equipment (GC/MS/MS, LC/MS/MS etc.), because of insufficient "cleanup step" of the sample. QuEChERS was used in order to detect residues of 36 pesticides in honey. Honey samples were extracted with acetonitrile. The extraction step was followed by the addition of acetic acid with the simultaneous addition of magnesium sulphate and sodium acetate. A mixture of primary/secondary amine (PSA) and magnesium sulphate was added as a second purification step. This step was followed by a change of solvent with a mixture of hexane and acetone. The quantification of organophosphorus compounds carried out using a nitrogen phosphorus detector (NPD), while an electron capture detector (ECD) was used for the determination of chlorinated hydrocarbons and pyrethroids. Recovery experiments were made at three levels (from 0.02 mg kg<sup>-1</sup> to 5 mg kg<sup>-1</sup>) and the results ranged from 70% to 120%. Experimental repeatability was satisfactory, as the RSD ranged from 1% to 22%. Finally, the expanded uncertainty

was relatively high (30%), but within the limit of 50% provided in pesticide residues analysis (Barakat, 2007).

- Solid Phase Extraction (SPE). It is the most widely used technique of last decades, in the case of analysis for pesticide and veterinary drug residues. The solid phase extraction is the perfect choice for most researchers, since it requires a small amount of organic solvent (and thus is environmentally friendly), is easily automated and requires no expensive equipment. The disadvantages are the more expensive consumables (solid phase extraction microcolumns), the specialized staff, the differences between lots of microcolumns and the possible absorption of some substances on the polypropylene used in cartridges. Specifically, the sample is dissolved in water (Jimenez et al., 2000; Bernal et al., 1996), alcohol (Bernal et al., 2000) or mixtures of them (Karazafiris et al., 2008; Jimenez et al., 2008), followed by activation of the microcolumn with the same solvent. Subsequently, the sample is passed through a microcolumn containing a suitable solid material, which captures the analyte. The bound substances are eluted with the passage of an appropriate organic solvent. In the case of honey acetone (Bernal et al., 1996), dichloromethane (Jimenez et al., 1998), ethyl acetate (Tsigouri et al., 2001), hexane (Gomis et al., 1996), methanol (Bernal et al., 2000), a mixture of hexane- ethyl acetate (Tsigouri et al., 2001) have been occasionally used. With regard to the types of substrates used occasionally, the reverse phase C<sub>18</sub> was the most appropriate and chosen by most researchers for the extraction of insecticides, acaricides, herbicides, fungicides and other pesticides (Jimenez et al., 2000; Bernal et al., 2000; Korta et al., 2001). Also microcolumn with Florisil gave good results in trials for determination of pyrethroid, OCP and OPP residues (Jimenez et al., 1998a) and C<sub>8</sub> in the determination of tau fluvalinate (Tsigouri et al., 2001). The pH adjustment proved particularly important for the good recovery of some active substances. For example, coumaphos is unstable in an alkaline environment, as opposed to amitraz, the recovery increases with increasing pH values up to 11 (Korta et al., 2001). A comparison of the effectiveness of SPE and SE in pesticide residue analysis was conducted in two publications by Bernal et al. (1996 & 2000). According to the results of the comparison, it should be noted that the recovery rate with both techniques was similar, but SPE proved superior to the purity of the chromatograms. The analysis of royal jelly using the technique of solid phase extraction was first mentioned, by Karazafiris et al., (2008b). The method proved efficient for the determination of acaricide and insecticide residues.

#### 2.3.4 Determination of analytes

The isolation of analytes from the matrix is followed by the necessary step of separation. The practices used on pesticide and veterinary drug residue analysis are based on chromatographic methods. The choice of gas or liquid chromatography is mainly based on the chemical properties of analytes. The technique of gas chromatography was proved suitable for the determination of volatile and low molecular weight compounds, in contrast to the technique of liquid chromatography, which was used in less volatile and high molecular weight substances. The methods of analysis are classified according to the number of compounds detected. The two main categories are multi residue methods (MRM) and single residue methods (SRM). The majority of compounds identified with multi residue methods.

The analyst is able to detect many different compounds by preparing and analyzing a sample only once. However, there are certain compounds, which can be identified individually and only with the use of complex techniques (e.g. amitraz in honey or pear after derivatisation). In recent years, due to the significant improvement of the equipment (GC-MS-MS, LC-MS-MS, etc.) the number of identified substances has increased considerably and many laboratories can identify the majority of active ingredients. The improvement of the quality and quantity of results issued by laboratories has been particularly important. The development of methods includes the use of chromatography described below.

