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Development of a Method for the Analysis of Neonicotinoid Insecticide Residues in Honey using LC-MS/MS and Investigations of Neonicotinoid Insecticides in Matrices of Importance in Apiculture

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1 Introduction

Neonicotinoid insecticides are a relatively new class of highly potential pesticides that act in a very specific way on the nervous system of insects. However, also beneficial non-target organisms such as honeybees can be affected by the use of these insecticides.

In many countries all over the world beekeepers have been reporting an uncommon increase of colony loss over the past years. Beside many different factors that could contribute to this phenomenon the poisoning of honeybees through pesticides applied to agricultural crops or for mite control may also play a major role (Environmental Protection Agency 2008a).

Depending on the application form of neonicotinoid insecticides different routes of exposure of honeybees to these pesticides can be envisaged. Neonicotinoids applied as chemical sprays can either contaminate the blossoms of plants on and beside agricultural fields or foraging honeybees during their flight. The same ways of contamination can also occur upon abrasion and environmental drift of neonicotinoids contained in seed dressings during the sowing process. Additionally, neonicotinoids applied in seed dressings are distributed in the plants and honeybees might come into contact with them through their presence in pollen, nectar and guttation liquid.

When honeybees come into contact with neonicotinoids the insecticides might be taken along into the beehive and contaminate bee products such as honey. Such a contamination of honey would stand in clear contrast to consumer expectations, for whom honey represents a natural product of highest purity.

Figure 1 shows the route of neonicotinoid insecticides from their agricultural application to honeybees and the subsequent transfer into honey. Both transfers, i.e. from the agricultural application to honeybees (first arrow) and from honeybees into honey (second arrow) were investigated in the present thesis by analysing appropriate matrices.

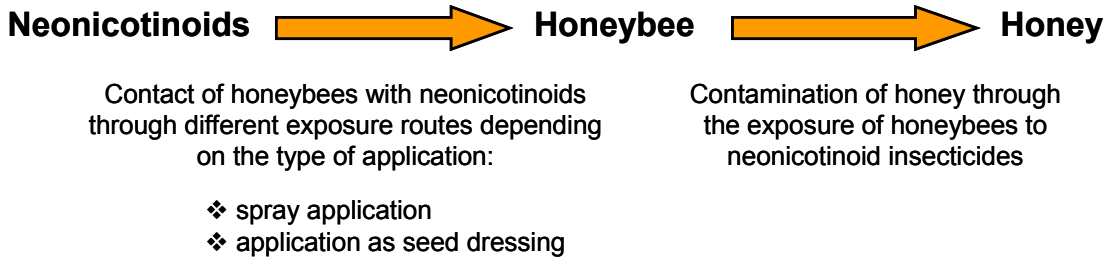

Figure 1: Route from the application of neonicotinoid insecticides to honeybees and transfer into honey

First investigations focused on the guttation liquid collected from plants grown from neonicotinoid-treated seeds to answer the question whether and in what quantities neonicotinoid insecticides can be transferred into the guttation liquid from seed dressings.

Concerning the contamination of honey with neonicotinoid insecticides only few investigations have been carried out so far. Up until now no multi-residue method for the determination of the whole class of neonicotinoid insecticides has been described. Thus, the main goal of this diploma thesis was the development and validation of a rapid and sensitive analytical method for the simultaneous identification and quantification of neonicotinoid insecticides and their metabolites in honey.

The subsequent analyses of different honey and nectar samples included suspicion honey and nectar samples from beehives with reported honeybee losses as well as flower and forest honey samples from different locations in Austria. The target of these analyses was to find out whether and to what extent neonicotinoid insecticide residues are present in Austrian honey and nectar samples.

2 Literature Survey

2.1 Honeybee – *Apis mellifera*

Figure 2: Most common honeybee in Austria: *Apis mellifera Carnica*

The honeybee is one of the most familiar insects and plays an important role in the human and natural world. Honeybees belong to the family *Apidae* (order *Hymenoptera*) and are characterised by a small number of species. The most common of the 25 different breeds of the species *Apis mellifera* in Austria is *Apis mellifera Carnica* (Figure 2). About 90 % of the Austrian beekeepers domesticate Carnica bees (Wawschinek 2004). Characteristic for this subspecies is their calmness, a high level of honey production and idleness to swarm. In Austria 22,000 beekeepers possess approximately 300,000 bee colonies (Lebensministerium 2009).

2.1.1 Role and Importance of the Honeybee

"If the bee disappeared off the surface of the globe then man would only have four years of life left. No more bees, no more pollination, no more plants, no more animals, no more man."

The descriptive content of this remark expressed by Albert Einstein indicates the importance of the honeybee for life on earth. The varied role of honeybees includes the pollination of a considerable number of wild and cultivated plants, the production of a wide range of different beehive products and the function as bioindicator.

Foraging honeybees collect nectar and pollen from flowers for nourishment, feed stock and brood development and pollinate various plants during this process. Honeybees therefore represent an essential species for the maintenance of natural and agricultural ecosystems. By pollination they contribute to the reproduction, fruit set development and dispersal of a majority

of plants and are thus also important for plant biodiversity (Food and Agriculture Organisation 2007). A total of 80 % of the economic plants are pollinated by honeybees (Del Vecchio 2009, Seefeld 2006). Considering this enormous pollination work accomplished by honeybees, their value for nature and for the production of foodstuffs reaches tremendous dimensions.

Beside honey honeybees supply humans also with a number of other beehive products such as pollen, bee wax, propolis and royal jelly. In Austria the annual honey production amounts to approximately 6,000 tons (Statistik Austria 2008). However, the value of the honey production is marginal compared to that of the pollination work performed by honeybees considering that the total economical value of the honeybee as pollinator is estimated to account for at least the ten- to twenty-fold value of all bee products (Pistorius 2009, Seefeld 2006).

A further special function of honeybees is their role as bioindicators. Through the honeybee's sensitivity to environmental parameters weakening and disappearing of honeybees as well as residues in bee products can indicate the presence of environmental pollution (Celli and Maccagnani 2003).

Due to the above-mentioned functions the honeybee reaches the third position in the ranking of the most important production animals for human-beings after cattle and pig (Brodschneider and Crailsheim 2009).

2.1.2 Colony Loss, Possible Causes and the Role of Pesticides

In many countries increasing losses and weakening of honeybee colonies were reported in recent years (Haubruge et al. 2006, Oldroyd 2007, Van Engelsdorp et al. 2008). Numerous studies carried out in Europe and the United States suggest that many factors and most probably a combination and interaction of these factors are responsible for the general loss of honeybee colonies (Haubruge et al. 2006, Oldroyd 2007). Possible influences are diverse including climatic conditions, environmental parameters such as missing food sources and diminishing biodiversity, parasites, diseases, handling mistakes, intended

and unintended poisoning, local pollution and the application of insecticides in agriculture (Haubruge et al. 2006).

Colony losses up to 10 % that occur mainly during winter are considered to be normal (Oldroyd 2007). However, in recent years a new phenomenon of unexplained honeybee losses gave cause of concern to beekeepers in the United States, Europe and elsewhere. Officially the syndrome of mysterious losses of a high number of honeybees was named colony collapse disorder (CCD). The main symptom is a low number of adult bees in the beehive which is most probably caused by the sudden death of adult worker bees in the fields (Oldroyd 2007). Usually the affected beehives are well supplied with honey and pollen and brood is present (Brodschneider and Crailsheim 2009). The absence of a known cause for this phenomenon led to extensive investigations and different monitoring programs (University of Marburg 2008, Van Engelsdorp et al. 2008). According to the U.S. Environmental Protection Agency (EPA) CCD might possibly be caused by the invasion of the varroa mite, emerging diseases or immune-suppressing stress on honeybees evoked through one or more factors such as poor nutrition, drought or migratory stress. Beside these potential causes EPA also mentioned the poisoning of honeybees through pesticides applied to agricultural crops or for mite control as possible contributing factor (Environmental Protection Agency 2008a).

Beside a possible contribution of pesticides to CCD, pesticides can also poison large numbers of honeybees as a consequence of accidental incidents during plant protection activities or misapplications of products with bee-toxic substances. Typical signs of poisoning are crawling, moribund and dead honeybees in front of the beehive entrance or on the surrounding ground. According to the Institute for Ecotoxicology and Ecochemistry in Plant Protection in Berlin investigations of the past 19 years indicate a clear decrease of damages to bees in the past ten years due to application of plant protection products (Seefeld 2006). In contrast, Pistorius (2009) stated comparable levels of damages in bees during the past ten years if the years 2003 and 2008 are excluded. However, in 2008 a significant peak in the loss of bees as a

consequence of the application of pesticides was observed. In spring 2008 large-scale losses were reported in the upper Rhine area in Southern Germany and the neonicotinoid insecticide clothianidin was identified as cause for the regional bee damages (see section 2.3.1).

2.2 Neonicotinoid Insecticides

The term neonicotinoids was chosen for this class of substances because of their new mode of action and structural differences in comparison to nicotinoids. In today's agriculture neonicotinoid insecticides constitute one of the most important and fastest-growing groups of pesticides (Jeschke and Nauen 2008, Schäfer 2008). The discovery of synthetic nitromethylene heterocycles in the 1970s established the basis for the long-lasting development of neonicotinoid insecticides with nithiazin as the main precursor substance. The successful history of neonicotinoids started in 1991 with the launching of imidacloprid by Bayer. Three more substances belong to the so-called first generation of neonicotinoids: nitenpyram from Takeda (1995), acetamiprid from Nippon Soda (1996) and thiacloprid from Bayer (2000). The second generation consists of two substances: thiamethoxam from Syngenta (1998) and clothianidin from Takeda/Bayer (2002). Dinotefuran from Mitsui Chemicals (2002) is the only substance of the third generation (Schäfer 2008). The insecticide flonicamid was developed by ISK in the late 1990s and is only sometimes assigned to the class of neonicotinoid insecticides depending on the individual study or register.

The neonicotinoids have reached an enormous economic value and represent one of the most important groups of insecticides on the current market of plant protection products. Imidacloprid is one of the best-selling insecticides worldwide and distributed in more than 120 countries (Maienfisch et al. 2001). In 2006 worldwide annual sales of neonicotinoids accounted for 1.56 billion US$ (Jeschke and Nauen 2008).

2.2.1 Chemistry

The neonicotinoid insecticides acetamiprid, imidacloprid, nitenpyram and thiacloprid (first generation) contain the heterocyclic 6-chloro-3-pyridyle group, clothianidin and thiamethoxam (second generation) the 2-chloro-5-thiazolyle group and dinotefuran (third generation) the 3-tetrahydrofuranyle group (Schäfer 2008). Flonicamid is characterised by the heterocyclic 4-trifluoromethyl-3-pyridyle group. The chemical structures of the neonicotinoids are shown in Figure 3.

With regard to chemical structure a common subdivision of neonicotinoids is into cyclic and open-chain compounds. Imidacloprid, thiacloprid and thiamethoxam belong to the cyclic neonicotinoids, while acetamiprid, clothianidin, dinotefuran, flonicamid and nitenpyram are open-chain compounds (Figure 3).

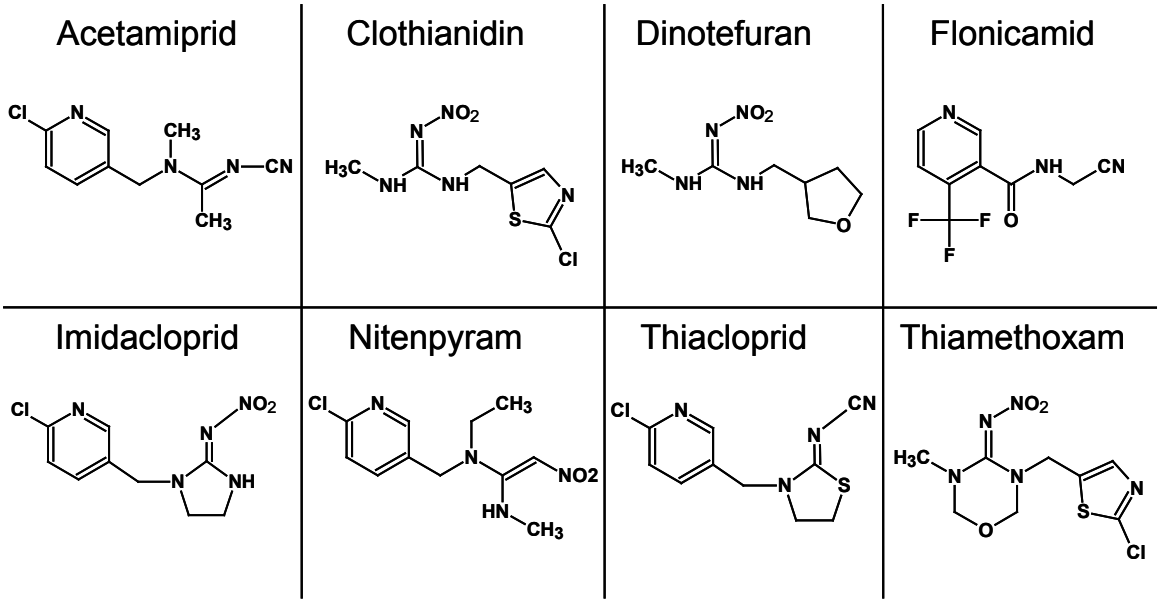

Figure 3: Chemical structures of the insecticides belonging to the group of neonicotinoids

The neonicotinoid insecticides share some chemical properties. All of them have a polar character and show moderate to very high solubility in water ranging from 185 mg/l to 840 g/l (CRL Datapool).

Ford and Casida (2008) carried out some studies about the metabolism of seven neonicotinoid insecticides in plants (spinach) and mammals (mice). The investigations revealed that neonicotinoids are converted to numerous and variable metabolites in plants as well as in mammals (Ford and Casida 2008). The metabolism of the parent compounds included various reactions such as nitro reduction, cyano hydrolysis, demethylation, sulfoxidation, imidazolidine and thiazolidine hydroxylation, olefin formation, oxadiazine hydroxylation and ring opening and chloropyridinyl dechlorination. In this context it is interesting to note that clothianidin is at the same time an applied neonicotinoid insecticide and a metabolite of thiamethoxam (Nauen et al. 2003).

2.2.2 Mode of Action

Neonicotinoid insecticides act as agonists on the postsynaptic nicotinic acetylcholine receptor of the insect's central nervous system. In the same manner as acetylcholine the binding of a neonicotinoid causes the opening of connected ion channels and leads to depolarisation. The crucial difference between the binding of acetylcholine and neonicotinoids to the receptor is that acetylcholine leaves the receptor after cleavage through the enzyme acetyl cholinesterase, whereas neonicotinoids remain bound to the receptor. Therefore, neonicotinoids in high dosages provoke an ongoing depolarisation and finally lead to the blocking of signal transmission (Schäfer 2008). In insects the actions of neonicotinoids cause excitations of the nerves and finally paralysis leading to death (Fishel 2005).

Nicotinic acetylcholine receptors exist not only in insects but also in vertebrates. However, the corresponding neuron pathway is more abundant in insects (Fishel 2005). There are at least 17 subtypes of nicotinic acetylcholine receptors characterised by differences in the subunits of the receptors (Schäfer 2008).

Neonicotinoid insecticides show a high selectivity for certain subtypes of receptors and exhibit differences in the actions on insect compared to vertebrate receptors (Matsuda et al. 2001 and 2009). This unique selectivity regarding the molecular target site is one key factor for the limitation of adverse effects on beneficial organisms and also for the assessment of risks posed by the presence of neonicotinoid residues in food.

Due to the neonicotinoids' specific mode of action there is no cross-resistance of neonicotinoids to longer-established insecticide classes such as carbamate, organophosphorous or synthetic pyrethroid insecticides (Fishel 2005, Jeschke and Nauen 2008). Many pests have developed resistances against these types of insecticides over the years. Neonicotinoids are a promising new class of pesticides for an effective and long-lasting protection of agricultural crops from pests with such resistances (Elbert et al. 2008).

Neonicotinoid insecticides are active against a wide range of sucking, biting and some chewing insects (Jeschke and Nauen 2008). Examples for pests against which neonicotinoids are applied include aphids, whiteflies, leaf- and planthoppers, thrips, micro lepidoptera and coleopteran insects (Elbert et al. 2008).

2.2.3 Application

The main reasons for the success of neonicotinoids in plant protection are their high efficacy, selectivity, plant systemicity as well as long-lasting effect and versatile application (Elbert et al. 2008).

In the European Union the use of pesticides is regulated on two levels. Prior to the authorisation of a plant protection product using a certain active substance the latter generally needs to be included in Annex I of the Directive 91/414/EEC. The subsequent authorisation and registration of the plant protection product is carried out by the individual member states. If the inclusion of an active substance in Annex I of the Directive 91/414/EEC is pending, plant protection

products containing this substance can be provisionally authorised in the member states.

Acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam are included in Annex I of the Directive 91/414/EEC, whereas dinotefuran and nitenpyram have never been included in Directive 91/414/EEC up to date (EU Pesticides Database). Consequently, no plant protection products with dinotefuran or nitenpyram as active substances are authorised in the EU. The inclusion of flonicamid in Directive 91/414/EEC is currently (January 2010) pending (EU Pesticides Database).

In Austria various plant protection products containing one of the five neonicotinoid insecticides acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam as active substance are authorised. One plant protection product containing flonicamid is authorised in Germany and the Netherlands on a provisional basis. §12 of the Austrian plant protection product law from 1997 regulates the use of plant protection products that are authorised in other member states. According to two decrees plant protection products with authorisations in either the Netherlands or Germany can be applied in Austria (decrees 109/1998 and 52/2002). The plant protection product containing flonicamid can therefore also be applied in Austria.

Table 1 shows the most relevant agricultural crops for the application of the different neonicotinoids (Register of Authorised Plant Protection Products). The versatile application of neonicotinoid insecticides covers many crops ranging from cereals and vegetables to various fruit cultures.

The nationwide level of application of each neonicotinoid insecticide depends on the range of crops that can be treated with the particular products and the share of cultivation of these crops on the total cropland. In this context the neonicotinoids that are used as active substances in authorised plant protection products (Table 1) can be expected to play the major role in Austria. Nevertheless, the illegal application of non-authorised plant protection products

containing dinotefuran or nitenpyram cannot be excluded and thus also needs to be taken into account.

Table 1: Authorisation status of plant protection products containing neonicotinoids in Austria and examples for commercial products and cultures on which these are used (Register of Authorised Plant Protection Products)

Active Substance	Authorisation of plant protection products containing this active substance	Examples for commercial products	Examples for cultures on which these products are used and type of application
Acetamiprid	currently authorised in Austria	Mospilan 20 SG	potato, rape, pome, cherry, plum etc. (spray application)
Clothianidin	currently authorised in Austria	Poncho	maize and poppy seed (seed dressing)
Flonicamid	provisionally authorised in the Netherlands and Germany	Teppeki	apple, peach, wheat, potato etc. (spray application)
Imidacloprid	currently authorised in Austria	Gaucho 600 FS	cereal, potato, maize, onion, pumpkin, fodder beet and sugar beet (seed dressing)
		Chinook	rape (seed dressing)
Thiacloprid	currently authorised in Austria	Biscaya	pea, barley, oat, potato, maize, poppy seed, rape, wheat, rye etc. (spray application)
		Calypso	Chinese cabbage, potato and pome (spray application)
Thiamethoxam	currently authorised in Austria	Cruiser 350 FS	maize (seed dressing)
		Cruiser 70 WS	fodder beet and sugar beet (seed dressing)
		Actara	potato (spray application)

Clothianidin, imidacloprid and thiamethoxam are used as active substances in seed dressings. These neonicotinoids protect the crops in several ways: The neonicotinoid-containing coat protects the seeds from being destroyed by insects in the soil and the uptake of the neonicotinoids through the roots and their systemic distribution in the entire plant protects the plant from biting, sucking and chewing insects during growth. Through these two modes of action the effect of neonicotinoids applied in seed dressings is long-lasting and covers all parts of the plants. In contrast, thiacloprid and acetamiprid are applied as sprays onto agricultural plants and supply direct and immediate but rather short-time protection. In addition to its use in seed dressings thiamethoxam can also be applied in the form of a spray.