#### 2.3.4.1 Gas chromatography (GC)

The gas chromatography was used more than any other method to determine pesticide and acaricide residues in bee hive products. As mentioned above, this technique is mainly used for determination of volatile and low molecular weight compounds, but there are cases where higher molecular weight compounds (e.g. amitraz), analyzed by gas chromatography after laborious and time consuming processes (e.g. derivatization). In these cases, the substances were converted into more volatile compounds and then analyzed using gas chromatographic system. Gas chromatographic systems consist of three main sections outlined below:

- a. The first main section is called inlet and ensure the entrance of the sample in a gas chromatograph. The types of inlets used in gas chromatography is the Cool On Column, purged packed and split/splitless. The type of inlet may be a problem for some classes of substances that are sensitive to high temperature (e.g. methamidophos and dichlorvos gave better results in Cool on Column inlets due to the lower temperature). The injection in a Cool on Column inlet takes place at low temperatures and benefits in repeatability and stability of analytes. The problem in this case is the more frequent maintenance of the column. Instead, the split/splitless inlet advantages in the purity of the sample, since the majority of high molecular weight substances are removed by a flow of gas and do not enter the column. The result is the extension of the lifetime of the column and the less frequent maintenance. In return, the above advantages of the split/splitless inlet may indicate the low reproducibility due to the removal of a quantity of analyte during cleaning.
- b. The second part consists of the oven and column at which the separation of analytes happens. The separation of substances achieved with the strictly programmed temperature and carrier gas flow within the oven and column respectively. The repeatability of retention time of an analyte depends on the repeatability of the above conditions. The column packing material is a very important factor for the separation and identification of various substances. Small to medium polarity columns are usually used for the detection of acaricides and pesticides. The use of more polar columns is necessary in some cases of single residue methods (e.g. determination of amitraz and its metabolites in honey and beeswax). Finally, a factor worth mentioning is the quality of the gases (carrier, auxiliary gas, etc.) that can substantially improve the sensitivity and lifetime of the column and the detector.
- c. The inlet and the column associated with a suitable detector. Detector achieves the visualization of the result. In the case of beehive products the following detectors have been used:



- Electron Capture (ECD), to detect pyrethroid, organochlorine and organophosphorus insecticide and acaricide residues (Baltussen et al., 1999; Barakat et al., 2007; Jimenez et al., 1996; Jimenez et al., 1998a; Karazafiris et al., 2008b; Menkissoglu-Spiroudi et al., 2000; Rissato et al., 2004).
- Nitrogen-Phosphorus (NPD), to detect pyrethroid and organophosphorus insecticide and acaricide residues (Balayannis, 2001; Baltussen et al. 1999; Jimenez et al., 1998b; Menkissoglu-Spiroudi et al., 2000).
- Flame Ionization (FID), to identify residual acaricides (Bernal et al., 2000).
- Atomic Emission (AED), to detect acaricide residues (Jimenez et al., 1996).
- Mass Spectrometry (MSD), to detect pyrethroid, organophosphate, carbamate and organochlorine insecticide or acaricide residues (Albero et al., 2004; Baltussen et al., 1996; Bernal et al., 1996; Chauzat et al., 2006; Rissato et al., 2004).
- Flame Photometric Detector (FPD) and Pulsed Flame Photometric Detector (PFPD), for detection of organophosphorus insecticide and acaricide residues (Yu et al., 2004).

Each chromatographic system may include components that automate the process and provide valuable assistance to the analyst. The most important component in optimizing the analytical procedure is the autosampler. The performance of a chromatographic system is maximizing by the use of autosampler, while it improves the reproducibility of injection volume and the number of samples, which can be analyzed daily.

#### 2.3.4.2 Liquid chromatography (LC)

Unlike gas chromatography, which is limited to determining the most volatile compounds, liquid chromatography is used for the isolation of a widespread group of compounds. These compounds may not be sufficiently volatile or heat-resistant to analysis by gas chromatography. The most common types of detectors used in liquid chromatography were Diode Array Detectors (DAD) (Atienza et al., 1993; Blasco et al., 2004; Jones & McCoy, 1997; Martel & Zeggane, 2002), Ultraviolet/Visible Detectors (UVD) (Jimenez et al., 2000) and Fluorescence Detector (FLD) (Bernal et al., 1997). The detection technique that is gaining ground is mass spectrometry (MS) (Blasco et al., 2004; Chauzat et al., 2006; Fernandez et al., 2002). In particular, the mass spectrometer with a triple quadrupole is concerned the most suitable detector for pesticide and veterinary drug residue analysis. The above technique enables determination of the majority of active substances, combined with excellent sensitivity (LOQ of about 0.001 mg kg<sup>-1</sup> for most of analyzed substances) and fewer requirements for the cleaning of the sample. The mass spectrometry is the ideal detector in conjunction with the QuEChERS method referred above. In each case, the high cost of the equipment and the need of qualified scientific staff should be noted. The packing material and the size of the column play an important role in the analysis with HPLC. The most widely used column is a C18 reverse phase with an internal diameter of 4.6 mm id. There are also columns with different packing material (C8, ODS, etc.) and columns with very small internal diameter (e.g. 2.1 mm id and 0.32 mm id), which help to increase the sensitivity and reduce the quantities of solvents used (Atienza et al., 1993). The mobile phase used in liquid chromatography is solvents such as water, methanol and acetonitrile or mixtures of them. Also, the adjustment of pH of the mobile phase plays an important role in the effectiveness of the method. In most cases a value of pH=9 is ideal for analysis of pesticide residues.

### 2.3.4.3 Thin layer chromatography (TLC)

This technique is used primarily for detecting drugs in biological samples. However, TLC was used to determine pesticide residues in food. More generally, the TLC requires sample extraction with a solvent mixture and separation of the components into blocks with a suitable coating material (e.g. Silica gel). The next step is an elution with suitable solvents. Special equipment is necessary in order to achieve the visualization and quantification of results. The TLC was used by Rezic et al. (2005) to detect residues of herbicides atrazine and simazine in honey. The recovery rate was estimated at 92.3% and 94.2% for atrazine and simazine respectively. The TLC was used in the above study in conjunction with the use of ultrasound during the extraction.

### 2.3.4.4 Matrix effect

A fact that has to be mentioned is that differences in the chemical synthesis of bee products may affect the efficiency of extraction (Blasco et al., 2004; Yu et al., 2004) and the response of the chromatographic systems to analytes (Volante et al., 2001; Jimenez et al., 1998; Karazafiris et al., 2008b). This is the reason why, solutions for calibration curve and recoveries should be prepared in an extract of the same sample analyzed. Specifically, the analyst applies the chosen technique in honey or other hive products containing no detectable residues of analytes. The final extract is derived from the overall process used in the construction of standard calibration curves. If it is not possible to find sample with no residues, an analyst can use an extract of the sample that gives a response 30% over the reference value. The response may be due to the presence of the analyte or an interference eluting at the same retention time.

## 2.4 Methods for the determination of volatile insecticide residues in bee products

The research on the detection of volatile insecticides residues from substances that are used against *Galleria mellonella* has been started twenty years ago. Various methods of isolation and analysis have been developed which are mainly based on chromatographic separation. Table 2 summarizes all those methods with some analytical information and the corresponding references.

During the first SMPE isolation method a small amount of honey was diluted with water and transferred to the vials. The p-DCB molecules were collected on PDMS-fiber (5 cm, 100  $\mu\text{m}$ ) and the adsorption process took place for 45 min at 20-25  $^{\circ}\text{C}$ . Desorption was performed by raising the fibre temperature to 250  $^{\circ}\text{C}$  for 15 min and the analytes transferred to the GC column (DB-5ms: 30m  $\times$  0,25mm, 0,25 $\mu\text{m}$ ). The detection was achieved with a MS detector at the level of 1  $\mu\text{g kg}^{-1}$  (Bogdanov et al., 2004). Tananaki et al. (2005) developed a sensitive method for the simultaneous determination of p-DCB, EDB and naphthalene residues in honey, using a purge and trap - gas chromatography - mass spectrometry system (P&T-GC-MS). In this research the analytes were extracted by He purging and then they absorbed onto the Tenax resin. With thermal desorption the isolated compounds were transferred to the GC - MS system. Separation was performed on a fused silica capillary column (30m $\times$ 0.25mm I.D., 0.25  $\mu\text{m}$  film thickness). The limits of detection were found to be 0.8, 0.15 and 0.05  $\mu\text{g kg}^{-1}$  honey, while the limits of quantification were 2.4, 0.5 and 0.125  $\mu\text{g kg}^{-1}$  for EDB, p-DCB and naphthalene respectively.