One example for a common application of neonicotinoid insecticides in Austria is maize: Maize represents the most relevant agricultural crop for the application of neonicotinoids because of its high proportion on agricultural cropland in certain areas and the existence of particularly damaging pests. About 22 % of the agricultural crop land in Austria (approximately 300,000 ha) are used for growing maize (Austrian Agency for Health and Food Safety 2009). The most dangerous pest for maize cultures is the Western corn rootworm (*Diabrotica virgifera virgifera*) which shows a fast geographical spread (15 to 50 km per year). The first occurrence of the Western corn rootworm in Austria was recorded in 2002 close to the Slovakian border and has since spread to large parts of the country (Austrian Agency for Health and Food Safety 2008). The European Union categorised the corn rootworm as quarantainable pest and released regulations for obligatory fighting measures (Ministerium für Ernährung und ländlichen Raum Baden-Württemberg 2008). The application of neonicotinoid-containing seed dressings on maize crops represents an essential part of the protection of maize crops from the Western corn rootworm.

2.3 Neonicotinoid Insecticides and Honeybees

The high efficacy of neonicotinoids in the protection of agricultural crops from pests insects can also affect beneficial organisms such as honeybees. Honeybees may come into contact with neonicotinoids through different exposure routes. Depending on the bee toxicity and the extent of exposure various effects are possible: no visible influence, honeybee losses, brood damages or contamination of bee products with insecticide residues.

A main factor for the exposure risk of honeybees to neonicotinoids is the proximity of areas of intensive agriculture and honeybee habitats. As a consequence of the large areas used for agriculture honeybees often forage on and close to cropland (Wallner 2009a). In Austria a high proportion of the total area is used as cropland so beehives are often placed in close proximity to the agricultural fields. Figure 4 shows an example for this proximity.

Figure 4: Beehives in close proximity to blooming rape fields

2.3.1 Exposure Routes

Considering the different application forms of neonicotinoid insecticides there are several possible routes how honeybees may come into contact with neonicotinoids. Neonicotinoids are either applied as chemical sprays in agricultural fields or affixed to the coats of seeds as a component of seed dressings (see section 2.2.3).

2.3.1.1 Spray Application

The spray application of neonicotinoid insecticides can lead to the contamination of blossoms of crops on the fields, non-target plants on and beside the fields as well as the foraging honeybees during their flight. The exact

time of treatment depends on the agricultural crops, the plant protection product and the target organisms of the application.

Thus, one way of contamination of honeybees with neonicotinoids is the contact of honeybees with insecticide-polluted blossoms on and beside the fields during the collection of nectar or pollen (Smodiš Škerl et al. 2009). A further possibility of contamination is the direct contact of honeybees with neonicotinoids if the spraying takes place simultaneously with the foraging activity of the bees. Considering these two exposure routes the contamination of honeybees with neonicotinoids can easily happen through the spraying of plant protection products containing neonicotinoids.

2.3.1.2 Seed Dressing

The application of neonicotinoid insecticides in the form of seed dressings avoids the spraying of these substances. Thus, the launch and use of such seed dressings led to a significant reduction of aerial pesticide pollution and amounts of toxic substances in the environment (Bonmatin et al. 2005). Nevertheless, also the application of neonicotinoid insecticides in the form of seed dressings can lead to an exposure of honeybees. Two main routes of exposure are possible: the contact between honeybee and active substance during the sowing process and through the presence of neonicotinoids in the treated agricultural plants.

<u>Exposure related to the Sowing Process</u>

In general the sowing of seeds is carried out using pneumatic single grain sowing machines. If abrasion of the seed dressing occurs dust containing neonicotinoids can drift into the environment and contaminate plants beside the fields or foraging honeybees during their flight. The use of sowing machines that emit discharged air upwards into the air leads to a more extensive distribution of dust and is therefore clearly disadvantageous for the environment compared to

sowing machines with direct soil emission (Ministry of Agriculture Forestry and Food Republic of Slovenia 2008). A further source of neonicotinoid-containing drift is the dust from the inside of the seed bags when poured into the sowing machine. In case of heavy wind the distribution of the dust can reach considerable distances. The extent of drift of neonicotinoid-containing dust and contamination of surrounding plants depends on the quality of seed treatment, the type of sowing machine and weather conditions. A recent incidence of dramatic losses of honeybees in Southern Germany clearly showed that the abovementioned possible exposure route related to the sowing process can indeed occur at a large scale in practice (Ministerium für Ernährung und ländlichen Raum Baden-Württemberg 2008). As a consequence of a considerable infestation of the corn rootworm in Bavaria and Baden-Württemberg in 2007, maize grown in and close to the affected areas was cultivated from seeds dressed with Poncho Pro containing clothianidin as active substance. As a result of abrasion during the sowing of the dressed maize seeds clothianidin-containing dust drifted into the air and contaminated surrounding blooming plants such as rape and apple cultures. The blossoms of these plants were attractive and highly frequented nectar sources for foraging honeybees at exactly that time. Immediate honeybee losses were observed during the sowing period at the end of April and the beginning of May 2008. More than 700 beekeepers were hit by the damage of approximately 12,000 beehives. The observed symptoms such as crawling, dying and dead honeybees in front of the entrance of the beehive were typical indicators for acute poisoning. Analytical investigations of plants, dead honeybees and beehive products of affected colonies confirmed the suspicion that the cause for the honeybee losses was intoxication. Clothianidin from the dressings applied onto the maize seeds was identified as the responsible substance (Ministerium für Ernährung und ländlichen Raum Baden-Württemberg 2008). Further honeybee losses and brood damages also occurred after the blossom periods of rape and fruit cultures due to the relatively high contamination of pollen and bee bread with clothianidin.

As a consequence of these incidents in the Upper Rhine Valley and similar cases reported in other countries such as Slovenia (Ministry of Agriculture, Forestry and Food Republic of Slovenia 2008) some precautions were taken to avoid such incidents in the future. In Germany and Slovenia the authorisation of neonicotinoid insecticide seed dressing products for maize such as Poncho (clothianidin), Gaucho 600 FS (imidacloprid) and Cruiser 350 FS (thiamethoxam) has been suspended for an indefinite time (Ministerium für Ernährung und ländlichen Raum Baden-Württemberg 2008, Ministry of Agriculture). In Austria the neonicotinoid-containing seed dressing products for maize are still authorised, however, additional requirements for the reduction of the risks for honeybees resulting from these products have been put into force. These preventive measures concern the seed treatment quality, the handling of dressed seeds and drift-reducing techniques of sowing machines (Kohl 2009).

Exposure related to the Presence of Neonicotinoids in the Plants

Neonicotinoids applied in seed dressings are systemic insecticides and are distributed in the entire plant through transportation in the xylem and phloem (Aliouane et al. 2009). Plant liquids and pollen of plants grown from neonicotinoid-treated seeds might contain these insecticides and are possible sources of contact for honeybees.

Bonmatin et al. conducted investigations about the uptake of imidacloprid in maize plants. In most of the leaf, blossom and pollen samples collected from maize plants grown from imidacloprid-treated seeds imidacloprid was detected. The results of this study confirmed the systemic character of imidacloprid in maize. In earlier investigations in the years 2002 to 2004 Bonmatin et al. found the same behaviour of imidacloprid in sunflowers (Bonmatin et al. 2005). Similar investigations of Chauzat et al. also showed the presence of imidacloprid in pollen and nectar (Chauzat et al. 2006a&b).

Beside the uptake of neonicotinoids in form of pollen or nectar another potential route of exposure has recently become increasingly discussed. Some vascular

plants such as maize and rape exude guttation liquid. Guttation is the botanical phenomenon of active or passive excretion of xylem liquid in the form of droplets on specific locations of the plant. The term guttation originates from the Latin word “gutta”, which means “drop”. Usually, the droplets appear on the tips and along the edges of the leaves (see Figure 5 to Figure 7).

Figure 5: Large guttation droplet and small dew drops on the leaf of an outdoor plant

Figure 6: Guttation droplets on the tip of an indoor-grown maize plant

Figure 7: Numerous guttation droplets on the leaves of a maize plant on the field

Guttation is characteristic for many vascular plants such as maize (*Zea mays*), barley (*Hordeum vulgare*), rape (*Brassica napus*) and wheat (*Triticum aestivum*) and occurs preferably under certain climatic circumstances. The main precondition for guttation activity is a high relative humidity, i.e. a high saturation of the atmosphere with water vapour. This atmospheric condition inhibits transpiration, the usual way of plants to eliminate a surplus of water (Nikolakis 2009). In this case plants can get rid of spare water by guttation. The exudation of the guttation droplets occurs actively through standard stomata or passively through special pores called hydathodes (Wallner 2009b). Since a decrease of air temperature increases relative humidity, guttation occurs mainly during nighttime and in the early morning. Guttation liquid is an aqueous solution containing 0.1-0.4 % inorganic and organic substances such as salts, amino acids, sugars, vitamins and hormones. Minerals of special importance to the plant are only exuded to a minimum extent or not at all (Bresinsky et al. 2008). If chemicals such as the active ingredients from seed dressings are located in the

root area of plants they can be taken up along with water and other compounds. Consequently, these substances can also pass into the guttation liquid. Guttation droplets of agricultural crops cultivated from dressed seeds can therefore represent a potential source of contact for honeybees with active substances of seed dressings.

This possible route of exposure has received little attention for a long time. Different investigations regarding neonicotinoid insecticides in guttation liquid were recently carried out at the Universities of Padua (Girolami et al. 2009) and Hohenheim (Wallner 2009b) as well as at Bayer CropScience (Nikolakis 2009). Girolami et al. analysed the concentrations of clothianidin, imidacloprid and thiamethoxam in the guttation liquid of maize plants cultivated from neonicotinoid-treated seeds and found neonicotinoid levels of more than 10 mg/l with maximum concentrations of up to 100 mg/l (clothianidin and thiamethoxam) and 200 mg/l (imidacloprid). The analyses of Wallner and Bayer CropScience focused on the detection of clothianidin in guttation liquid and showed concentrations of more than 1 mg/l (Wallner 2009b) and 5 to 133 mg/l (Nikolakis 2009), respectively.

The described experiments clearly showed that neonicotinoid insecticides that are applied in the seed dressings are able to pass into the guttation liquid of the plants. The concentrations of the detected neonicotinoid insecticides in guttation liquid lay in the ppm range (Girolami et al. 2009). Through the incorporation of these guttation droplets as water source honeybees can come into contact with neonicotinoids. Shawki et al. (2006) reported foraging honeybees to collect guttation liquid from rape plants in spring. However, there is still no conclusive evidence about the use of guttation liquid as water source by water-foraging honeybees, the transportation of neonicotinoids into the beehives via guttation liquid as well as its impact on the beehive (Wallner 2009b).

2.3.2 Impact of Neonicotinoid Insecticides on Honeybees

According to Jeschke and Nauen (2008) neonicotinoid insecticides represent a relatively low risk for the environment as well as for mammals and other non-target organisms due to their high selectivity. However, most of the neonicotinoid compounds are moderately or highly toxic for honeybees, whereby the toxicity depends on the kind of exposure (oral or contact).

In high doses the exposure of honeybees to neonicotinoids leads immediately to death, whereas smaller doses result in various symptoms. According to Aliouane et al. (2009) the oral and contact exposure of honeybees to sublethal doses of acetamiprid and thiamethoxam results in limited effects on motor, sensory as well as cognitive function. In two studies Medrzycki et al. reported an influence of sublethal doses of imidacloprid on honeybee mobility, communication ability, homing rate and foraging activity (Medrzycki et al. 2003a&b). A further risk of the exposure of honeybees to sublethal doses of insecticides is the possible accumulation of the substances in the beehive and the exposure of young honeybees to the insecticides (Aliouane et al. 2009). During the incidence of intoxication of honeybees with clothianidin in Germany in 2008 beekeepers observed considerable brood damages (Koch and Heuvel 2009). Especially the contact of honeybees with contaminated pollen can have negative impacts on the whole bee colony. Since pollen represents the only source of proteins for honeybees the contamination of pollen can affect all developmental stages of bees and all members of a bee colony (Bonmatin et al. 2005, Vighi et al. 2000).

Regarding the risk of neonicotinoids in guttation liquid for honeybees, Girolami et al. (2009) conducted some trials to investigate the direct effects of neonicotinoid-containing guttation liquid on honeybees upon its incorporation. For this purpose honeybees were fed with the collected neonicotinoid-containing droplets of guttation liquid. Further trials investigated the dose-response effects of the different neonicotinoids by feeding honeybees with solutions of neonicotinoids in water with 15 % of honey. These investigations

showed that the consumption of neonicotinoid-containing guttation liquid (concentrations in the range of 50 to 100 mg/l) provokes two symptoms of intoxication (arching of the abdomen and paralysis of the thorax muscle) before leading to death within 2 to 15 minutes after feeding. After the consumption of guttation liquid containing clothianidin or thiamethoxam wing paralysis occurred within a shorter period of time compared to guttation liquid containing imidacloprid in similar concentrations. The dose-response experiments showed that concentrations of 1.5 mg/l of clothianidin or thiamethoxam or 6.25 mg/l of imidacloprid, respectively, cause the abovementioned symptoms within one hour (Girolami et al. 2009). Considering the high concentrations of neonicotinoid insecticides that were detected in the guttation liquid of maize plants from dressed seeds the guttation liquid represents a potential threat to honeybees. However, the actual risk for honeybees strongly depends on the effective use of guttation liquid as water source by honeybees (Nikolakis 2009).

Bee toxicity is expressed as the LD50 (lethal dose 50) value which represents the required amount of a substance to kill 50 % of a sample population. LD50 values are determined for both acute oral and acute contact toxicity. Table 2 displays the acute oral and contact bee toxicities of the neonicotinoid insecticides.

Clothianidin, imidacloprid, thiamethoxam, dinotefuran and nitenpyram show the highest levels of oral and contact toxicity for honeybees with LD50 values below 1 µg/bee. Acetamiprid and thiacloprid are moderately toxic to honeybees whereas flonicamid constitutes the least toxic of the neonicotinoids. Comparing the acute oral and contact bee toxicity of the neonicotinoids the LD50 value is always lower for acute oral toxicity (with the exception of flonicamid). Consequently, the health risk for honeybees is higher when neonicotinoids are incorporated.

Table 2: Acute oral and contact bee toxicities of neonicotinoid insecticides expressed as LD50 values

Substance	LD50 Acute oral toxicity	LD50 Acute contact toxicity
Acetamiprid	8.85 µg/bee[1]	9.26 µg/bee[1]
Clothianidin	0.00379 µg/bee[1]	0.04426 µg/bee[1]
Dinotefuran	0.023 µg/bee[2]	0.047 µg/bee[2]
Flonicamid	53.3 mg/bee[1]	51.1 mg/bee[1]
Imidacloprid	0.0037 µg/bee[3]	0.0179 µg/bee[4]
Nitenpyram	0.138 µg/bee[5]	No data
Thiacloprid	17.32 µg/bee[1]	38.82 µg/bee[1]
Thiamethoxam	0.005 µg/bee[1]	0.024 µg/bee[1]

[1] Circa "List of end points", [2] Environmental Protection Agency b, [3] Halm et al. 2006, [4] Iwasa et al. 2004, [5] Footprint pesticide properties database

The differences in acute bee toxicity of the individual neonicotinoids are predominantly the result of differences in their chemical structure. LD50 values are in the ng/bee range for nitro-substituted compounds (clothianidin, dinotefuran, imidacloprid, nitenpyram and thiamethoxam), whereas they are in the µg/bee range for cyano-substituted neonicotinoids (acetamiprid and thiacloprid). The overall toxicity of a pesticide is not only determined by that of the compound itself but also by the toxicity of its metabolites. In an investigation of different plant metabolites of acetamiprid (IM 2-1, IM-O and IC-O) no mortality was observed at doses of 50 µg/bee (Iwasa et al. 2004). The results of that study showed the oxidation through cytochromes P450 to be an important mechanism for the detoxification of acetamiprid and thiacloprid and a reason for their low toxicity to honeybees (Iwasa et al. 2004).

The exposure of honeybees to multiple types of pesticides can possibly lead to toxic interactions of the substances (Frazier et al. 2008). The effect of some neonicotinoid insecticides on honeybees can for example be influenced by the presence of other pesticides such as fungicides. Laboratory studies of Iwasa et al. (2004) showed certain fungicides (e.g. triflumizole, propiconazole and triadimefon) to increase the bee toxicity of acetamiprid and thiacloprid 1100-fold. Only little is known about all the possible interactions of different pesticides and the resulting impact on honeybees.

2.4 Honey

Honey is one of the oldest foods and played an important role in nutrition, healing and ritual ceremonies in most cultures during thousands of years (Khan et al. 2007). Prehistoric illustrations of honey harvest estimated to have an age of 10,000 to 15,000 years were found in a Spanish cave (Frank 2005). With regard to nutrition honey was the only known or available sweetener for a long time in the history of mankind (Bogdanov 2009a). Until today honey represents a highly valued food among consumers of all ages and origins (Bechthold 2009a). Furthermore, honey is a product with a high medicinal value. Already the ancient Egyptians, Greeks and Romans utilized honey for medical treatments and also today honey still plays an important role as alternative remedy for wound healing (Bechtold 2009b). A review of 19 studies reported a more rapid wound healing for some types of wounds through the application of medicinal honey (Jull et al. 2008). In another review of 43 studies honey was concluded to be a suitable alternative treatment for various skin conditions, burns and wound healing (Bardy et al. 2008). A further medical application for honey is the support of the treatment of cough and colds through the intake in combination with tea or milk with the World Health Organisation mentioning honey as potential remedy for the treatment of respiratory infections in young children (World Health Organisation 2001).

2.4.1 Definition and Composition of Honey

The Austrian honey regulation (Bundesministerium für Gesundheit und Frauen 2004) provides the following definition, classifications and composition of honey:

Honey is the naturally sweet substance produced by honeybees of the species *Apis mellifera* that collect nectar from plants, excretions of living plant parts or secretions located on the surface of living plant parts excreted by plant-sucking insects, mingle the collected material with species-specific substances and

transform, stock, dehydrate, store and maturate it in the honeycombs of the beehives.

Depending on the origin of the basic material, honey can be divided into two classes. Flower honey refers to honey produced from the nectar of plants, while honeydew or forest honey is honey produced from excretions of living plants or plant-sucking insects (*Hemiptera*). Further classifications and differentiations are based on kind of production, colour, consistency, flavour as well as botanical, topographical and geographical origin.

The basic material for flower honey is nectar, the sugar-rich liquid produced in floral or extrafloral nectaries of plants. Honeybees are attracted by the nectar, collect it and store it in the honeycombs of the beehive. The nectar turns into honey through the decrease of the water content from initially 70 to 75 % to a maximum of 20 %.

Honey is a saturated solution of various carbohydrates in water. The carbohydrates account for 80 to 85 % of the weight, while the water content varies between 16 and 20 %. Further plant and bee specific compounds in honey are enzymes, vitamins, flavour and colour substances, waxes, acids, proteins and minerals. Honey consists of about 200 different substances (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2008).

The exact composition depends mainly on the origin of the basic material of the honey. Since honey represents a natural product neither the addition of any other substances, nor the removal of substances characteristically contained in honey is allowed (Bundesministerium für Gesundheit und Frauen 2004).

In the last three years the annual Austrian honey production amounted to 6,000 tons on average. In recent years the trend of honey consumption in Austria has been slightly degressive. In 2002/2003 the per-capita consumption accounted for 1.5 kg/year, whereas the average consumption of 2007/2008 was 1.2 kg/year. About 60 % of the Austrian consumption of honey is covered by domestic production (Statistik Austria 2009).

2.4.2 Relevance of Honey in Human Nutrition

Honey represents a source of rapidly available energy due to its high content of simple carbohydrates (about 80 %). 100 g of honey supply approximately 1360 kJ (320 kcal) (Bechthold 2009a). Honey only contains small amounts of mineral nutrients and vitamins and makes no considerable contribution to the coverage of nutrient requirements (Bogdanov 2009a). If one considers the moderate annual per capita consumption of honey in Austria the relevance of honey in the average Austrian nutrition is small.