Table 2. Methods for the determination of volatile insecticides residues

Bee product	Analytes	Isolation	Determination	Analytical informat
Honey	p-DCB	Head space sampling	GC - MS	Column: Rtx-624 LOD: 1 $\mu\text{g kg}^{-1}$
		SPME (PDMS-fiber)	GC - MS	Column: J & W DB5ms LOD: 1 $\mu\text{g kg}^{-1}$
	DBE, p-DCB, naphthalene	Purge & Trap (Tenax TA)	GC - MS	Column HP-5MS LOD: 0.5, 0.15, 0.05 $\mu\text{g kg}^{-1}$ LOQ: 2.4, 0.5, 0.125 $\mu\text{g kg}^{-1}$
	p-DCB	Acid-induced liquid-liquid phase separation of anionic surfactants	HPLC - UV	Column : LiChrospher-18 LOD: 2.5 $\mu\text{g kg}^{-1}$ LOQ: 7.5 $\mu\text{g kg}^{-1}$
	p-DCB, naphthalene	SPME (VB/carboxen/PDMS)	GC - MS	Column: J & W DB5ms LOD: 1, 0.1 $\mu\text{g kg}^{-1}$ LOQ: 5, 1 $\mu\text{g kg}^{-1}$
	DBE, p-DCB, naphthalene	HS-SPME (PDMS)	GC - MS	Column: DB5 LOD: 2, 1, 0.1 $\mu\text{g kg}^{-1}$ LOQ: 5, 4, 0,3 $\mu\text{g kg}^{-1}$
	Naphthalene		HPLC-DAD GC-MS	LOD: 0.023 $\mu\text{g kg}^{-1}$ LOQ: 0.078 $\mu\text{g kg}^{-1}$
Beeswax	p-DCB	ethanol, SPE (C18)	GC - MS	LOD: 0.7 mg $\text{kg}^{-1}$
Royal jelly	p-DCB	Purge & Trap (Tenax TA)	GC - MS	Column HP-5MS LOD: 0.3 $\mu\text{g kg}^{-1}$ LOQ: 0.9 $\mu\text{g kg}^{-1}$

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The acid-induced liquid-liquid phase separation of anionic surfactants in aqueous solutions and its applicability to cloud point extraction methodology were applied as a tool for the extraction of 1,4-dichlorobenzene (p-DCB) from aqueous honey samples. The analyte is extracted into the micelles of sodium dodecane sulfonate. For the separation of p-DCB a high-performance liquid chromatographic equipped with a UV detector system (225 nm) was used (Paleologos, et al. 2006). Dichlorobenzene and naphthalene residues in honey were investigated by solid-phase microextraction (SPME) coupled to gas-chromatographic/mass spectrometry from Harizanis et. al (2008). The equilibration time and the sampling time for the extraction of the analytes by the fibre was 30 min and 60 min respectively, while the honey solution was kept at 60 °C. The LOD and LOQ for the p-dichlorobenzene was 1 µg kg<sup>-1</sup> and 5 µg kg<sup>-1</sup>, while for naphthalene 0.1 µg kg<sup>-1</sup> and 1 µg kg<sup>-1</sup> respectively.

Tsimeli et al. (2008) developed a method for the determination of DBE, p-DCB and naphthalene based on SMPE extraction. Commercially available 100 µm film thickness polydimethylsiloxane (PDMS) fiber was employed for the extraction. The fibre was exposed to the headspace above the sample for 30 min, while the sample was kept at 40±2 °C and stirred at 900 rpm. The separation and detection are carried out using gas chromatography-mass spectrometry (GC/MS) in selected ion monitoring mode (SIM).

For the determination of naphthalene in honey, a high-performance liquid chromatography with a diode array detector method was also used. The compound was detected at 220 nm and the limit of detection and the limit of quantification were 0.023 µg g<sup>-1</sup> and 0.078 µg g<sup>-1</sup> respectively (Beyoğlu & Omurtag 2007).