However, some experts attribute positive health effects to honey due to the presence of flavonoids. The total content of flavonoids in honey varies from 5 to 20 mg/kg and includes substances from the groups of flavonols, flavanols and flavonones (Frank et al. 2007). All of these flavonoids are known for their antioxidative effects, some of them also for antibacterial, anticancerogen and heart protective actions (Frank et al. 2007). The substitution of sugar with honey might therefore possibly provide a higher level of antioxidative capacity within human nutrition. One of the few nutritional studies with focus on honey in human nutrition was carried out in Austria in 2007. In this study Frank et al. (2007) investigated the effects of an additional daily intake of 50 grams of honey during eight weeks on different health parameters of 50 test persons. The results of this investigation showed a significant reduction of the test persons' exposure to free radicals and positive effects on the immune system. Further improvements were reported for sleeping behaviour, digestion, muscular cramps and the frequency of headaches. No positive effects were reported for the blood parameters cholesterol, triglycerides, uric acids and ferritin (Frank et al. 2007). Nevertheless, an additional intake of honey or the partial replacement of a balanced diet through honey is not advisable (Bechthold 2009a). However, the replacement of commercial sugar with honey might lead to positive effects since honey, compared to cane or beet sugar, contains a wide range of different substances.

2.4.3 Pesticide Residues in Honey

Among consumers honey is considered to be a natural, healthy and clean product of a particularly high quality (Bogdanov 2006b). Therefore, the expectations of the consumers regarding the pureness of honey are very high. As a consequence of this consumer attitude honey is subject to strict quality regulations and regular laboratory analysis (Wallner and Potyka 2006).

The contamination sources of honey can basically be divided into two main groups: apicultural and environmental. Apicultural contamination of honey is predominantly the result of the application of specific substances in the beehive in the fight against the varroa mite (acaricides) or different bee diseases such as the foul brood (antibiotics). Environmental contamination of honey includes pesticides, other organic pollutants, heavy metals, radioactivity, organic compounds, pathogenic bacteria and pollen from genetically modified plants (Bogdanov 2006b).

Pesticide residues can be present in honey if honeybees come into contact with pesticides and transport them into the beehive in the form of contaminated nectar, pollen, water or in the pelage of their body. Concerning the transfer of pesticides into honey honeybees act as natural filters (Bogdanov 2006b). If foraging honeybees encounter highly bee-toxic pesticides they die before they can return to the beehive. Consequently, highly bee-toxic pesticides are neither transported into the beehive nor transferred into the honey. Honeybees can come into contact with moderate or non-bee toxic pesticides in larger quantities without harmful consequences. Therefore such pesticides can reach the beehive in significant amounts and be transferred into the honey in detectable quantities. In addition to this filtering effect of honeybees, the amount of lipophilic pesticides often decreases significantly during the transformation of nectar into honey (Wallner 2009a). In contrast the amount of hydrophilic pesticides substances shows no such decrease (Wallner 2009a). Through the decrease of the water content during the transformation of nectar into honey one might even expect the accumulation of such residues in the honey.

Various analyses in Europe showed a generally low level of pesticide residues in honey (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2008). For example, in a recent analysis of 500 pesticides in 64 honey samples in Germany 82 % of the samples contained no quantifiable residues. The positive samples contained residues only in traces (Chemisches und Veterinäruntersuchungsamt Stuttgart 2008b). According to Wallner and Potyka (2006) honey is one of the foodstuffs with the lowest level of environmental residues. In a review of contaminations of bee products Bogdanov concluded that there are no toxicological safety problems for consumers from pesticide residues in honey (Bogdanov 2006b).

Even though the overall levels of pesticide residues in honey are very low in Europe, already the detection of small amounts of residues can impair the good reputation of honey due to the very high consumer expectation and sensitivity towards such contaminations. Additionally the presence of pesticides in foodstuffs always raises the question whether the residues might pose a health risk for the consumer. In order to protect consumers from possible negative effects of pesticides in food the residues are not allowed to exceed certain maximum residue limits (MRL). In the European Union specific MRLs are defined for distinct pesticides in individual commodities (EU Pesticides Database). For some pesticides not only the original substance but also the metabolites have to be taken into account when checking for compliance with a MRL. The substances that have to be considered are encompassed in the so-called residue definition. For all pesticides without specific MRLs a general MRL was set at 0.01 mg/kg.

Table 3 displays the current MRLs for neonicotinoid insecticides in honey in the European Union. Specific MRLs for neonicotinoids in honey are defined for clothianidin, flonicamid, imidacloprid, thiacloprid and thiamethoxam. For acetamiprid, dinotefuran and nitenpyram no specific MRLs are listed for honey.

Table 3: Current neonicotinoid residue definitions and maximum residue limits in honey in the European Union (EU Pesticides database)

Substance (Residue definition)	Maximum residue limit in honey [mg/kg]
Acetamiprid	0.01*
Clothianidin	0.01
Dinotefuran	0.01*
Flonicamid (sum of flonicamid +TFNA-AM)	0.05
Imidacloprid	0.05
Nitenpyram	0.01*
Thiacloprid	0.2
Thiamethoxam (sum of thiamethoxam + clothianidin)	0.01

* indicates the absence of a specific MRL for the substance in honey

The residue definitions of flonicamid and thiamethoxam in honey include more than one substance. The residue definition for flonicamid comprises also the metabolite TFNA-AM (4-trifluoromethylnicotinamide). The residue definition for thiamethoxam includes also the metabolite clothianidin. Since clothianidin is at the same time a thiamethoxam metabolite and a distinctly applied neonicotinoid insecticide, the residue definition for thiamethoxam can lead to judgement problems.

Only few data are available about the presence of neonicotinoid residues in honey. Analyses of pesticide residues within honey monitoring programs are usually performed using multi-residue methods that include a large number of pesticides. The small number of publications that include the analysis of neonicotinoids in honey may indicate an absence of neonicotinoids in these multi-residue methods. The fact that neonicotinoids represent a relatively new group of pesticides and differ from many other pesticide classes in terms of their chemical properties would explain the absence of neonicotinoids in multi-residue methods. However, some investigations of several neonicotinoid insecticides in honey were conducted in Germany in 2008. In May 2008 the CVUA Stuttgart analysed 24 honey samples from Southern Germany with focus

on the following neonicotinoid insecticides: acetamiprid, clothianidin, imidacloprid, nitenpyram, thiacloprid and thiamethoxam. In 75 % of the samples, thiacloprid was detected in concentrations ranging from 2 to 110 µg/kg. Traces of thiamethoxam (1 µg/kg) were found in one honey sample while no other neonicotinoids insecticides were detected in the samples (Chemisches und Veterinäruntersuchungsamt Stuttgart 2008a). In June to August 2008 the CVUA Stuttgart analysed another 67 honey samples for pesticide residues. Only 18 % of them contained pesticide residues. Thiacloprid was found in four honey samples at concentrations of 7 to 45 µg/kg (Chemisches und Veterinäruntersuchungsamt Stuttgart 2008b). Further, the content of clothianidin was analysed in 65 honey samples from areas affected by honeybee colony losses in Southern Germany. In seven samples clothianidin was detected in the range of 1.1 to 2.3 µg/kg. The found concentrations were very low and represented no danger for the consumer (Ministerium für Ernährung und ländlichen Raum Baden-Württemberg 2008). In all mentioned analyses of neonicotinoid residues none of the samples exceeded the MRL. Nevertheless, the detection of neonicotinoids in some honey samples indicates the usefulness of the inclusion of neonicotinoid insecticides in multi-residue methods and monitoring programs.

3 Analysis of Guttation Liquid

3.1 Introduction

The topic of neonicotinoid insecticides in guttation liquid has only very recently received significant attention. Detailed studies were reported by several institutions in 2009 (see section 2.3.1.2). As little information was available so far, some investigations with the focus on neonicotinoids in guttation liquid were performed in the framework of this thesis in the Austrian Agency for Health and Food Safety in spring 2009. The investigations were carried out using maize and rape plants since in Austria the seeds of these agricultural crops in Austria are dressed with neonicotinoids to a large extent. The target of the performed investigations was the answering of the following questions:

- To what extent do cultivated maize and rape plants excrete guttation liquid?
- Do neonicotinoids pass into the guttation liquid?
- If so, what concentrations of neonicotinoids can be found in the droplets and how do these change over time?
- Are there differences between guttation liquid from indoor (controlled parameters) and outdoor (natural conditions) plants?

For this purpose the investigations included the cultivation of different neonicotinoid-dressed and untreated maize and rape seeds under indoor and outdoor conditions, the observation of their guttation activity, the collection of guttation liquid and the analytical determination of the concentrations of the neonicotinoid insecticides in the samples.

3.2 Experimental

3.2.1 Chemicals

The chemicals employed in the studies are listed in Table 4.

Table 4: Chemicals used for the analyses of the guttation liquid

Substance	Supplier	Purity
Acetonitrile	Merck, Germany	HPLC grade
Methanol	LGC Standards, Germany	HPLC grade
Deionized water	In-house water purification system (Millipore, USA)	100 %
Ammonium formate	Sigma Aldrich, Germany	≥ 99 %
Clothianidin	Bayer, Germany	99.5 %
Thiazolylmethylurea	Bayer, Germany	98.3 %
Thiazolylnitroguanidine	Bayer, Germany	98.6 %
Imidacloprid	Dr. Ehrenstorfer, Germany	99.5 %
Thiamethoxam	Riedel de Haën, Germany	99.4 %

3.2.2 Samples

The treated and untreated grains of four types of maize seeds and one type of rape seeds that were employed in the investigations are listed in Table 5.

Table 5: Maize and rape seeds used for the cultivation experiments. Neonicotinoid-containing dressings are marked in orange and the application rates of the neonicotinoids in the seed dressings are given in parentheses.

Seeds	Dressings	Active substances and application rates of neonicotinoids
Maize indoor		
Amato DKC 5143	*Poncho*	*Clothianidin (0.5 mg/seed)*
	Maxim XL	Fludioxonil, Metalaxyl M
	Morkit	Anthrachinon
	Flowsan FS	Thiram
Masetto	*Cruiser 350*	*Thiamethoxam (0.63 mg/seed)*
	Maxim XL	Fludioxonil, Metalaxyl M
PR 39H84	*Gaucho 600 FS*	*Imidacloprid (1.08 mg/seed)*
	Maxim XL	Fludioxonil, Metalaxyl M
Maize outdoor		
Arido	*Poncho*	*Clothianidin (0.5 mg/seed)*
Rape indoor		
Castille	*Chinook*	*Imidacloprid (200.2 mg/100g seed);* β-Cyfluthrin
	Flowsan FS	Thiram

The seeds for the indoor cultivation experiments were obtained from the Institute for Seed of the Austrian Agency for Health and Food Safety. All neonicotinoid-containing dressings that were used in the indoor and outdoor cultivation experiments were registered in the Austrian plant protection product register in 2009.

3.2.3 Cultivation

The cultivation of the indoor maize plants was carried out by the Centre for Agricultural Experimentation in Vienna in a closed greenhouse.

The maize seeds were planted in round Goettinger pots (10 cm diameter, 360 ml, 1 seed/pot) using a soil mixture of 1/3 silica sand and 2/3 torboton 2. Torboton 2 is a universal substrate for pot planting consisting of 96 Vol% highmoor turf and clay granulate material (Gartenhilfe Grünsiedl GesmbH, Austria). The pots of every plant group (different seed dressings and treated and untreated plants) were placed in separate storage racks and distributed evenly across a sliding table. The plants were watered manually once a day using a spray gun. The environmental conditions maintained in the greenhouse during the entire period of cultivation and sample collection are given in Table 6.

Table 6: Environmental conditions in the greenhouse

Parameter	Day-time	Night-time
Temperature	25 °C	18 °C
Relative humidity	65 %	35 %
Day-Night rhythm	08.30 - 04.00	04.00 - 08.30

The cultivation of the clothianidin-treated maize seeds on the field was carried out in Vienna at the end of April 2009 in the framework of a project by the Institute of Plant Health. The seedlings were grown in lines at intervals of 20 cm.

3.2.4 Collection

The collection of guttation liquid took place in the early morning between 6 and 7 am. For the collection of the guttation droplets a simple construction consisting of a glass capillary tube and a plastic Pasteur pipette was used (see Figure 8). The sample liquid of the individual plant groups was directly transferred into plastic vials from Eppendorf. Depending on the cultivation and experiment the collection of sample material was carried out on seven, nine or fifteen sequenced days. Immediately after collection the vials were stored in the freezer at -18 °C until analysis.

Figure 8: Collection of guttation liquid of indoor-grown maize plants

3.2.5 Analysis of Guttation Liquid by LC-MS/MS

The guttation liquid samples were diluted with methanol and analysed using LC-MS/MS. If the concentration of the investigated analytes exceeded the upper limit of calibration, the sample was re-diluted and analysed again. Samples with concentrations below the lower limit of calibration were analysed in a more concentrated form. Control samples collected from maize plants grown from untreated seeds and samples from maize plants grown from thiamethoxam-treated seeds were measured without previous dilution or injector program (dilution factor 1). Standard solutions contained clothianidin, imidacloprid and thiamethoxam and covered a calibration range of 5 to 100 µg/l.

Additional analysis included two metabolites of clothianidin: thiazolylmethylurea (TZMU) and thiazolylnitroguanidine (TZNG). Defined portions of all samples collected on a specific day from the first cultivation of indoor-grown maize plants were pooled. The analyses were performed after appropriate dilution employing

standard solutions containing clothianidin, TZMU and TZNG over a range of 2 to 100 µg/l.

The LC-MS/MS analyses of guttation liquid samples were carried out using an Agilent 1100 HPLC coupled to an Applied Biosystems API 2000 triple-quadrupole mass spectrometer. During the injection of the sample an injector program diluted the injected volume of 5 µl 1:5 (v/v) with water. This additional dilution is already included in the stated dilution factors. Chromatography was performed using a Synergi Fusion column (50 x 2 mm, 5 µm particle size). Mobile phase A consisted of an 80/20 (v/v) mixture of water/acetonitrile with 5 mmol/l ammonium formate and mobile phase B of a 10/90 mixture of water/acetonitrile with 5 mmol/l ammonium formate. The employed gradient is given in Table 7.

Table 7: Chromatographic gradient of the guttation liquid analyses

Time [min]	Mobile phase A [%]	Mobile phase B [%]
0.0	100	0
11.00	0	100
23.00	0	100
25.00	100	0
40.00	100	0

The flow rate was 200 µl/min and the temperature of the column was held at 20 °C.

Mass spectrometric detection was performed in multiple reaction monitoring (MRM) mode using electrospray ionization in positive ion mode. The first transition (MRM 1) was used as quantifier, the second transition (MRM 2) as qualifier. The source temperature of the mass spectrometer was 400 °C and the dwell time for the analytes 100 msec. The gas flows were set as follows: ion source gas 1 (GS1) 30, ion source gas 2 (GS2) 70, curtain gas (CUR) 30 and collision gas (CAD) 5. Further experimental parameters for the individual analytes are given in Table 8.

Table 8: MS parameters for both MRM transitions of all analytes

Substance	Q1[1]	Q3[2]	DP[3]	EP[4]	CEP[5]	CE[6]	CXP[7]
Clothianidin	250	132	31	5	14.77	19	4
	250	169	74	10	14.77	19	8
Clothianidin metabolite TZMU	206	175	31	10	13.67	27	22
	206	132	31	10	13.67	23	16
Clothianidin metabolite TZNG	236	132	26	8.5	14.42	17	12
	236	155	26	8.5	14.42	19	10
Imidacloprid	256	209	51	9	14.92	21	10
	256	175	49	9	14.93	25	8
Thiamethoxam	292	211	21	10	15.82	17	6
	292	181	54	8.5	15.82	31	10

[1] m/z precursor ion [2] m/z product ion [3] declustering potential [4] entrance potential [5] cell entrance potential [6] collision energy [7] cell exit potential

3.2.6 Analysis of Neonicotinoid-treated Seeds

The analysis of neonicotinoid-treated seeds was performed on a qualitative basis. The sample preparation of the seed samples was carried out as follows:

For every sample 100 grains (dug out seeds: 10 grains) were put into a flask and weighed. After the addition of 100 ml (dug out seeds: 10 ml) of a 1/1 (v/v) mixture of acetonitrile and water (with 0.1 % acetic acid), the flask was placed in an ultrasonic bath for 30 minutes. A defined portion of the supernatant solution was then filtered into a vial using a 0.45 µm membrane filter (Schleicher & Schuell Micro Science, Germany).

The analysis of the samples was performed using a HP 1090 HPLC equipped with a DAD detector. A RP-18e LIChrospher 100 column (250 mm x 4 mm, 5 µm particle size) was used and isocratic elution was performed with a 60/40 (v/v) mixture of water containing 0.1 % acetic acid and acetonitrile at 1 ml/min. The injection volume was 25 µl, the oven temperature 40 °C and the selected wavelength 270 nm. Mixed standard solutions at two concentration levels (5 mg/l and 50 mg/l in a 1:1 mixture of acetonitrile/water (v/v)) containing all three analytes were used for identification.

3.3 Results and Discussion

3.3.1 Cultivation, Guttation Liquid Sampling and Analysis

The trials with seeds treated with different neonicotinoid insecticides were conducted from April to June 2009 at the Austrian Agency for Health and Food Safety in Vienna. The experimental design consisted of two indoor cultivation experiments of treated and untreated maize plants and one indoor cultivation experiment of rape plants. In addition, the outdoor cultivation of clothianidin-treated maize plants within the framework of another project provided the opportunity to collect and analyse guttation liquid of field-grown maize plants. The following table gives an overview of the kind, number and treatments of the cultivated plants.

Table 9: Overview and details of indoor and outdoor cultivation experiments

Experiment	Plants	Neonicotinoid Insecticide	Number of treated plants	Number of control plants (untreated)
1st indoor cultivation experiment	maize	clothianidin	100	100
	rape	imidacloprid	100	100
2nd indoor cultivation experiment	maize	clothianidin	50	50
	maize	imidacloprid	60	20
	maize	thiamethoxam	60	20
Outdoor cultivation experiment	maize	clothianidin	> 100	9

For the optimal collection of the guttation liquid different instruments were tested on a houseplant. The leaves of the plant were sprayed with water for the simulation of guttation droplets. The collection trials were carried out using glass and plastic pipettes, capillary tubes in different sizes connected to a small air pump and a Hamilton syringe. Finally, a simple construction consisting of a capillary tube and a Pasteur micropipette (see Figure 9) proved to be most suitable for the collection of the guttation liquid (see Figure 10).

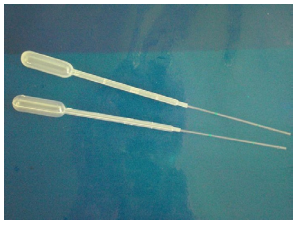

Figure 9: Instrument of choice for the collection of guttation liquid

With regards to LC-MS/MS analysis, guttation liquid represents a very clean matrix, so that no special sample preparation had to be undertaken. It was only necessary to dilute the samples appropriately depending on the actual concentrations of the analyte in order to obtain measurement samples with concentrations that were within the calibration range. Dilution factors for all guttation liquid samples ranged from 1 to 5000.

Figure 10: Collection of guttation liquid on the field

The results for each investigated analyte are reported and discussed in sections 3.3.2 to 3.3.4.

3.3.1.1 Indoor Cultivation

Figure 11: Maize and rape plants of the 1st indoor cultivation

Indoor cultivation (Figure 11 and Figure 12) of experimental plants has the advantage of providing the possibility to control environmental parameters in order to create identical conditions during the whole period of investigation. The chosen day-night rhythm (day-time: 08.30 - 04.00, night-time: 04.00 - 08.30) with a lowering of the relative humidity setting from 65 % to 35 % between 04.00 and 08.30 a.m. averted a possible dilution of the guttation droplets through the water of the vaporiser prior to the collection between 6 and 7 a.m. Environmental data logging showed that the actual value of relative humidity during the night-time was above 35 %, thus confirming that the vaporiser did not come into action during that period.

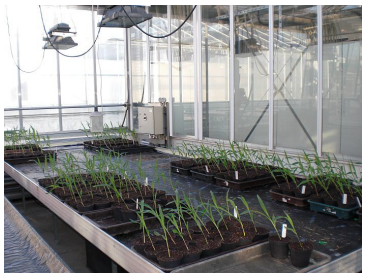

Figure 12: Maize plants of the 2nd indoor cultivation

The even distribution of the separate racks with the pots on the sliding table prevented the contamination of the plant groups among each other and between treated and untreated plants during watering and sample collection (see Figure 11 and Figure 12).

In the indoor cultivation experiments the maize plants showed high guttation activity in their early stages of growth. Guttation liquid of indoor-grown maize plants was exuded until the tenth day after emergence. Beginning the collection on the first day after emergence, guttation liquid could be sampled over a period of nine days. These findings are in accordance with a report of Bayer CropScience (Nikolakis 2009). Girolami et al. (2009) even reported guttation activity during three weeks for indoor maize plants. On the contrary, the rape plants did not show any formation of guttation droplets. As a consequence it was not possible to collect and analyse samples of rape plants. Therefore, further investigations were restricted to maize plants exclusively.

An interesting observation during the first indoor cultivation experiment concerned the exact positions of the guttation droplets on the leaves. In agreement with botanical textbooks droplets were predominantly found at the tips and the edges of the leaves. The leaves of young maize plants formed a kind of cone at the junction of leaves and stipe. Due to the concave shape of the surfaces of the leaves, guttation droplets could roll off along the leaf axis and gather in the cones.

In the first indoor cultivation experiment the collected samples included guttation liquid from all positions: tips, edges and cones. The LC-MS/MS analyses of the guttation liquid samples of the maize plants grown from clothianidin-treated seeds showed clothianidin to be present in considerable quantities (see below). This finding raised the question concerning the concentrations of the active substance in the guttation liquid from the different positions of the plant. To evaluate whether differences existed between the concentrations of clothianidin in the guttation liquid from the three positions of the plants, maize plants were cultivated from clothianidin-treated seeds again in a second experiment for separate collection of guttation liquid from these different positions.

Further, the detection of clothianidin in the guttation liquid samples of the first cultivation experiment led to the question, whether other neonicotinoid insecticides are translocated into the guttation liquid as well and if to what extent. To answer these questions maize seeds treated with imidacloprid and thiamethoxam were also cultivated in the second experiment.

3.3.1.2 Outdoor Cultivation

In the trial with outdoor cultivation it was of particular interest whether field-grown maize plants actually exude guttation liquid at all and if to what extent. A further question was if there are differences in the translocation of neonicotinoids into the guttation liquid of field-grown plants compared to indoor-grown maize plants.

Figure 13: Growing maize plants on an open field

In contrast to the indoor-grown maize plants samples from field-grown plants provided valuable information on the exudation of guttation liquid and its content of active substance under natural circumstances with varying environmental conditions. Moreover, the investigations of the guttation liquid of maize plants on the field pictured the real situation encountered by honeybees.

On the field some hundred maize plants cultivated from clothianidin-treated seeds were disposable for sample collection. Nine of the cultivated plants were grown from untreated seeds and used for the collection of control samples. Three samples of guttation liquid from maize plants cultivated from dressed seeds and one control sample of guttation liquid grown from untreated seeds was collected on each collection day. In contrast to indoor plants, field-grown maize plants showed guttation activity during more than two weeks, so the collection took place over 15 days. Girolami et al. (2009) even reported

guttation activity during three weeks for both indoor and outdoor plants. During the collection it was noted that maize plants on the field seemed to exude larger volumes of guttation liquid compared to indoor maize plants.

Figure 14: Fly drinking from a guttation droplet

During the sampling of the guttation liquid it was regularly observed that different insects (lady bugs, flies) used the guttation droplets on the leaves and in the cones of the plants as a water source (see Figure 14 and Figure 15).

On four collection days dead insects such as lady bugs, mosquitoes and flies were found next to the maize plants or even in the cones of the maize plants. These observations may indicate a possibly negative impact of the clothianidin-treated maize plants and their guttation liquid on insects in general. However, these observations should not be overstated as the exact cause for the death of these insects is not known. Table 10 shows details concerning the sampling, weather conditions and observations during the collection of guttation liquid on the field.

Figure 15: Lady bug drinking water from the cone of a maize plant

In the context of outdoor cultivation it is important that guttation droplets are not confused with dew water. Dew droplets on the plant surface are the result of condensation of atmospheric moisture and predominantly appear during the night hours after a warm day (Shawki et al. 2006). Observations on the field showed that dew and guttation droplets can be differentiated in practice on the basis of the volume of the droplets by comparing them with those on the leaves of surrounding plants without guttation activity. Guttation droplets are substantially larger than dew droplets (see also Figure 5).

Table 10: Overview of time of sampling, weather conditions and observations on the different collection days

Collection Day	Date and Time of Collection	Weather Conditions	Observations
1	12.05.2009, 7.00 h	12°C, no dew, no rain, no wind	
2	13.05.2009, 7.00 h	12°C, no dew, no rain, moderate wind	Drinking fly (inactive behaviour)
3	14.05.2009, 7.00 h	10°C, no dew, no rain, moderate wind	drinking fly and bug, dead lady bug in the cone of a plant
4	15.05.2009, no collection	10°C, continuous rainfall	no collection possible
5	16.05.2009, 8.00 h	12°C, no dew, no rain, wind	
6	17.05.2009, 8.00 h	12°C, little dew, no rain, no wind	
7	18.05.2009, 7.00 h	15°C, a lot of dew, no rain, moderate wind	dead mosquito in the cone of a plant
8	19.05.2009, 7.00 h	14°C, no dew, rain during night, wind	dead insect in the cone of a plant
9	20.05.2009, 7.00 h	15°C, very little dew, no rain, no wind	drinking lady bug in the cone of a plant
10	21.05.2009, 7.00 h	14°C, a lot of dew, no rain, no wind	
11	22.05.2009, no collection	14°C, heavy wind	no collection possible
12	23.05.2009, 7.00 h	14°C, no dew, no rain, wind	only very few guttation droplets
13	24.05.2009, no collection	12°C, rainfall	no collection possible
14	25.05.2009, 7.00 h	19°C, no dew, no rain, no wind	dead mosquitoes in the cone of a plant, dead lady bug next to a plant
15	26.05.2009, 7.00 h	20°C, very little dew, no rain, heavy wind	only very few guttation droplets

3.3.2 Clothianidin

3.3.2.1 Indoor-grown Maize Plants

Two cultivation experiments of indoor-grown maize plants grown from clothianidin-treated seeds were performed. The first experiment consisted of 100 plants grown from treated seeds and 100 plants grown from untreated seeds. The plants grown from treated and untreated seeds were each divided into five groups of 20 plants. The collection of the guttation droplets started on the third day after emergence and took place on seven sequenced days.

The analyses of the guttation liquid samples of the first experiment showed the presence of clothianidin in substantial quantities. Average contents of clothianidin from the five plant groups on the seven collection days are illustrated in Figure 16. It can be seen that the concentration of clothianidin decreased over time. A maximum value of 103.8 mg/l was found on the second day of collection.

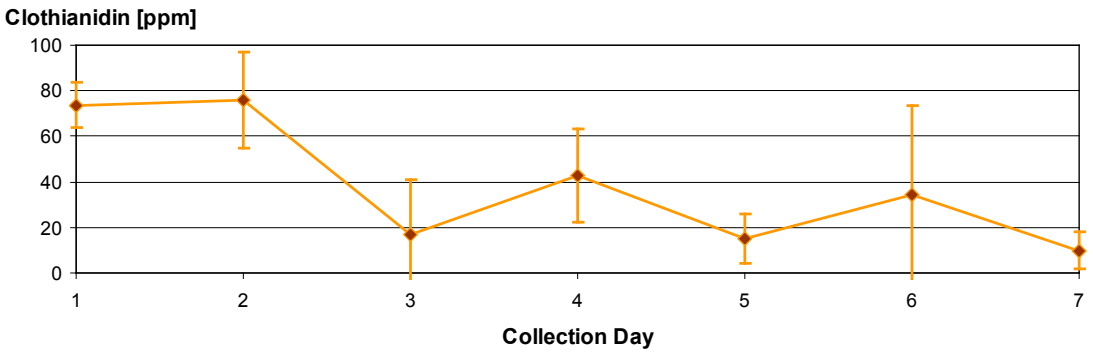

Figure 16: Average concentrations of clothianidin in guttation liquid of the maize plants from the first indoor cultivation. Error bars give standard deviations of the collected samples (n=5).

There are several possible reasons for the progressive decrease of the concentration of clothianidin in the guttation liquid. On the one hand, the total amount of the active substance in the inside of the plant may become smaller due to natural degradation. On the other hand, less substance may be taken up by the plant since the growing roots reach further soil layers where there is no active substance of the seed coating available.

The amount of guttation liquid of each plant group was determined on every day of collection. Observations during the whole collection period showed a large variability in the amount of guttation liquid exuded by the individual plants. In contrast to the concentration of clothianidin the average amount of exuded guttation liquid showed no clear trend over time. Thus, no clear correlation between the concentration of clothianidin and the exuded amount of guttation liquid was observed.

In the guttation liquid of the control plants no clothianidin could be detected, thus proving that the experimental setup was devoid of any cross-contamination.

The determined concentrations of clothianidin in the guttation liquid of indoor-grown maize plants ranged from 0.2 to 104 mg/l which is in good agreement with similar trials from Bayer CropScience; in those investigations concentrations of clothianidin between 5 and 133 mg/l were found (Nikolakis 2009).

A further target of investigation was to explore, whether two metabolites of clothianidin, TZMU and TZNG, would be detectable in the guttation liquid samples of plants grown from clothianidin-treated seeds. To obtain information on the content of TZMU and TZNG in the guttation liquid samples from the first indoor cultivation experiment were pooled for every collection day. Utilizing previously measured concentrations of clothianidin in these samples individual dilutions resulting in 100 µg/l clothianidin were prepared. However, with these dilutions the two metabolites were below the lower limit of the calibration range in all cases. Consequently, the pooled samples were injected in a more concentrated form (dilution factors ranging from 5 to 80 instead of 50 to 800). The measurement of TZMU and TZNG in these dilutions indicated the presence of the two metabolites in the guttation liquid. TZMU was found in the range of 0.14 to 0.83 % relative to the concentration of clothianidin. TZNG showed slightly higher values of 0.85 to 2.1 %. For both metabolites an increase of concentration over time could be observed. Generally it can be said that the metabolites of clothianidin are present in the guttation liquid of maize plants cultivated from seeds dressed with neonicotinoids, albeit at low concentrations compared to the parent compound.

3.3.2.2 Tips, Edges and Cones of the Maize Plants

For the investigation of possible differences in the concentration of clothianidin in guttation liquid from different locations on the plant, guttation droplets were

collected separately from tips, edges and cones of the leaves. In this experiment 50 plants grown from treated and 50 plants grown from untreated seeds were cultivated. Starting already on the first day after emergence the guttation liquid was collected over a period of nine days separately from three different positions of the plants (tip, edge and cone).

Figure 17 displays the concentrations of clothianidin in the guttation liquid samples from the different plant positions.

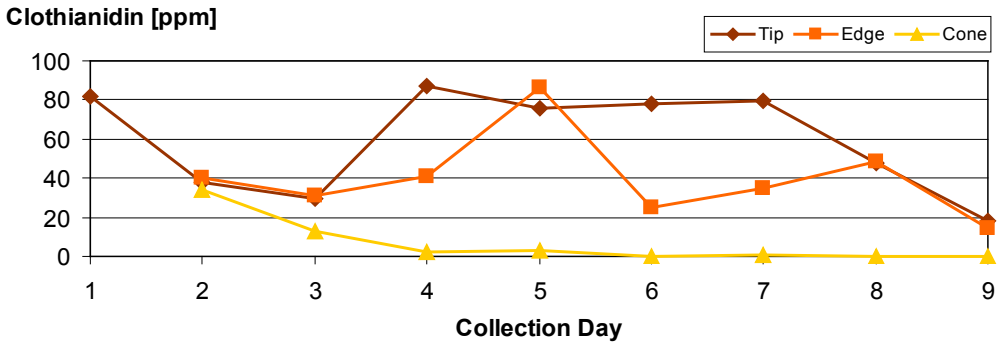

Figure 17: Comparison of the concentrations of clothianidin in the guttation liquid from tips, edges and cones of maize plants

It is clearly visible that the guttation droplets on the tips and edges of the leaves contained higher amounts of clothianidin than the guttation liquid from the cones. This might be due to the regular watering of the plants from above: the water remains in the cones during night, does not evaporate entirely and then becomes mixed with the guttation liquid in the early morning.

The content of clothianidin in the samples from the tips and edges of the leaves remained on a rather high level for several days. By contrast, the concentration of clothianidin in the guttation liquid from the cones of the leaves showed a progressive decrease at a generally much lower level.

3.3.2.3 Field-grown Maize Plants

The maize plants cultivated on the field exuded guttation liquid in considerable quantities, so that sample collection could take place over 15 sequenced days. It was observed that the maize plants on the field developed at a slower rate compared to the plants in the greenhouse. This might be due to the fact that the indoor plants had constant and ideal environmental conditions for growth and development, whereas the field-grown plants were exposed to natural circumstances and changing environmental conditions. This circumstance can be expected to have some considerable effects on the results of the investigations.

An overview of the average concentrations of clothianidin in the guttation liquid of all three samples over time indicated again a decrease of the concentrations of clothianidin during plant development (see Figure 18). The guttation liquid samples contained clothianidin in the range of 0 to 55 mg/l.

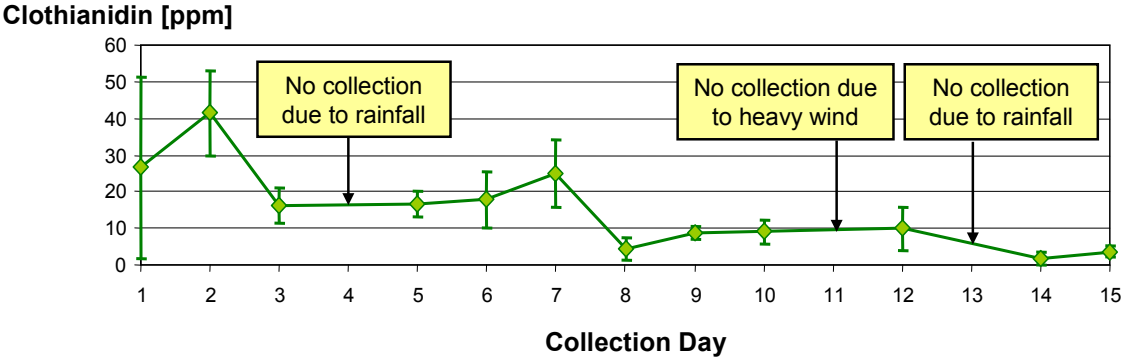

Figure 18: Average concentrations of clothianidin in the guttation liquid of field-grown maize plants. Error bars indicate standard deviations of the collected samples (n=3).

Even though the environmental parameters as determined by the weather conditions (temperature, humidity, sunshine, wind etc.) were not constant (see Table 10), the change in the concentration of clothianidin in the guttation liquid of field-grown plants over time showed a similar pattern compared to plants in the greenhouse. Whereas the concentrations of clothianidin in the guttation

liquid of field-grown maize plants were smaller than those of the maize plants in the greenhouse, they remained at a more or less constant level over a longer period of time. This correlates well with the slower development of the plants on the field.

In the measurements of guttation liquid samples of untreated maize plants no clothianidin could be detected, so that a cross-contamination can also be excluded for plants growing on the same field close to each other.

In a similar study Girolami et al. (2009) recently conducted trials with guttation liquid of field-grown maize plants that were cultivated from clothianidin-dressed seeds and detected an average of 23.3 mg/l of active substance in the samples over a period of three weeks. These findings are in good agreement with the results of the current investigations which ranged from 0 to 55 mg/l. The higher maxima of up to 100 mg/l found by Girolami et al. can, besides biological variations and differing environmental conditions, probably also be explained by the higher application rates of the dressing (1.25 mg per kernel vs. 0.5 mg per kernel in the present study).

3.3.3 Imidacloprid

The plants cultivated from seeds treated with imidacloprid were divided into three groups with 20 plants per group. In addition there was one control group with 20 plants grown from untreated seeds. The collection of the guttation liquid started on the first day after emergence over a period of nine sequenced days. High concentrations of imidacloprid were found in the guttation liquid samples of the seed-treated maize plants. Especially on the first four days of collection the guttation liquid contained large amounts of imidacloprid. One extremely high concentration (374 mg/l) of imidacloprid in plant group two on collection day four was an exceptional case and therefore treated as outlier and excluded from the results. Considerable amounts of imidacloprid in guttation liquid could only be measured for the first five days of collection. After that the level of imidacloprid dropped to almost zero (Figure 19).

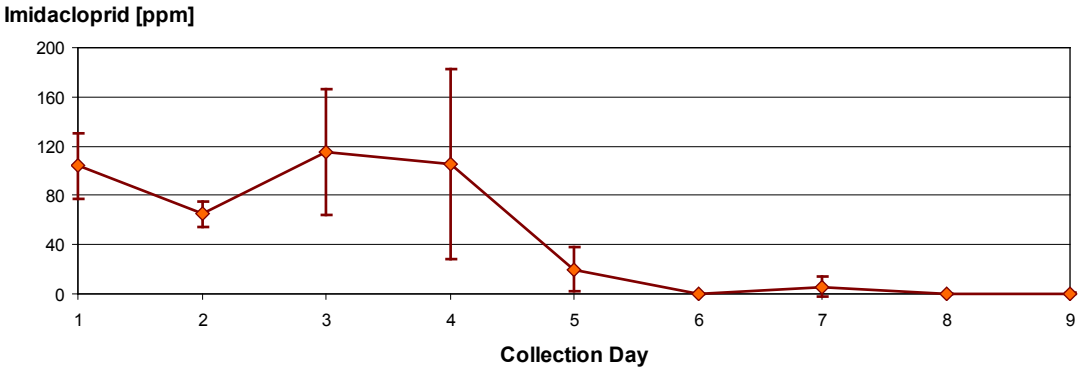

Figure 19: Average concentrations of imidacloprid in the guttation liquid of treated maize plants. Error bars give standard deviations of the collected samples (n=3).

Figure 19 clearly shows the same kind of trend over time regarding the concentration of neonicotinoid present in the guttation liquid as the results from maize plants grown from clothianidin-treated seeds.

The concentration of imidacloprid in the guttation liquid did not depend on the amount of exuded guttation liquid, since the amount of produced guttation liquid stayed on a more or less constant level over the whole period of collection (Figure 20).

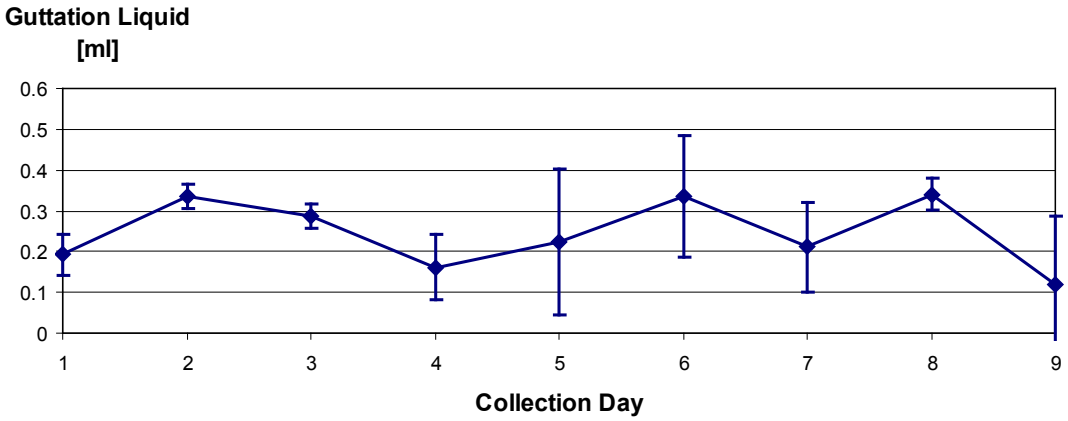

Figure 20: Amounts of guttation liquid produced by maize plants grown from imidacloprid-treated seeds. Error bars give standard deviations of all collected samples (n=3).