The p-DCB molecules were extracted from the bee wax with ethanol and the sample clean up was accomplished by solid-phase extraction (C<sub>18</sub> columns), while the determination was achieved by capillary GC and FID detector. The detection limits of the method were 0.7 mg kg<sup>-1</sup> while average recovery was 74.8±5.5% (Bogdanov et al., 1998; 2004). For the isolation of p-DCB from the royal jelly a Purge and Trap system was used (Tananaki et al., 2009). The molecules of this compound extracted from the aqua royal jelly solution by He purging at 40 ml min<sup>-1</sup> for 40 min keeping the sample temperature at 40 °C and were absorbed on Tenax resin. For the separation a fused silica capillary column (HP-5MS) has been used, while the detection was achieved using a mass spectrometer detector. The LOD and LOQ of the method was 0.3 µg kg<sup>-1</sup> and 0.9 µg kg<sup>-1</sup> respectively.

### 3. Conclusion

To maximize the production of agricultural products, extended amount of insecticides, herbicides, fungicides and bactericides are used which eventually lead to contamination of water, soil, crops, animals, even humans. Many environmental studies are concerned with the bioavailability of these pollutants and their subsequent introduction into food chain. Pesticides, Persistent Organic Pollutants (OCs, PCBs, PBBs), toxins and heavy metals have been investigated worldwide as substances that contaminate man's food. Chemicals contaminate hive products like honey, wax, pollen, propolis and royal jelly, while residues may exceed the established MRLs, either because of the improper use of the products or the utilization of unauthorized products by the beekeepers.

Honeybees forage over a circular area, with radius more than 6 Km, visiting numerous plant species and various sources of water and are notorious for collecting materials contaminated with chemicals and bringing them back to the hive. In anyway, pollutants may reach the

hive products and this justifies consumers' concern on this subject. According to research studies, the risk for bee products contamination with pesticides from the environment is low. Concentrations of pesticide residues detected are below LOQs in most studies, while there are only few cases that high concentrations of pesticides were detected in bee products. Moreover, antibiotics used as plant protection products can contaminate bee products, but the concentrations detected are low.

Besides the above indirect method of pesticides transferring into bee's nest, the bigger risk for bee products contamination is the beekeeping practices. Diseases attack bee colonies and the beekeepers use acaricides, antibiotics, fungicide and other chemicals inside the hive to control them.

Antibiotics played an important role as effective chemotherapeutics for bee diseases and have been used until recently. However, the use of antibiotics against any bee disease is not permitted in Europe anymore, because pharmaceutical companies did not apply and support the experimental data for MRLs in bee products as required by the European Medicinal Evaluation Agency (EMA). Despite of this forbiddance, monitoring results indicate that antibiotic residues are still present in European honeys, but the detection frequency is decreasing after the European ban. Antibiotic residues are usually detected in honey and royal jelly, while the concentrations are very low comparing to other products such as milk, eggs etc.

Another source of contamination that is caused by beekeepers is the chemicals that they use against Varroaosis, a disease caused by the parasitic mite *Varroa destructor* Anderson and Trueman. Varroas' presence causes many troubles to the bees including appearance of other diseases like sacbrood, American and European foulbrood. If it is left untreated it could destroy the whole colony within 2-3 years period. Varroaosis is actually the only disease of bees against which the use of pharmaceutical products within the hive is permitted. In European countries, there are authorized chemicals that can be used and limits (MRLs) that should not be exceeded. The contamination of bee products by acaricides can be minimized by careful use of the chemotherapeutic products. As far as we know the percentage of honey samples containing residues exceeding MRLs is low. A major problem could be the use of unauthorized products in order to control Varroaosis.

Additional problem for the quality of bee products was the volatile insecticides and other chemicals that beekeepers used in storehouses to protect bee combs from the larvae of the insect *Galleria mellonella* (wax moth). This insect attacks the honeycombs during storage and can even damage the wooden frames in which they hang. The devastating activity of these insects is known to beekeepers all over the world. To save the combs, beekeepers use several chemical fumigants that are incorporated into wax and from there they are readily translocated into bee products. Some of those like PDCB, DBE and naphthalene pose a potential health hazard. Although beekeepers stopped using these compounds, residues were still in old combs for many years and readily transferred into honey. Volatile insecticide residues detected in bee products are below LOQ in countries like Greece, which had a major contamination problem the previous decade.

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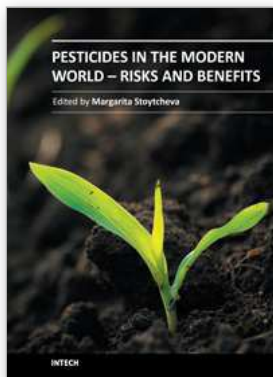
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## **Pesticides in the Modern World - Risks and Benefits**

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