The findings of these investigations were in good agreement with the results of a similar study that was recently carried out by Girolami et al (2009). The trials of Girolami et al. were conducted with indoor maize plants grown from imidacloprid-treated seeds with an application rate of 0.5 mg per kernel. Their results showed average contents of 82.8 mg/l imidacloprid in the guttation liquid with a maximum value of more than 110 mg/l. The measured concentrations of imidacloprid in the current investigations ranged from 0 to 160 mg/l. The guttation liquid samples for these analyses were collected from maize plants from seeds treated with 1.08 mg imidacloprid per kernel. The difference in the application rates of imidacloprid in the seed dressings of the two investigations corresponds well with the measured concentrations of imidacloprid in the guttation liquid samples.

In the analyses of the guttation liquid samples that were collected from maize plants grown from imidacloprid-treated seeds not only imidacloprid, but also thiamethoxam was detectable (see 3.3.4).

3.3.4 Thiamethoxam

The plants treated with thiamethoxam were divided into three groups with 20 treated plants per group. In addition there was one control group with 20 plants grown from untreated seeds. Guttation liquid could be collected on nine sequenced days.

Astonishingly, no thiamethoxam was detectable in the guttation liquid of the treated maize plants. This finding raised the questions whether a translocation of thiamethoxam into the guttation liquid of the plants is possible at all. Another explanation for the results would be a deficiency in the seed dressing of the applied maize seeds. Some information concerning the translocation of thiamethoxam into guttation liquid could be gathered as, surprisingly, the guttation liquid of maize plants cultivated from imidacloprid-dressed seeds contained not only imidacloprid but also considerable amounts of thiamethoxam. The concentrations of thiamethoxam reached approximately one

tenth of the imidacloprid content. Figure 21 shows the concentration of thiamethoxam in guttation liquid samples of maize plants grown from imidacloprid-dressed seeds.

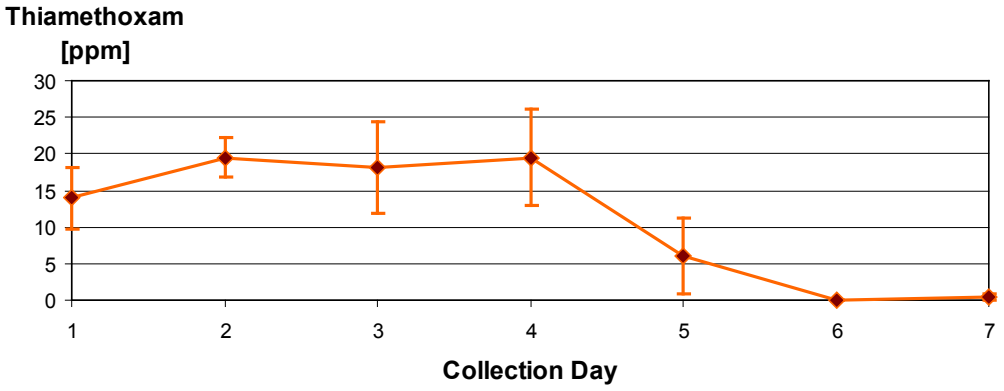

Figure 21: Concentrations of thiamethoxam in the guttation liquid samples of maize plants grown from imidacloprid-treated seeds. Error bars give standard deviations of all collected samples (n=3).

The detection of thiamethoxam in the guttation liquid indicates that thiamethoxam is distributed in the plant and can be transferred into the guttation liquid like the other investigated neonicotinoid insecticides.

As a consequence of these results the quality of the neonicotinoid-treated seeds that were utilized for the cultivation of the maize plants of the indoor cultivation experiments was investigated.

3.3.5 Neonicotinoid-treated Seeds

In order to enlighten the unexpected results of the analyses of guttation liquid from maize plants grown from seeds that should have been treated with imidacloprid or thiamethoxam (only) respectively, the seeds used for cultivation of the plants were analysed in an additional investigation.

The following samples of seeds were investigated:

- Three samples of original seeds of each treatment type (Amato DKC 5143-clothianidin, PR 39H84-imidacloprid and Masetto-thiamethoxam) (see Figure 22)
- One control sample of untreated seeds for every seed type (Amato DKC 5143, PR 39H84 and Masetto) (see Figure 22)
- One sample of dug out seeds per seed type (see Figure 23)

Figure 22: Treated and untreated seed samples

Figure 23: Sample consisting of dug out seeds

The chromatograms and spectra of standards, original seed samples, 1:10 diluted samples, dug out samples, control samples as well as a spiked sample in case of thiamethoxam were compared for every analyte.

Maize Seeds treated with Clothianidin:

On the maize seed which was declared to be treated with clothianidin, the compound was detected. Clothianidin was found in the dug out plant sample as well as in the seed samples. Spectra of clothianidin and standard samples showed high similarity. Consequently, it can be said that the treatment of clothianidin-treated maize seed was all right.

Maize Seeds treated with Imidacloprid:

On the maize seed which was declared to contain imidacloprid in the seed coat, imidacloprid was detected. However, beside imidacloprid the maize seed additionally contained thiamethoxam.

Figure 24 shows the overlapping chromatograms of a sample of imidacloprid-treated PR 39H84 seeds, a 1:10 dilution of this sample and a mixed standard containing clothianidin, imidacloprid and thiamethoxam (50 mg/l). The peaks of the seed samples indicating the presence of imidacloprid and thiamethoxam are clearly visible. The imidacloprid peak of the undiluted seed sample is split as a result of analyte overload.

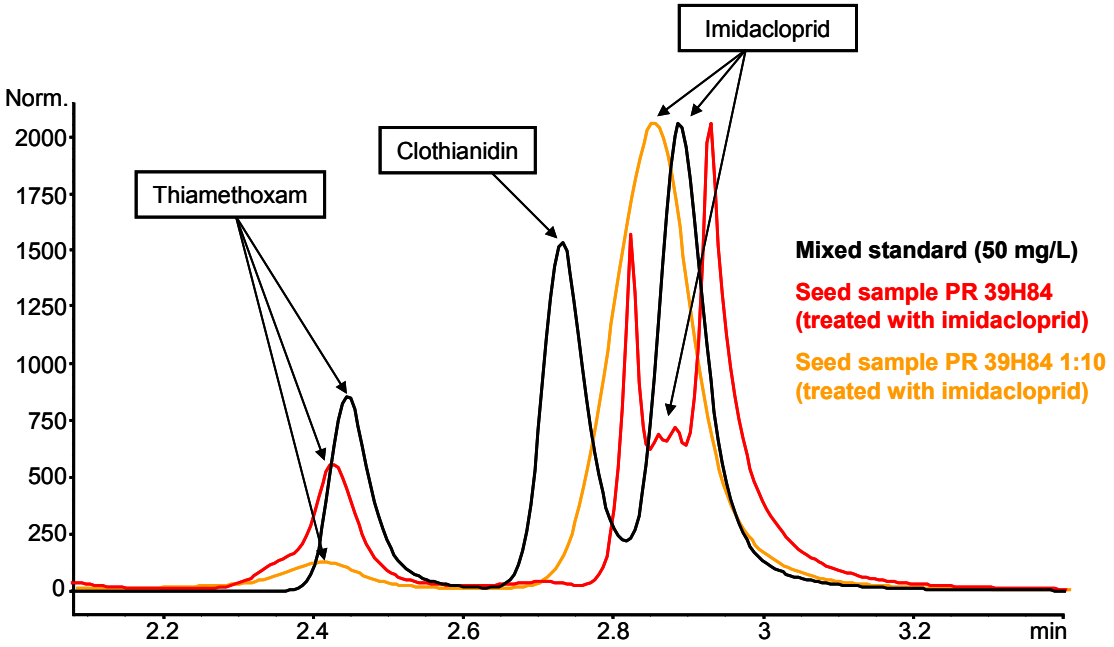

Figure 24: Overlapping chromatograms of mixed standard (50 mg/l), PR 39H84 seed sample (treated with imidacloprid) and 1:10 dilution of PR 39H84 seed sample (treated with imidacloprid) showing the presence of thiamethoxam in imidacloprid-dressed seeds

Supplementary to the identical retention times of standards and samples, comparable spectra delivered a further proof for the presence of thiamethoxam in the seeds (Figure 25).

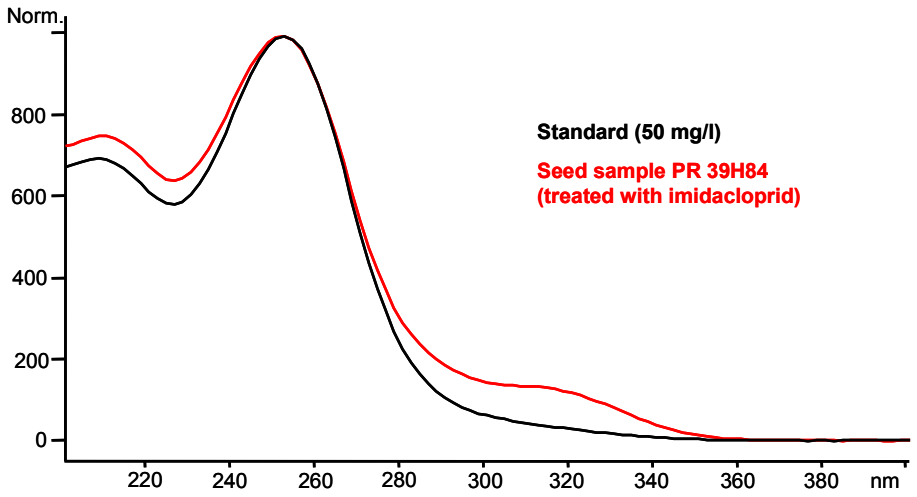

Figure 25: Overlapping spectra of thiamethoxam for standard and imidacloprid-treated PR 39H84 seed sample

Imidacloprid and thiamethoxam were also found in the dug out plant samples. Quantitatively, the concentration of thiamethoxam accounted for about one tenth of the concentration of imidacloprid. The presence of thiamethoxam in both the guttation liquid samples from plants grown from PR 39H84 seeds and the PR 39H84 seeds themselves indicates that there was no contamination during cultivation, guttation liquid collection or LC-MS/MS analysis but that the seed material was additionally treated with thiamethoxam in contrast to the manufacturer's statement. This result reveals a deficiency during the process of the seed treatment.

Maize Seeds treated with Thiamethoxam:

On the maize seed which was declared to be dressed with thiamethoxam the active substance could not be detected. Neither the dug out plant sample nor the seed samples showed a peak indicating the presence of thiamethoxam. The chromatograms of the control and the seed samples were more or less identical (Figure 26). In order to verify these results, a thiamethoxam-spiked seed sample (10 mg/l) was measured. In Figure 26 it is clearly visible that only the spiked sample contained thiamethoxam.

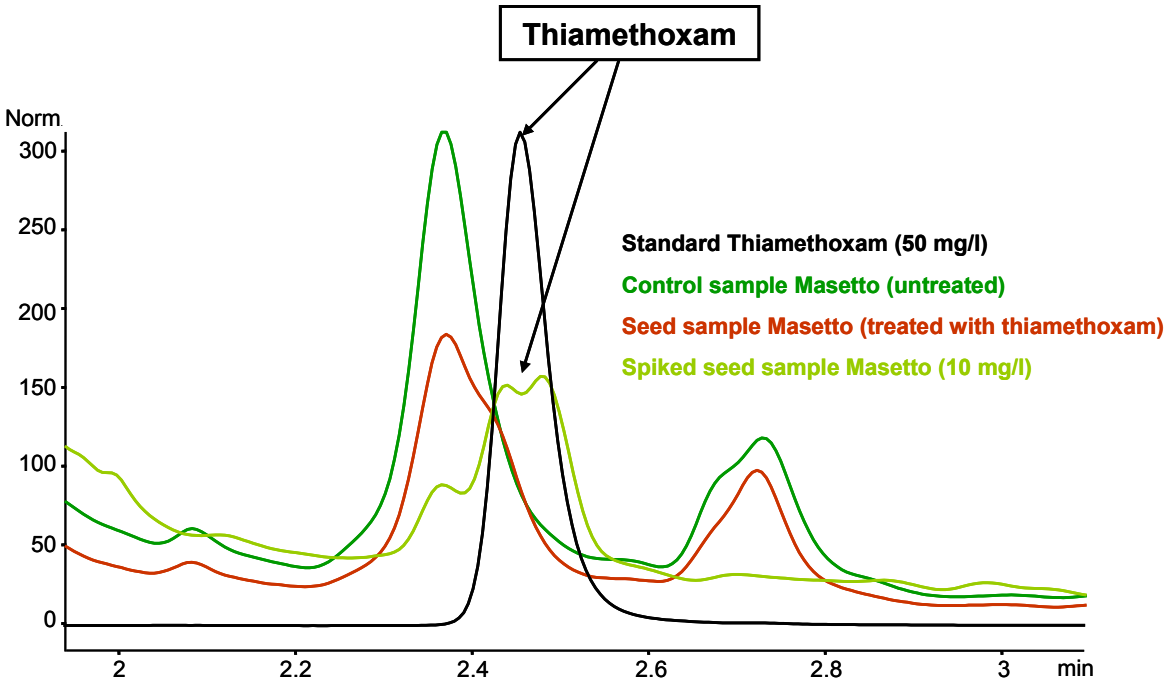

Figure 26: Overlapping chromatograms of thiamethoxam standard, control sample, seed sample and spiked seed sample showing the absence of thiamethoxam in the seed sample

Thus, the results of the seed quality tests confirmed the findings of neonicotinoids in the guttation liquid samples. Upon consultation with the seed producer, it was admitted that the concerned lots of seed were deficient.

4 Analysis of Honey and Nectar

4.1 Introduction

The high content of carbohydrates in honey and nectar poses a challenge for the analysis of trace substances such as pesticide residues in these matrices. Therefore, an optimal separation of the investigated residues from the honey or nectar matrix by suitable sample preparation combined with a robust and sensitive detection and quantification is obligatory for a successful analysis.

In recent years numerous publications have reported analytical methods for the analysis of different pesticide residues in honey. A review of chromatographic methods from Rial-Otero et al. (2007) provided an overview of the relevant approaches for the determination of pesticides in honey. The mainly employed techniques for the extraction of pesticides from honey include solvent extraction (SE), supercritical fluid extraction (SFE), solid-phase extraction (SPE), solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE). The chromatographic determination is usually conducted with GC (gas chromatography) or HPLC (high performance liquid chromatography). The choice of extraction, chromatographic method and detector depends on the chemical properties of the investigated pesticides. Chemically the most common pesticides examined in honey are organochlorines, organophosphorus pesticides and carbamates (Bogdanov 2006b).

In terms of neonicotinoid insecticides only a small number of published methods focused on the detection of neonicotinoid residues in honey (Fidente et al. 2005, Schöning and Schmuck 2003). Additionally, no more than four neonicotinoid insecticides were included in a single method.

A method for the simultaneous analysis of residues of acetamiprid, imidacloprid, thiacloprid and thiamethoxam in honey was published by Fidente et al. in 2005. Sample preparation included the blending of the honey samples with Milli-Q water and subsequent clean-up with Extrelut NT20 cartridges with dichlormethane as elution solvent. After evaporation of the eluate the residue

was redissolved in methanol. Analysis was carried out with LC-MS equipped with an electrospray source operating in the positive ionization mode. The limits of detection (LODs) for the investigated analytes were in the range of 10 to 100 µg/kg, the limits of quantitation (LOQs) ranged from 40 to 300 µg/kg (Fidente et al. 2005).

Schöning and Schmuck (2003) described a method for the determination of imidacloprid and the two plant metabolites 5-hydroxy-imidacloprid and olefin-imidacloprid in some honeybee-related matrices such as pollen, nectar, honey, wax and bees. Analytes were extracted with methanol/water followed by liquid liquid extraction (LLE) using ChemElut cartridges and cyclohexane/ethyl acetate as eluent. Analysis was carried out with LC-MS/MS in multiple reaction monitoring mode using positive electrospray ionization. For imidacloprid the LOD and LOQ were 1.5 and 5 µg/kg, respectively. An unpublished method for the determination of clothianidin and two of its metabolites, TZMU and TZNG, of Schöning employed a similar analytical procedure as the imidacloprid method (Schöning 2001).

Several publications dealt with the determination of neonicotinoid insecticides in other matrices such as fruits and vegetables (Di Muccio et al. 2006, Obana et al. 2003). These publications served as additional input for the present method development. Obana et al. (2003) and Di Muccio et al. (2006) developed and validated methods for the simultaneous analysis of five (acetamiprid, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) and four (acetamiprid, imidacloprid, thiacloprid and thiamethoxam) neonicotinoid pesticide residues, respectively, in fruit and vegetables. The method of Obana et al. included the extraction with methanol and clean-up by SPE using a graphitized carbon cartridge. Analysis was performed with LC-MS, using positive atmospheric pressure chemical ionization. Di Muccio et al. extracted the neonicotinoid residues with water-acetone followed by a liquid-liquid extraction with dichlormethane using Extrelut–NT20 cartridges. The neonicotinoids were then analysed by LC-ESI-MS. LC-MS was found to be sufficiently selective for the analysis due to the simplicity of the fruit and vegetable matrices.

Since no analytical method for the simultaneous determination of the entire group of neonicotinoid insecticides in honey has been published so far, the development and validation of such a method was the target of the present work.

4.2 Experimental

4.2.1 Chemicals

The tables below list the chemicals employed for sample preparation and LC-MS/MS analysis (Table 11) as well as the substances used for the preparation of the standard solutions (Table 12).

Table 11: Chemicals used for sample preparation and LC-MS/MS analysis

Substance	Supplier
Acetonitrile	Merck, Germany
Methanol	LGC Standards, Germany
Cyclohexane	LGC Standards, Germany
Ethyl acetate	LGC Standards, Germany
Deionized water	In-house water purification system Milli-Q Plus (Millipore, USA)
Formic acid	Riedel de Haën, Germany
Magnesium sulphate anhydrous coarsely grained	Sigma Aldrich, Germany
Sodium chloride	Merck, Germany
Disodium hydrogencitrate sesquihydrate	Merck, Germany
Trisodium citrate dihydrate	Merck, Germany
PSA 40µm	Supelco, USA
Celite	Merck, Germany

Table 12: Origin and purity of the substances used to prepare the standard solutions

Substance	Supplier	Purity [%]
Acetamiprid	Nippon Soda, Japan	100
Acetamiprid metabolite IM 2-1	Nippon Soda, Japan	99.7
Clothianidin	Bayer, Germany	99.5
Clothianidin metabolite TZMU	Bayer, Germany	98.3
Clothianidin metabolite TZNG	Bayer, Germany	98.6
Dinotefuran	Dr. Ehrenstorfer, Germany	98
Flonicamid	Dr. Ehrenstorfer, Germany	98.5
Flonicamid metabolite TFNA-AM	Fluorochem, UK	97
Nitenpyram	Dr. Ehrenstorfer, Germany	99
Imidacloprid	Dr. Ehrenstorfer, Germany	99.5
Thiacloprid	Dr. Ehrenstorfer, Germany	99.5
Thiamethoxam	Riedel de Haën, Germany	99.4

The substances used for the preparation of the standard solutions were stored in a freezer at -18 °C. Prefabricated stock solutions in acetonitrile of clothianidin-d_3 (0.312 g/l), TZMU-d_3 (1 g/l) and TZNG-$^{13}C^{15}N$ (1 g/l) were provided by Bayer CropScience.

4.2.2 Standard Solutions

Analyte Stock Solutions

Stock solutions of the 12 analytes (Table 12) with a concentration of 1 g/l were prepared in acetonitrile taking into account the purity of the standard substances. 5 to 10 mg were weighed accurately into a screw-cap glass tube and the approximate amount of acetonitrile was added gravimetrically taking into account the density of acetonitrile. The stock solutions were stored protected from light in a fridge at +6 °C.

Analyte Mixture Working Solutions

A 10 mg/l analyte mixture of the neonicotinoids and their metabolites (see Table 12) was prepared by mixing 100 µl of each stock solution in a volumetric flask and filling up to 10 ml with acetonitrile.

By 1:10 and 1:100 dilution of this 10 mg/l analyte mixture with acetonitrile, further working standard solutions of **1 mg/l** and **0.1 mg/l** were prepared.

Internal Standard Solutions

A 10 mg/l internal standard mixture of isotopically labelled forms of clothianidin and two of its metabolites was prepared by mixing 100 µl of the stock solutions of TZMU-d_3 and TZNG-$^{13}C^{15}N$ as well as 320.5 µl of the stock solution of clothianidin-d_3 in a volumetric flask and filling up to 10 ml with acetonitrile.

The 10 mg/l internal standard mixture was diluted 1:10 with acetonitrile for the preparation of a **1 mg/l** internal standard mixture working solution.

Solvent Standards

Solvent standards in methanol with concentrations of 2, 5, 10, 25, 50 and 100 µg/l were prepared using the 1 mg/l or 0.1 mg/l working standard mixture. All standards contained the internal standard substances at a level of 50 µg/l.

Matrix-matched Standards

Organic flower honey collected at an altitude above 2000 m was used as blank matrix for the preparation of matrix-matched standards. Blank honey was first prepared according to the QuEChERS sample preparation method (see 4.2.6) whereby no internal standard solution was added at the beginning. The appropriate amounts of internal standard mixture and analyte mixture working solutions for each standard level were added prior to the evaporation of the solvent. Matrix-matched standards were prepared at concentration levels of 2, 5, 10, 25, 50 and 100 µg/l with the internal standard substances at 50 µg/l.

4.2.3 Spiked Honey Samples

For the preparation of spiked honey samples that were used for the method validation and as quality control samples organic flower honey collected at an altitude above 2000 m was used as blank matrix. The spiking of honey samples was performed by adding a defined amount of the 10 mg/l analyte mixture working solution to the honey which was then stirred for 30 minutes under slight heating at a maximum temperature of 45 °C.

Spiked honey samples were prepared at three concentration levels:

10 µg/kg: 100 g honey + 100 µl of 10 mg/l working solution

50 µg/kg: 100 g honey + 500 µl of 10 mg/l working solution

100 µg/kg: 100 g honey + 1 ml of 10 mg/l working solution

1000 µg/kg: 25 g honey + 25 µl of 1g/l stock solution of each analyte

The spiked honey samples were stored in a laboratory fridge at +6 °C.

4.2.4 Austrian Honey and Nectar Samples

All investigated honey and nectar samples originated from beehives in Austria and were collected either in the scope of the Melissa project (Project title: Investigations of the occurrence of honeybee losses in maize and rape cultivation areas in Austria and possible correlations with bee diseases and the application of plant protection products, Moosbeckhofer 2009) or the Austrian residue control program. The nectar samples were also collected within the Melissa project and consisted of nectar that was freshly brought into the hives by the honeybees and was obtained through shaking out the honeycombs onto a clean plastic foil. All honey and nectar samples were stored in a laboratory cupboard at room temperature protected from light.

4.2.5 Sample Preparation Method ChemElut

This sample preparation approach was based on an unpublished method for the determination of the residues of clothianidin, TZNG and TZMU in honey, nectar and pollen developed by R. Schöning from Bayer CropScience (Schöning 2001) which is a modified version of the so-called ChemElut method by Alder and Klein (Alder and Klein 2003).

The preparation of honey and nectar samples consisted of the following procedure: 1 g honey was weighed into a 150 ml beaker. After the addition of 10 ml water and 10 µl of internal standard (1 mg/l) the sample was placed in an ultra-sonic bath for 2 minutes. 20 ml of methanol were added and the sample was homogenised for 1 minute with an Ultra-Turrax T25 (IKA Labortechnik, Germany) at 8000 rpm. The sample was then filtered through a filter paper (Ø 55 mm, Schleicher & Schuell, Germany) using a Büchner funnel and employing 2.5 g of celite as filter aid. Subsequently, the filter was washed with 20 ml of a 75/25 mixture of methanol/water (v/v). The filtrate was then transferred to a 250-ml round bottom flask and concentrated to the aqueous remainder employing a rotary evaporator with a bath temperature of 50 °C. The aqueous remainder was transferred onto a ChemElut 1020 column. After 15 minutes elution was carried out with 80 ml of a 50/50 mixture of cyclohexane/ethyl acetate (v/v). The eluate was collected in a 250-ml round bottom flask and evaporated to dryness using a rotary evaporator with a bath temperature of 50 °C. The residues in the flask were re-dissolved in 2 ml methanol by adding four portions of 500 µl each. This solution was transferred to a pyrex tube and evaporated to dryness using a stream of nitrogen and a temperature of 30 °C. Finally, the residues were re-dissolved in 200 µl methanol and transferred into vials for LC-MS/MS measurement.

4.2.6 Sample Preparation Method QuEChERS

The second sample preparation procedure used in this study was based on a multi-residue method for the analysis of pesticide residues in low-fat products called QuEChERS (Anastassiades et al. 2003, Anastassiades 2005).

In a first step the following mixture of salts was weighed into a pyrex tube:

- 4 g ± 0.2 g magnesium sulphate anhydrous
- 1 g ± 0.05 g sodium chloride
- 1 g ± 0.05 g trisodium citrate dihydrate
- 0.5 g ± 0.03 g disodium hydrogencitrate sesquihydrate

A mixture of 900 mg magnesium sulphate anhydrous and 150 mg PSA was prepared in another pyrex tube. After these preparatory steps 5 g ± 0.05 g of honey or nectar were weighed into a 50 ml screw cap centrifuge tube. 25 µl of internal standard solution (10 mg/l), 10 ml (honey) or 6.5 ml (nectar) of water as well as 10 ml acetonitrile were added and the tube was vigorously shaken by hand until a homogenous solution was obtained. The previously prepared mixture of four salts was then added to the centrifuge tube with the aid of a powder funnel. The tube was shaken vigorously by hand for at least one minute and centrifuged for 5 minutes at 3000 g and 10 °C. An aliquot of 6 ml of the supernatant was transferred into the pyrex tube containing the magnesium sulphate and PSA. The tube was vigorously shaken by hand for 30 seconds and centrifuged for 5 minutes at 3000 g and 10 °C. 2 ml of the liquid phase were transferred to a pyrex tube and the solvent was entirely removed using a stream of nitrogen at 30 °C. The residue was re-dissolved in 200 µl methanol and transferred into a vial for LC-MS/MS measurement.

4.2.7 Analysis by LC-MS/MS

The analyses were performed using an Agilent 1100 HPLC coupled to an API 2000 triple-quadrupole mass spectrometer from Applied Biosystems. A volume of 5 µl of the samples was injected using an injection program that included a dilution of a factor 5 with water. The analytes were separated on a Synergi Fusion RP column (50 x 2 mm, 4 µm particle size, 80 Å pore size). A guard column containing the same stationary phase was employed. Both columns were kept at 20 °C. Mobile Phase A consisted of water, mobile Phase B of methanol, both containing 5 mmol/l ammonium formate. The flow rate was 200 µl/min. The final gradient that was applied after optimization (see 4.3.1.3) is shown in Table 13.

Table 13: Chromatographic gradient of the LC-MS/MS method, Mobile Phase A consists of water, Mobile Phase B of methanol, both with 5 mmol/l ammonium formate

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
0.00	90	10
7.00	38	62
12.00	10	90
12.10	0	100
17.00	0	100
18.00	90	10
33.00	90	10

The triple-quadrupole mass spectrometer was equipped with an electrospray ion source which was operated in positive ionization mode. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with two transitions per compound: the first transition (MRM 1) was used as quantifier, the second transition (MRM 2) as qualifier. The source temperature was kept at 400 °C and the employed dwell time was 50 msec. The employed gas flows were chosen as follows: ion source gas 1 (GS1) 30, ion source gas 2 (GS2) 70, curtain gas (CUR) 30 and collision gas (CAD) 5. The LC-MS/MS system was controlled by the Analyst software 1.5.

For unambiguous identification retention times and ion ratios of the samples were compared with those of the standards. The ion ratios were calculated for every substance detected in a sample using the peak areas. Subsequently, the relative difference of the ion ratio of the sample and the average one of the standards was calculated and expressed as the percentage of the average ion ratios of the matrix standards. According to the SANCO/10684/2009 document a relative tolerance of ± 50 % is acceptable for the confirmation of the result, if the relative intensity of MRM 2, expressed as the percentage of the MRM 1, is smaller or equal to 10 %. If this relative intensity is 10 to 20 %, 20 to 50 % or above 50 %, the corresponding recommendations for maximum relative tolerances are ± 30 %, ± 25 % or ± 20 %, respectively (European Commission DG SANCO 2009).

Quantification of the compounds was based on 4-, 5- or 6-point calibrations (10 to 100 µg/l, 5 to 100 µg/l or 2 to 100 µg/l) using matrix-matched standards. Calibration curves were obtained by linear regression using no or 1/x weighting. Clothianidin-d_3 was applied as internal standard for the compensation of possible losses during sample preparation for all analytes with the exception of TZMU and TZNG for which the following isotopically labelled forms were used as internal standards: TZMU-d_3 and TZNG-^{13}C-^{15}N.

4.3 Results and Discussion

The main target was to develop a method for the simultaneous determination of all currently existing neonicotinoids (acetamiprid, clothianidin, dinotefuran, flonicamid, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) as well as the metabolites that are included in the residue definitions of the individual substances (clothianidin, TFNA-AM) for honey (see Table 3). Three additional metabolites were included in the method due to their availability (TZMU, TZNG, IM 2-1 (N-demethyl acetamiprid)).

The development and validation of an analytical method requires samples spiked with known concentrations of the analytes. For their preparation it is essential to obtain a blank matrix, i.e. in the present case to find a honey that is completely devoid of all of the investigated analytes. Since neonicotinoid insecticides are exclusively applied on agricultural fields, an organic flower honey that was collected above an altitude of 2000 m was chosen as blank matrix.

As honey has a high viscosity special care had to be taken in the preparation of the spiked samples to ensure a homogenous distribution of the analytes within the matrix. To this end the honey was slightly heated after the addition of the spiking solutions to lower its viscosity, followed by extended stirring at the elevated temperature. Blank honey was spiked at three fortification levels: 10 µg/kg, 50 µg/kg and 100 µg/kg.

For the best possible identification and quantification of neonicotinoid insecticides in honey it was essential to find the most appropriate analytical tool. LC-MS/MS is a particularly sensitive and selective analytical technique and represents the tool of choice for the quantification of thermally unstable or non-volatile pesticide residues. The linking of liquid chromatography and tandem mass spectrometry has essential advantages compared to other analytical techniques. The liquid chromatography effects the separation of analytes that are part of a complex mixture. Even though liquid chromatography provides information about retention times of the substances, an unequivocal identification of the analytes is usually not possible because of numerous compounds that may elute at the same retention time. The certain identification of compounds must therefore be achieved by connecting the liquid chromatography to a highly specific and sensitive detector for which a tandem mass spectrometer is an excellent choice. Compounds of similar retention times usually can be unequivocally discriminated upon their mass spectrometric behaviour, so the combination of LC and MS/MS allows the differentiation between these substances.

All multi-residue methods with the main focus on neonicotinoid insecticides used liquid chromatography for the separation of the compounds (Di Muccio et al. 2006, Fidente et al. 2005, Obana et al. 2003, Schöning and Schmuck 2003). LC is the separation tool of choice for neonicotinoids because of their high polarity and low volatility which makes them less amenable to GC analysis.

For the simultaneous determination of the 12 analytes (eight neonicotinoids and four metabolites) in the complex matrix honey LC in combination with tandem mass spectrometry was chosen for analysis. LC and MS/MS were connected through an electrospray interface operating in positive ionization mode (ESI+). The main functions of this interface included the removal of the mobile phase solvent (desolvation) and the formation of a quasi-molecular ion [M+H]+ (ionization). Analyses were performed in multi reaction monitoring (MRM) mode to obtain maximum selectivity and high sensitivity.

Figure 27 shows the LC-MS/MS apparatus used and the important stations passed by the samples.

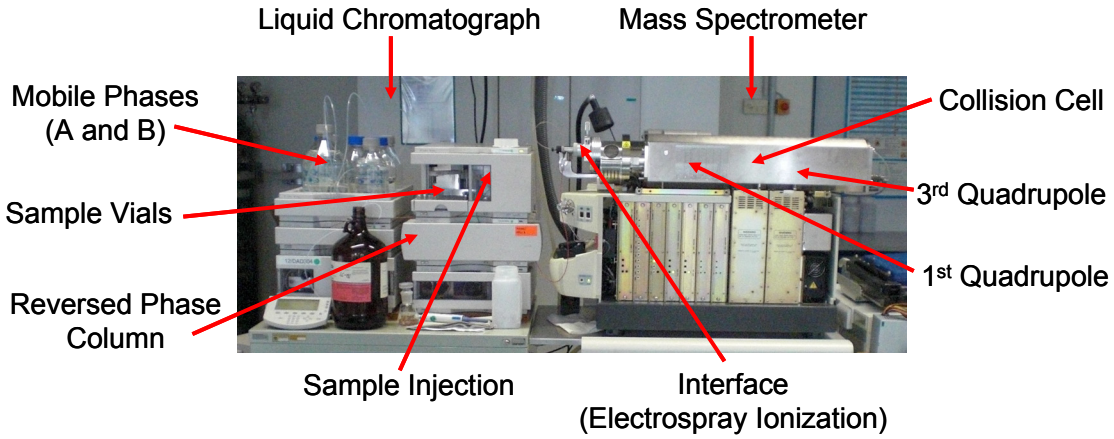

Figure 27: LC-MS/MS system employed for the analyses

In order to minimize the possible matrix effects of honey samples during LC-MS/MS analysis matrix-matched standards were used for calibration. In addition the use of an internal standard compensated for losses during sample preparation.

4.3.1 Method Development

4.3.1.1 Tuning

Within the development of other multi-residue methods conducted on the same LC-MS/MS setup, the neonicotinoid insecticides acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam were tuned. Hence, the MS parameters of these substances were already optimized.

The other three neonicotinoid insecticides dinotefuran, nitenpyram and flonicamid as well as the four metabolites IM 2-1, TFNA-AM, TZMU and TZNG still needed to be tuned. For tuning solutions of 0.1 mg/l, 1 mg/l and 10 mg/l were prepared in 50/50 (v/v) methanol/water with 5 mmol/l ammonium formate for each substance. The substances were infused into the MS instrument with a Hamilton syringe starting with the smallest concentration of 0.1 mg/l. The optimization of the MS parameters was carried out automatically with the tuning function of the Analyst 1.5 software. The two most suitable MRM transitions of each substance were chosen.

The main goal of the individual optimization of parameters regarding ionization and fragmentation was to obtain signals of highest possible intensities. Declustering potential (DP) and entrance potential (EP) are responsible for the desolvation of the solvent cluster and the transfer of the parent ion into the vacuum chamber and are applied in front of the first quadrupole. Cell entrance potential (CEP), collision energy (CE) and cell exit potential (CXP) are optimized in order to create ideal conditions for an effective fragmentation of the parent ion into product ions and are applied around the second quadrupole.

The optimized MS parameters are given in Table 14.

Table 14: Optimized MS parameters for both MRM transitions of all analytes

Substance	Q1[1]	Q3[2]	DP[3]	EP[4]	CEP[5]	CE[6]	CXP[7]
Acetamiprid	223	126	36	12	14.1	27	6
	223	90	34	12	14.1	45	4
Acetamiprid metabolite IM 2-1	209	126	26	10	13.75	23	14
	209	90	26	10	13.75	43	12
Clothianidin	250	132	31	5	14.77	19	4
	250	169	31	5	14.77	19	8
Clothianidin- d_3	253	132	31	5	14.85	19	4
	253	172	31	10	14.85	19	8
Clothianidin metabolite TZMU	206	175	31	10	13.67	27	22
	206	132	31	10	13.67	23	16
TZMU- d_3	209	175	31	10	13.75	27	22
	209	132	31	10	13.75	23	16
Clothianidin metabolite TZNG	236	132	26	8.5	14.42	19	16
	236	155	26	8.5	14.42	17	12
TZNG-^{13}C-^{15}N	238	132	26	8.5	14.47	19	16
	238	157	26	8.5	14.47	17	12
Dinotefuran	203	129	16	10	13.62	17	12
	203	113	16	10	13.62	15	4
Flonicamid	230	203	31	10	14.27	21	16
	230	148	31	10	14.27	39	16
Flonicamid metabolite TFNA-AM	191	148	26	10	13.3	31	18
	191	98	26	10	13.3	43	4
Imidacloprid	256	209	51	9	14.92	21	10
	256	175	49	9	14.92	25	8
Nitenpyram	271	126	26	9	15.3	45	8
	271	225	26	9	15.3	17	12
Thiacloprid	253	126	81	12	14.85	29	6
	253	186	79	12	14.85	19	10
Thiamethoxam	292	211	21	10	15.82	17	6
	292	181	21	8.5	15.82	31	10

[1] m/z of parent ion [2] m/z of product ion [3] declustering potential [4] entrance potential [5] cell entrance potential [6] collision energy [7] cell exit potential

4.3.1.2 Sample Preparation

Considering recent investigations of residues in honey or nectar one can find various sample preparation procedures. However, they all are based on a solvent extraction as first step followed by a purification and concentration step to remove matrix compounds and obtain the required sensitivity, respectively.

The first analyses of spiked honey samples were carried out using the ChemElut sample preparation method. Spiked samples at levels of 10, 100 and 1000 µg/kg were worked up. Quantification was based on solvent standards

containing the internal standard. The recovery rates for all substances on the three concentration levels ranged from 0 to 149 %. Dinotefuran showed low recovery rates of 0 to 45 % whereas the substance nitenpyram entirely failed to be recovered in these trials. Even though the other substances could be recovered to a larger extent the recoveries were clearly unsatisfactory.

In order to investigate the reasons for the loss of dinotefuran and nitenpyram different trials were carried out. In a first experiment the target was to explore whether the substances might get lost during the analyses with LC-MS/MS. Therefore, a standard solution containing dinotefuran and nitenpyram as well as the internal standards (clothianidin-d_3, TZMU-d_3 and TZNG-^{13}C-^{15}N) was added to a worked-up blank honey sample. The final concentrations of dinotefuran and nitenpyram in the sample were 100 µg/kg. The recovery rates were ≥ 100% for both substances. Conclusively, the loss of dinotefuran and nitenpyram seemed to take place sometime during the sample preparation procedure.

The next step was to find out which specific part of the preparation process was responsible for the loss of the substances. For this purpose two spiked honey samples (100 µg/kg) as well as two samples of spiked honey (100 µg/kg) that were additionally fortified with 100 µg/kg dinotefuran and nitenpyram were worked up. To investigate possible effects of the temperature of the water bath of the rotary evaporator (50 °C), one spiked sample and one spiked sample with additional fortification underwent evaporation at a maximum temperature of 40 °C. Dinotefuran showed recovery rates between 16 and 42 % and no difference was observed concerning the temperature of the water bath of the rotary evaporator (40 or 50 °C). Nitenpyram was again not detectable in any of the analysed samples. It was concluded that the evaporation of the solvent at 50 °C did not lead to any significant losses of the analytes.

The liquid-liquid extraction of the analytes on the ChemElut cartridge as a possible source of substance loss was investigated in a next step. A mixture of nitenpyram standard solution (100 µg/l), internal standard solution and water was placed on the top of a ChemElut cartridge. Elution and the further steps of the method were carried out as before. In the LC-MS/MS measurement no

nitenpyram was detectable. It was concluded that nitenpyram was retained in the ChemElut cartridge during sample preparation.

In the course of finding an explanation for the loss of nitenpyram in the ChemElut cartridge, the octanol-water partition coefficients were compared for the different investigated analytes. The octanol-water partition coefficient (P) describes the distribution of a compound between two phases, viz. water and 1-octanol. P is > 1 if a substance is better soluble in 1-octanol (representing lipophilic solvents), whereas P is < 1 if the substance is better soluble in water (representing hydrophilic solvents). Correspondingly, log P is positive for lipophilic substances and negative for hydrophilic substances. While nitenpyram and dinotefuran have negative log P values of -0.66 and -0.55, respectively, the logaritmic octanol-water partition coefficients of the other neonicotinoids are in the range of -0.13 to 1.26.

Table 15: Octanol-water partition coefficients (log P) of the neonicotinoid insecticides

Substance	Log P
Acetamiprid	0.8
Clothianidin	0.9
Dinotefuran	-0.55
Flonicamid	0.3
Imidacloprid	0.57
Nitenpyram	-0.66
Thiacloprid	1.26
Thiamethoxam	-0.13

The high hydrophilicity of nitenpyram represents a probable explanation for its loss during liquid-liquid extraction. It can be assumed that nitenpyram remains in the methanol-water phase that is adsorbed onto the diatomaceous earth of the ChemElut cartridge and does not partition into the cyclohexane/ethylacetate eluent. The same situation also applies to dinotefuran to a lesser extent, explaining its low recoveries. Obana et al. (2003) investigated nitenpyram and other neonicotinoids in fruits and vegetables and noted also unsatisfactory recovery rates of nitenpyram after the extraction with acetonitrile. Consequently an alternative extraction method had to be chosen. By using SPE (graphitized

carbon loaded with 20 % of methanol) Obana et al. finally achieved recoveries between 70 and 85 % in the different matrices (Obana et al. 2003).

From the above experiments it was concluded that the sample preparation approach employing a clean-up step using a LLE on a ChemElut cartridge was not suitable for the whole range of analytes present in the study. Therefore, the utility of a different sample preparation procedure, QuEChERS, was investigated.

A first experiment using the QuEChERS method included the sample preparation and subsequent analysis of five spiked honey samples for each of the three fortification levels (10, 50 and 100 µg/kg). Solvent standards were used for calibration and internal standards were employed to compensate for possible analyte losses during sample preparation. The recovery rates obtained ranged from 54 % to 164 % and were thus clearly superior to the recoveries of the ChemElut method. The recoveries of nitenpyram at the three fortification levels ranged from 54.5 to 78.5 %. Even though the recovery rates were much better with the QuEChERS sample preparation they were still not sufficient to entirely meet the expectations of validation. According to the EU validation guideline for pesticide residues SANCO/10684/2009 mean recovery values should be within the range of 70 to 120 % at each spiking level (European commission DG SANCO 2009).

The observed recovery rates result from losses during sample preparation and possibly also from matrix effects during LC-MS/MS measurements, as “ideal” internal standards were only present for clothianidin and its metabolites, whereas clothianidin-d_3 was also used as internal standard for all other analytes. Differing matrix effects for clothianidin-d_3 compared to an analyte would also add to the overall recovery rate and might influence it negatively.

In order to optimize the determination of the neonicotinoid insecticides and their metabolites in the honey matrix matrix-matched standards were prepared. Subsequent analyses of spiked honey samples with matrix-matched standards for calibration finally showed very satisfactory recovery rates (Table 20).

Nevertheless, recovery rates of nitenpyram were still not optimal due to its high hydrophilicity. This can be explained by the employed internal standard clothianidin-d_3 experiencing significantly different losses during sample preparation compared to nitenpyram. The only solution would be to use an isotopically labelled form of nitenpyram for recovery correction for this analyte. However, such an internal standard was not available. Nevertheless, nitenpyram still achieved recoveries of 60 % which is acceptable.

Besides its clear superiority in terms of analyte recoveries the QuEChERS method proved to be much less laborious than the ChemElut method. The QuEChERS method allowed a high number of samples to be worked up in a short period of time. Up to 20 samples could be prepared for analysis each day. Thus, the QuEChERS sample preparation lived up to the characteristics that are attributed to it: **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, **S**afe.

4.3.1.3 LC Optimization

The target of the LC optimization was to find a well-suited chromatographic gradient for the analyses of the neonicotinoid insecticides and their metabolites in honey. Particular goals of the optimization were the elution of all substances within a reasonable period of time (15 min) and a certain distribution of the individual retention times of the substances. A further objective was to obtain a sufficient retention of the first eluting analyte in order to avoid possible interferences with highly polar matrix compounds. Table 16 shows the detailed chromatographic gradients that were tried out in the course of the LC-optimization. The length and the steepness of the different gradient sections were varied.

C0 was the original gradient that was already used for the determination of the neonicotinoid insecticides in the guttation liquid of maize plants. After the analysis of a matrix-matched standard solution using gradient C0 the distribution of the retention times of the individual substances was examined. Taking into account their agreement with the above-mentioned goals some

modifications were undertaken in order to create a new gradient C1, which was again tried out by a further measurement of a matrix-matched standard solution. In this manner the gradient was optimized step by step until the retention times of the standard solutions matched the previously defined goals. A graphical comparison of the different gradients is shown in Figure 28.

Table 16: Investigated chromatographic gradients

Gradient	Time [min]	MeOH [%]
C0	0.0 11.0 23.0	20 90 90
C1	0.0 12.5 17.5	10 80 80
C2	0.0 1.5 13.1 18.1	15 15 80 80
C3	0.0 2.5 12.5 17.5	15 15 80 80
C4	0.0 1.5 12.0 17.0	12 12 90 90
C5	0.0 12.0 17.0	12 90 90
C6	0.0 7.0 12.0 12.1 17.0	10 62 90 100 100

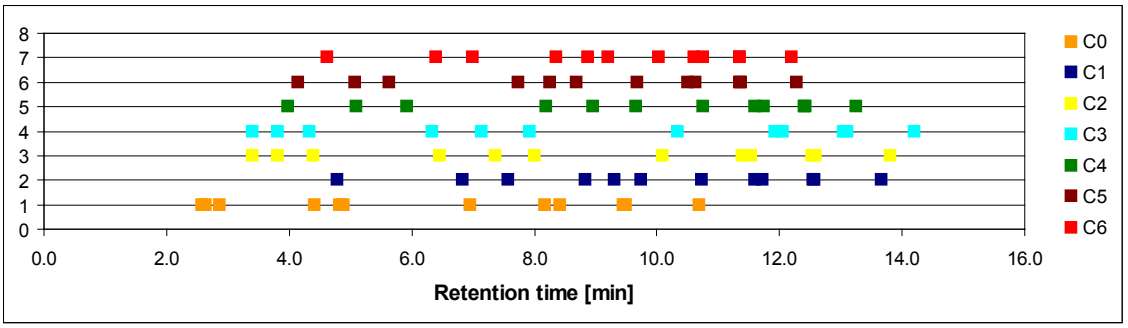

Figure 28: Elution behaviour of the analytes applying different chromatographic gradients. Each square corresponds to an analyte.

The optimization process was terminated with gradient C6 which nicely fulfilled all aims for all analytes. The main difference of gradient C6 in comparison to the other gradients is that it consists of two sections with increasing percentage of methanol prior to the isocratic section at high organic content instead of one. This modification resulted in a more regular distribution of the analytes over the elution period. Employing gradient C6 the first substance flonicamid metabolite TFNA-AM elutes after approximately 4.6 minutes. Acetamiprid and its metabolite IM 2-1 have practically the same retention times due to their chemical similarity: they elute at 11.4 minutes. Imidacloprid and clothianidin have similar retention times of 10.61 and 10.76 minutes, respectively but are still distinguishable. Of course, unequivocal differentiation of all analytes, also the closely eluting ones, was provided by the MS/MS detection (Figure 29).

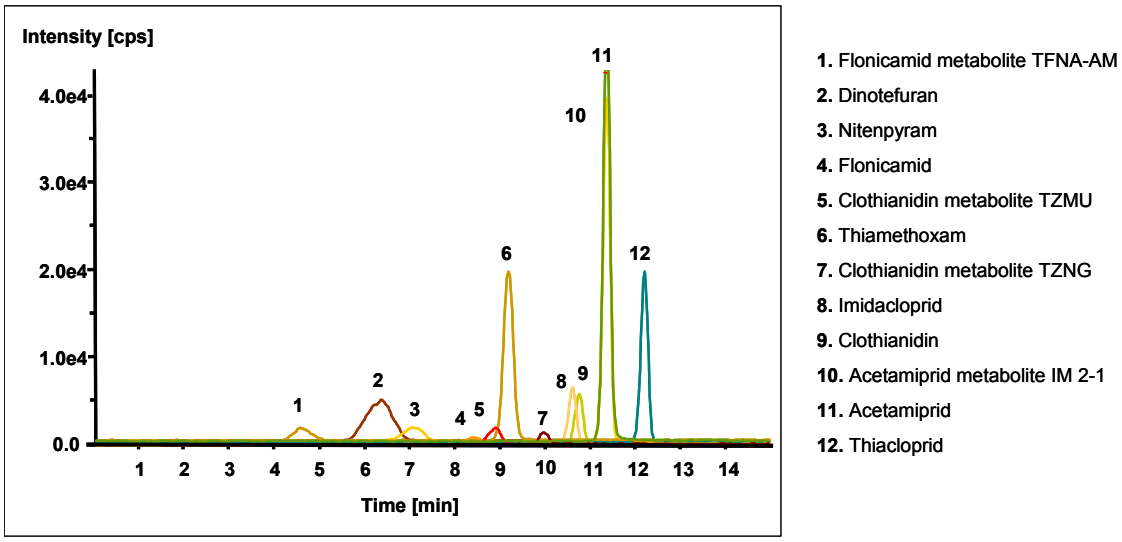

Figure 29: Chromatogram of the neonicotinoid insecticides and their metabolites after optimization of the LC conditions. Shown are the traces of the respective first MRM transition.

In order to test the reproducibility of the retention times five matrix-matched standards of 10 µg/kg and 50 µg/kg were measured alternately. The results are shown in Table 17. The obtained relative standard deviations ranged from 0.17 to 1.43 % for all analytes and were thus very satisfactory.

Table 17: Reproducibility of the retention times applying the final chromatographic gradient

Substance	Average retention time [min]*	RSD [%]*
Flonicamid-metabolite TFNA-AM	4.61	1.43
Dinotefuran	6.34	1.26
Nitenpyram	7.08	1.10
Flonicamid	8.45	0.60
Clothianidin-metabolite TZMU	8.87	0.38
Thiamethoxam	9.19	0.17
Clothianidin-metabolite TZNG	10.02	0.51
Imidacloprid	10.61	0.27
Clothianidin	10.80	0.19
Acetamiprid-metabolite IM 2-1	11.38	0.39
Acetamiprid	11.40	0.18
Thiacloprid	12.14	0.34

* n= 10

4.3.2 Validation

The validation of an analytical method is necessary to ensure the suitability of the procedure for the requested application. The performance of validation includes the determination of the limit of detection, limit of quantitation, sensitivity, mean recovery and precision.

4.3.2.1 LOD and LOQ

An essential part of the validation of an analytical method is the determination of the limit of detection (*LOD*) and the limit of quantitation (*LOQ*). LOQ is the minimum concentration of an analyte that can be quantified with acceptable accuracy and precision, whereas LOD refers to the minimum concentration of an analyte that can be detected with acceptable certainty, although not being quantifiable with acceptable precision (European Commission DG SANCO 2009). Naturally, the limit of quantitation is higher than the limit of detection. One aim of the present study was the development of a multi-residue method for neonicotinoids in honey with maximum LOQs of 10 µg/kg for all analytes.

The limit of 10 µg/kg results from the maximally tolerated level of synthetic pesticides in organic products.

Since the definitions of LOD and LOQ in the validation guideline (European Commission DG SANCO 2009) use general and broad terms there exist some recommendations for the practical determination of the limits of detection and quantification: the S/N ratio (signal-to-noise ratio) at the LOD should be at least **3:1**, whereas the minimum S/N ratio for the LOQ is **10:1**.

Thus, in order to determine the LOD and LOQ of the developed method, the S/N ratios of all analytes were calculated using the analytical data of matrix-matched standards. The S/N values of the two MRM transitions of every substance were compared with each other. The smaller S/N ratio of each analyte was then divided by the required S/N ratio for the LOD or LOQ, respectively, to yield the extrapolation factor for calculating the LOD and LOQ. In any case the “reporting” LOQ was not set below the lowest level of calibration (2 µg/kg) and hence the LOD not below 0.6 µg/kg.

Example for the determination of LOD and LOQ for thiacloprid:

The S/N ratios for the first and second MRM transition of thiacloprid in a matrix standard of 2 µg/kg were 175 and 96.3 respectively. In this case the lower S/N ratio was 96.3 of the second transition. The division of 96.3 by 10 (from the minimum S/N ratio for the LOQ of 10:1) gives an extrapolation factor of 9.6. The analytical limit of quantitation is then calculated by dividing the concentration of the standard, 2 µg/kg, by 9.6 yielding a value of 0.21 µg/kg. However, as mentioned above, the “reporting” LOQ was finally set at 2 µg/kg. The LOD for thiacloprid was calculated in a similar manner. The S/N ratio of 96.3 was divided by 3 (from the minimum S/N ratio for the LOD of 3:1) giving an extrapolation factor of 32. This led to an analytical LOD of 0.063 µg/kg. Nevertheless, the “reporting” LOD was set at 0.6 µg/kg as described above.

The LODs and LOQs for all analytes are listed in Table 18:

Table 18: Limits of detection and quantification for all analytes encompassed by the developed method

Analyte	Limit of detection [µg/kg]	Limit of quantitation [µg/kg]
Acetamiprid	0.6	2
Acetamiprid metabolite IM 2-1	0.6	2
Clothianidin	0.6	2
Clothianidin metabolite TZMU	2	5
Clothianidin metabolite TZNG	5	10
Dinotefuran	2	5
Flonicamid	2	5
Flonicamid metabolite TFNA-AM	2	5
Imidacloprid	0.6	2
Nitenpyram	5	10
Thiacloprid	0.6	2
Thiamethoxam	0.6	2

Those neonicotinoid insecticides (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam) that constitute the active ingredients of plant protection products that are currently registered in Austria (see Table 1) all had a limit of detection of 0.6 µg/kg and a limit of quantitation of 2 µg/kg. For dinotefuran, flonicamid, nitenpyram, the clothianidin-metabolites TZMU and TZNG as well as the flonicamid-metabolite TFNA-AM the limits of detection and quantitation were slightly higher being between 2 and 5 µg/kg for the LOD and 5 to 10 µg/kg for the LOQ. Nevertheless, half of the analytes are detectable at 0.6 µg/kg. Thus, the present method can be considered as highly sensitive and allows the detection and quantitation of very low concentrations of neonicotinoid insecticides in honey.

4.3.2.2 Linearity

For the acquisition of the calibration curves matrix-matched standards were used. Calibrations were performed using 6 levels ranging from 2 to 100 µg/kg for all analytes with the exception of nitenpyram and TZNG. For the latter substances that had LODs of 5 µg/kg the calibrations were based on 5

concentration levels (5 to 100 µg/kg). As an example, Figure 30 shows the 6-point calibration curve for thiacloprid.

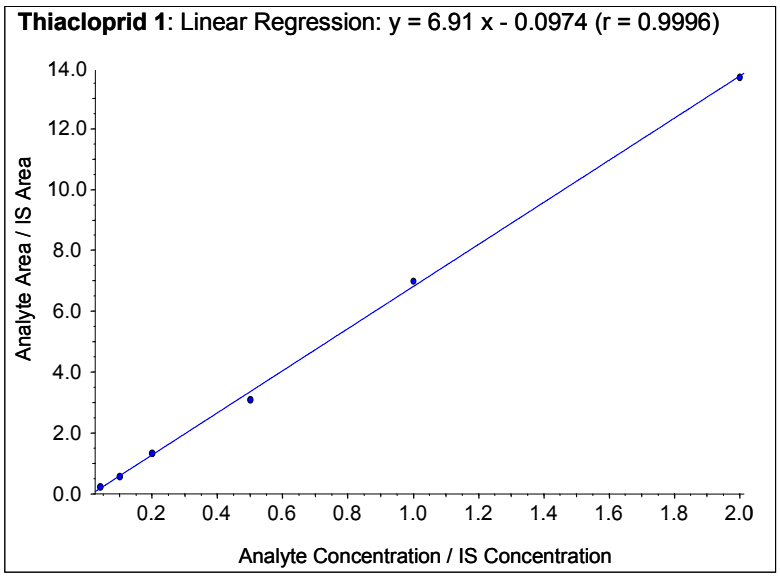

Figure 30: Calibration curve of the first MRM transition of thiacloprid

As shown in Table 19, the regression coefficients of the calibration curves of the first MRM transitions used for quantification were > 0.99 for all substances, indicating very good linearity.

Table 19: Regression coefficients of the first MRM transitions

Analyte	Regression coefficient
Acetamiprid	0.9979
Acetamiprid met. IM 2-1	0.9977
Clothianidin	0.9989
Clothianidin met. TZMU	0.9999
Clothianidin met. TZNG	0.9985
Dinotefuran	0.9991
Flonicamid	0.9998
Flonicamid met. TFNA-AM	0.9989
Imidacloprid	0.9982
Nitenpyram	0.9938
Thiacloprid	0.9996
Thiamethoxam	0.9979

The regression coefficient was lowest for nitenpyram, indicating again the existing analytical challenge of this particular substance. The linear regression coefficients of the second MRM transitions of all analytes were also > 0.99.

4.3.2.3 Recovery Rates and Precision

According to the SANCO/10684/2009 document for method validation and quality control procedures for pesticide residues analysis in food and feed (European Commission DG SANCO 2009) recoveries for all analytes included in a method should be within a range of 70 to 120 % for all fortification levels with a relative standard deviation≤ 20 %. For the determination of the recovery rates and precision a blank honey sample was spiked at three concentration levels (10 µg/kg, 50 µg/kg, 100 µg/kg) and each was analysed five times.

The obtained validation data are listed in Table 20 and are also depicted in Figure 31.

Table 20: Recovery rates and precision data for all analytes

	10 ppb		50 ppb		100 ppb	
Substance	**RR [%][1]**	**RSD [%][2]**	**RR [%][1]**	**RSD [%][2]**	**RR [%][1]**	**RSD [%][2]**
Acetamiprid	102.1	7.6	89.0	8.2	87.4	4.8
Acetamiprid metabolite IM 2-1	99.2	7.5	91.5	7.5	86.6	4.1
Clothianidin	99.7	8.1	93.0	3.5	94.3	4.0
Clothianidin metabolite TZMU	96.4	6.9	95.7	6.2	95.5	5.1
Clothianidin metabolite TZNG	96.6	4.4	102.6	6.5	101.1	6.8
Dinotefuran	87.3	4.8	83.9	11.8	83.9	9.1
Flonicamid	103.5	2.7	94.5	8.1	96.8	6.2
Flonicamid metabolite TFNA-AM	114.2	8.2	83.7	9.9	82.9	6.4
Imidacloprid	100.7	12.8	101.8	8.9	107.0	7.9
Nitenpyram	76.5	7.7	67.3	8.3	60.0	9.2
Thiacloprid	98.5	4.1	88.4	8.6	86.3	6.0
Thiamethoxam	93.3	9.7	83.5	12.3	82.0	10.1

[1] RR = recovery rate [2] RSD= relative standard deviation

The recovery rates for all analytes were within the required range except nitenpyram, which was partially lost during sample preparation due to its high

hydrophilicity. On average the best recovery rates were obtained for the 10 µg/kg fortification level.

All relative standard deviations were smaller than 13 % and thus fulfilled the requirement of the SANCO/10684/2009 guideline. The relative standard deviations were on average smaller for the higher fortification levels.

Clothianidin and its two metabolites TZMU and TZNG exhibited recoveries close to 100 % at very high levels of precision, which can be attributed to the application of an ideal internal standard, viz. an isotopically labelled form of the respective analyte. For all other analytes clothianidin-d_3 was employed as internal standard to compensate for losses during sample preparation. Due to differences in the physico-chemical properties between clothianidin-d_3 and the respective analyte, despite belonging to the same class of pesticides, the internal standard cannot fulfill its role in an optimal way.

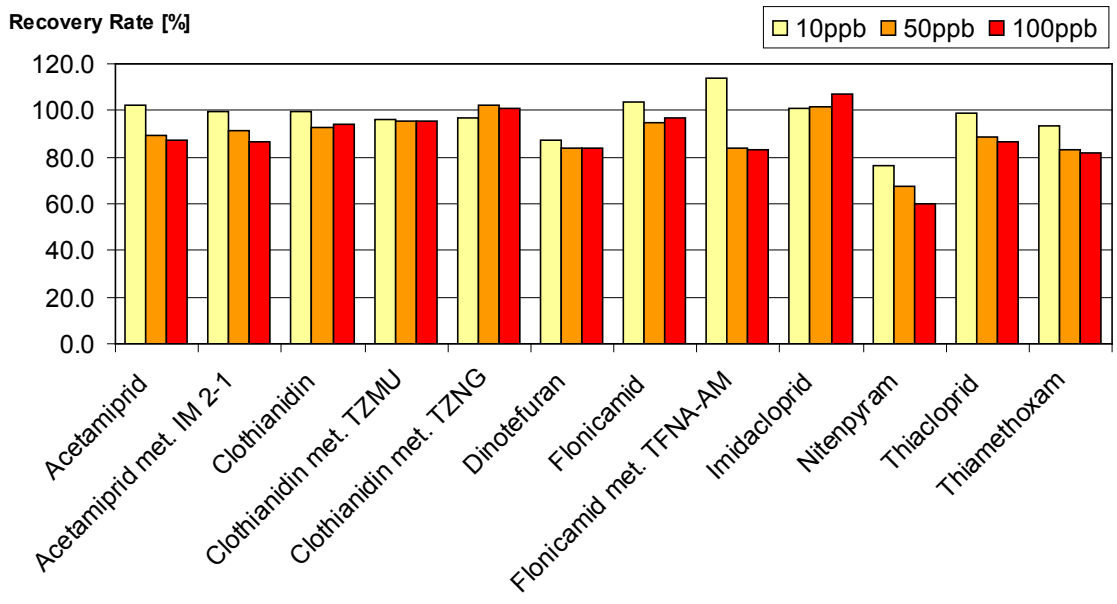

Figure 31: Average recovery rates of all analytes shown for the three fortification levels

4.3.3 Analysis of Honey and Nectar Samples

A total of 52 Austrian honey and nectar samples were analysed in the context of this thesis. Thirteen suspicion honey and eleven suspicion nectar samples originated from the ongoing Melissa project from the Institute for Apiculture of the Austrian Agency for Health and Food Safety (Moosbeckhofer 2009). This project focuses on investigating the occurrence of honeybee losses in regions with high proportions of maize and rape cultivation in Austria and possible correlations with honeybee diseases and the application of plant protection products. These samples were collected in beehives that were affected by honeybee losses and/or were located close to neonicotinoid-treated maize or rape fields. Additionally, 19 flower honey samples and nine forest honey samples from different locations within Austria were analysed. The collection of these samples was part of the regular Austrian residue control program.

In the course of the analysis of these honey and nectar samples the following questions should be investigated:

- Do honey and nectar samples contain residues of neonicotinoid insecticides and in which quantities?
- Do suspicion samples and other samples differ concerning the residue concentrations of neonicotinoid insecticides?
- Do differences exist between flower and forest honey?
- Do the neonicotinoid residues correlate with the local crop cultivation patterns in the vicinity of the samples' origin?

The collection of all honey and nectar samples took place in the first half of 2009. The processing and analyses of the samples took place from August to October 2009. All samples were analysed in duplicate for the verification of the obtained results in case of the detection of one of the analytes.

All suspicion honey samples were flower honeys and were made available by Austrian beekeepers. The detailed results of the analyses of the suspicion honey samples are shown in Table 21. In the thirteen suspicion honey samples thiacloprid and thiamethoxam were the only detectable analytes. Seven out of thirteen samples contained thiacloprid in concentrations above the LOD, of which one additionally showed traces of thiamethoxam.

Figure 32 shows a chromatogram of a honey sample containing thiacloprid. No matrix peaks disturbed the clear identification and quantification of this substance.

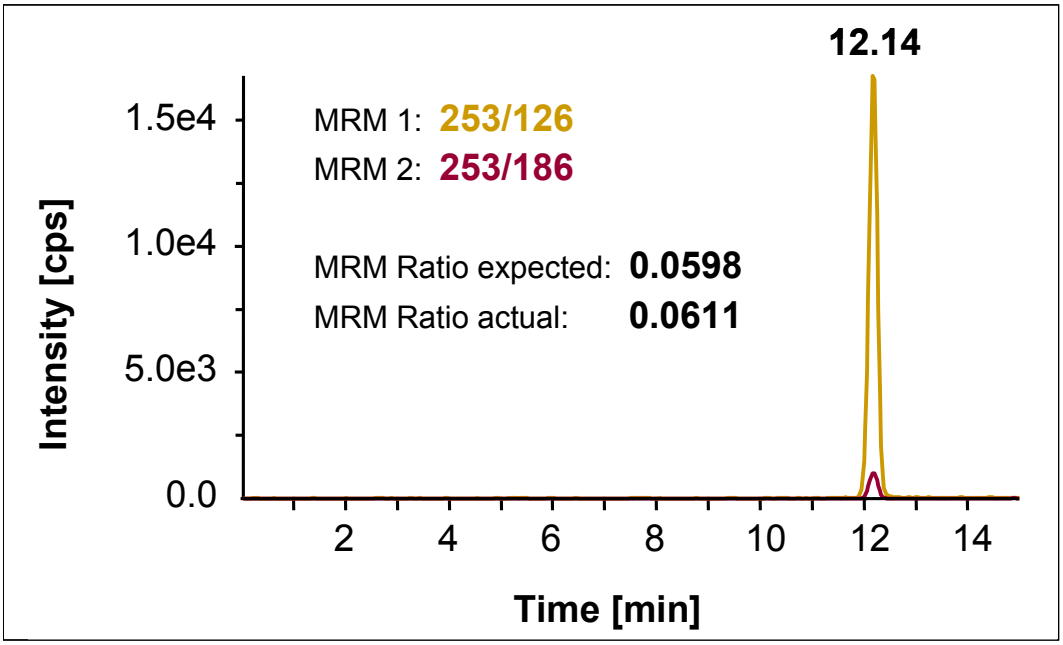

Figure 32: Chromatogram of a suspicion honey sample containing thiacloprid (27.4 µg/kg)

Table 21: Results of the analyses of suspicion honey samples

Sample information		Analytes*	
Sample number	**Origin**	**Thiacloprid [µg/kg]**	**Thiamethoxam [µg/kg]**
Suspicion honey sample **1**	Upper Austria Perg	**26.0**	<LOD
Suspicion honey sample **2**	Upper Austria Schärding	**27.4**	<LOD
Suspicion honey sample 3	Styria Deutschlandsberg	<LOD	<LOD
Suspicion honey sample 4	Styria Radkersburg	<LOD	<LOD
Suspicion honey sample 5	Styria Deutschlandsberg	<LOD	<LOD
Suspicion honey sample 6	Styria Feldbach	<LOD	<LOD
Suspicion honey sample **7**	Upper Austria Perg	**8.6**	<LOD
Suspicion honey sample **8**	Upper Austria Ittensam	**detectable**	<LOD
Suspicion honey sample **9**	Styria Fürstenfeld	**6.2**	<LOD
Suspicion honey sample **10**	Upper Austria Reichersberg	**19.6**	**detectable**
Suspicion honey sample **11**	Lower Austria Großmugl	**11.2**	<LOD
Suspicion honey sample 12	Unknown	<LOD	<LOD
Suspicion honey sample 13	Upper Austria Peilstein	<LOD	<LOD

* All other investigated analytes were <LOD for all samples

The determined quantities of thiacloprid ranged from 6.2 to 27.4 µg/kg. Five of the seven positive samples were from Upper Austria, one from Styria and one from Lower Austria. Most of the negative samples were collected in Styria (four samples), one in Upper Austria. The sample containing traces of thiamethoxam had its origin in Upper Austria.

The analysis of flower honey included nineteen samples from six different Austrian provinces. The results are listed in Table 22. Out of the twelve investigated analytes only thiacloprid and acetamiprid could be detected. Seven samples contained thiacloprid, two samples acetamiprid.

Table 22: Results of the analyses of Austrian flower honey samples

Sample information		Analytes*	
Sample number	**Origin**	**Thiacloprid [µg/kg]**	**Acetamiprid [µg/kg]**
Flower honey sample 1	Upper Austria Perg	<LOD	<LOD
Flower honey sample 2	Lower Austria Nonndorf	<LOD	<LOD
Flower honey sample 3	Styria Weiz- Göttelsberg	<LOD	<LOD
Flower honey sample 4	Styria Weiz- Göttelsberg	<LOD	<LOD
Flower honey sample **5**	Styria Weiz	**5.5**	<LOD
Flower honey sample **6**	Styria Puch bei Weiz	<LOD	**2.2**
Flower honey sample **7**	Styria Puch bei Weiz	**5.0**	**15.2**
Flower honey sample 8	Lower Austria Fischamend	<LOD	<LOD
Flower honey sample 9	Carinthia Velden	<LOD	<LOD
Flower honey sample **10**	Styria Brodersdorf	**detectable**	<LOD
Flower honey sample 11	Upper Austria Kirchdorf am Inn	<LOD	<LOD
Flower honey sample **12**	Upper Austria Reichersberg	**detectable**	<LOD
Flower honey sample **13**	Upper Austria Engerwitzdorf	**12.3**	<LOD
Flower honey sample 14	Styria St. Lorenzen am Wechsel	<LOD	<LOD
Flower honey sample **15**	Styria Söchau-Aschbach	**detectable**	<LOD
Flower honey sample 16	Burgenland St. Margarethen	<LOD	<LOD
Flower honey sample **17**	Lower Austria Völlendorf	**detectable**	<LOD
Flower honey sample 18	Lower Austria Kirchstetten	<LOD	<LOD
Flower honey sample 19	Lower Austria Kirnberg an der Mank	<LOD	<LOD

* All other analytes were <LOD for all samples

Thiacloprid was detected in samples from three Austrian provinces, namely Styria (four samples), Upper Austria (two samples) and Lower Austria (one sample). In contrast, acetamiprid was exclusively found in honey samples from Styria. Thiacloprid was found between the LOD (0.6 µg/kg) and 12.3 µg/kg, while acetamiprid concentrations were 2.2 and 15.2 µg/kg.

Geographically the origins of the samples three to seven were located quite closely. Two of these samples were free from thiacloprid and acetamiprid while the other three samples contained one or both of these analytes.

Nine forest honey samples from six Austrian provinces were analysed. None of the investigated neonicotinoid insecticides except for thiacloprid was detectable in the samples. Four samples contained small amounts of thiacloprid below or at the LOQ (Table 23).

Table 23: Results of the analyses of Austrian forest honey samples

Sample information		**Analytes***
Sample number	**Origin**	**Thiacloprid [µg/kg]**
Forest honey sample 1	Tyrol Söll	<LOD
Forest honey sample **2**	Carinthia Millstatt	**2.1**
Forest honey sample 3	Upper Austria Zell am Moos	<LOD
Forest honey sample 4	Styria Friedberg	<LOD
Forest honey sample 5	Styria Sebersdorf-Rohrbach	<LOD
Forest honey sample **6**	Carinthia St. Michael im Lavanttal	**detectable**
Forest honey sample **7**	Lower Austria Saubersdorf	**detectable**
Forest honey sample **8**	Salzburg Saalfelden	**detectable**
Forest honey sample 9	Salzburg Maria Alm	<LOD

* All other analytes were <LOD for all samples

Two of the positive samples were from Carinthia, one from Lower Austria and one from Salzburg.

Beside the abovementioned honey samples, the investigations also included eleven suspicion nectar samples which were supplied by Austrian beekeepers. The results of the analyses of the nectar samples are shown in Table 24.

Table 24: Results of the analyses of the suspicion nectar samples

Sample information		Analytes*
Sample number	**Origin**	**Thiacloprid [µg/kg]**
Suspicion nectar sample **1**	Upper Austria Ried	**18.7**
Suspicion nectar sample **2**	Upper Austria Ried	**81.2**
Suspicion nectar sample **3**	Upper Austria Reichersberg	**19.8**
Suspicion nectar sample **4**	Lower Austria Großmugl	**29.5**
Suspicion nectar sample **5**	Lower Austria Großmugl	**29.2**
Suspicion nectar sample **6**	Lower Austria Großmugl	**11.1**
Suspicion nectar sample 7	Lower Austria Dorfstetten	<LOD
Suspicion nectar sample 8	Lower Austria Dorfstetten	<LOD
Suspicion nectar sample **9**	Upper Austria Eizendorf	**13.9**
Suspicion nectar sample **10**	Upper Austria Eizendorf	**18.8**
Suspicion nectar sample **11**	Upper Austria Eizendorf	**24.4**

* All other analytes were <LOD for all samples

It has to be noted that the nectar samples were stored in a laboratory cupboard at room temperature. At the time of sample preparation and analysis the nectar had already started to ferment. It is unknown whether this degradation process had any influence on the content of neonicotinoid residues in the nectar samples. In any case it was found that nine out of eleven samples contained thiacloprid in considerable quantities ranging from 11.1 to 81.2 µg/kg. All samples collected in Upper Austria contained thiacloprid, while from the samples originating from Lower Austria three were positive and two negative.

The investigation of neonicotinoid insecticides in the different Austrian honey and nectar samples showed the presence of three neonicotinoids: thiacloprid (27 positive samples), acetamiprid (two positive samples) and thiamethoxam (one positive sample). Thiacloprid was detectable in concentrations up to 27.4 µg/kg in honey and 81.2 µg/kg in nectar, respectively. The maximum concentration of acetamiprid in honey was 15.2 µg/kg, whereas thiamethoxam was only detectable in traces.

These results are in good agreement with recent investigations in Germany regarding the presence and concentrations of neonicotinoid insecticide residues in honey. In 2008 the CVUA Stuttgart conducted several analyses of honey samples from Southern Germany with focus on neonicotinoid insecticides (Chemisches und Veterinäruntersuchungsamt Stuttgart 2008a&b). In a first investigation two neonicotinoids, thiacloprid and thiamethoxam, were detected in the analysed honey samples. Thiacloprid was found in 75 % of the samples in concentrations between 2 and 110 µg/kg whereas only one honey sample contained traces of thiamethoxam (1 µg/kg). None of the other investigated neonicotinoids insecticides (acetamiprid, clothianidin, imidacloprid and nitenpyram) was detectable in the samples (Chemisches und Veterinäruntersuchungsamt Stuttgart 2008a). In further investigations of pesticide residues by analysing for 500 substances in 67 honey samples thiacloprid was detectable in four honey samples in concentrations of 7 to 45 µg/kg (Chemisches und Veterinäruntersuchungsamt Stuttgart 2008b).

Comparing the different categories of honey samples (suspicion honey samples, flower honey samples and forest honey samples) suspicion honey samples showed the largest share of positive samples as well as the highest concentrations of thiacloprid. While seven of the thirteen suspicion honey samples contained thiacloprid (54 %), the ratio was seven out of 19 for the flower honey samples (37 %).

As expected forest honey showed no or only very low residues of neonicotinoid insecticides. The production of forest honey is based on the incorporation and further processing of sugar-rich secretions of aphids by honeybees. Forest honey might contain a small percentage of flower honey, if some honeybees of the beehive collect nectar from flowering plants growing in and close to the forest.

Thiacloprid was the neonicotinoid insecticide mainly detected in the investigated honey and nectar samples. One reason for this might be the low bee-toxicity of thiacloprid. Insecticides of lower bee-toxicity are generally more likely to be transported into the beehive since honeybees endure higher doses of these

compounds. Thiacloprid is moderately toxic to honeybees with an acute oral LD50 of 17.32 µg/bee (Circa “List of end points”). Due to this comparably low bee-toxicity honeybees are not exposed to lethal doses of the substance during their foraging activities, and are thus able to transport thiacloprid into the beehive. This reasoning can also be applied to the finding that the moderately bee-toxic neonicotinoid acetamiprid (acute oral LD 50: 8.85 µg/bee) was also detected above the LOQ in two of the analysed samples, while only traces of one highly bee-toxic neonicotinoid (thiamethoxam, acute oral LD 50: 0.005 µg/bee) were detected in a single sample.

Another reason for the more frequent detection of thiacloprid compared to the other investigated neonicotinoid insecticides might as well be the wide and versatile application of plant protection products containing thiacloprid as active ingredient. Thiacloprid is the active substance of the widely used plant protection product Biscaya. Biscaya is a pesticide spray applied in different agricultural crops such as barley, maize, rape, poppy seed, rye, oat, wheat and potato (Register of Authorised Plant Protection Products). Both the application form and the applicability to a large number of different crops might be possible explanations for the wide-spread presence of thiacloprid in honey and nectar samples.

The potential application of thiacloprid in maize and rape crops which constitute large proportions of the overall Austrian crop area (cultivation in 2009: maize 178,000 ha, rape 56,000 ha (Landwirtschaftskammer Österreich 2009a&b)) and the frequent proximity of these fields to beehives raised the question if the results of the investigations show a geographical correlation with the cultivation of maize and rape. Figure 33 and Figure 34 show maps of Austria displaying the proportion of maize or rape of the total cropland. The origins and concentration levels of thiacloprid of the various analysed samples are marked with different colours and symbols.

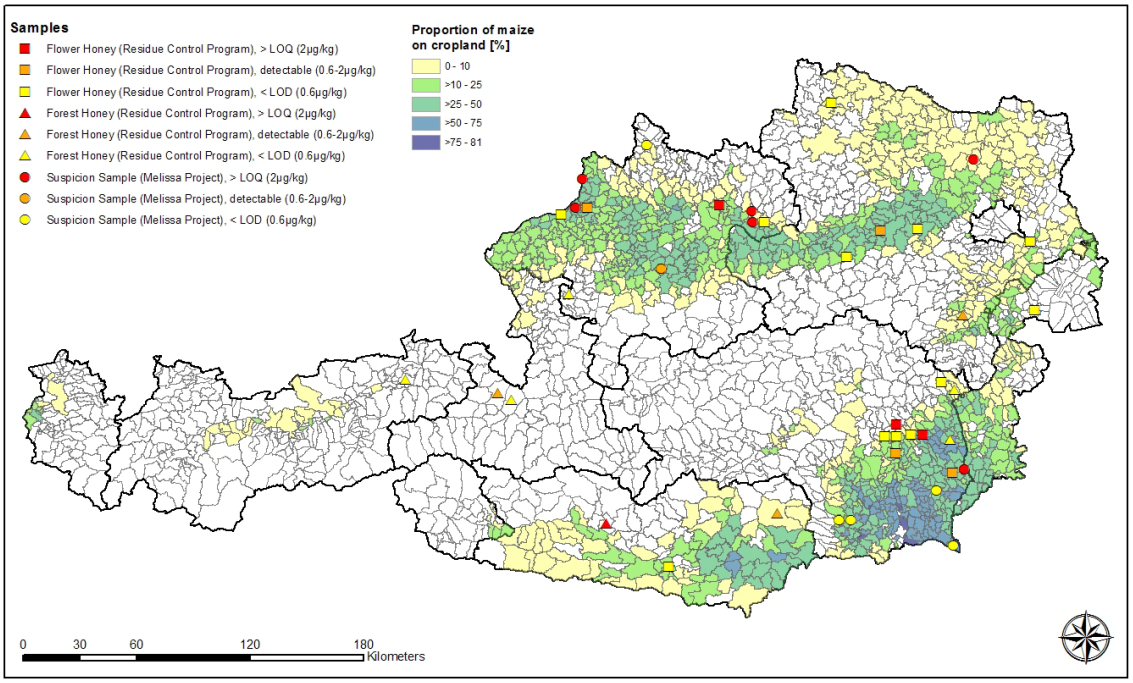

Figure 33: Map of Austria showing maize cultivation areas, origins of the analysed samples and concentration levels of thiacloprid in the samples

The main regions of maize cultivation are located in the South-Eastern part of Styria, in Southern Burgenland, in central Upper and Lower Austria as well as in Eastern Carinthia. Most of the investigated honey samples originated from one of these regions. All suspicion and flower honey samples were collected in Austrian provinces with substantial areas of maize cultivation.

No clear correlation of the geographical origin of the samples and the intensity of the corresponding local maize cultivation is visible. On the one hand samples containing thiacloprid residues were found in regions with high as well as low proportions of maize cultivation. On the other hand negative samples could also be found in regions with a high proportion of maize cultivation.

An equivalent picture was observed for the comparison of the analytical results with the cultivation of rape (Figure 34).

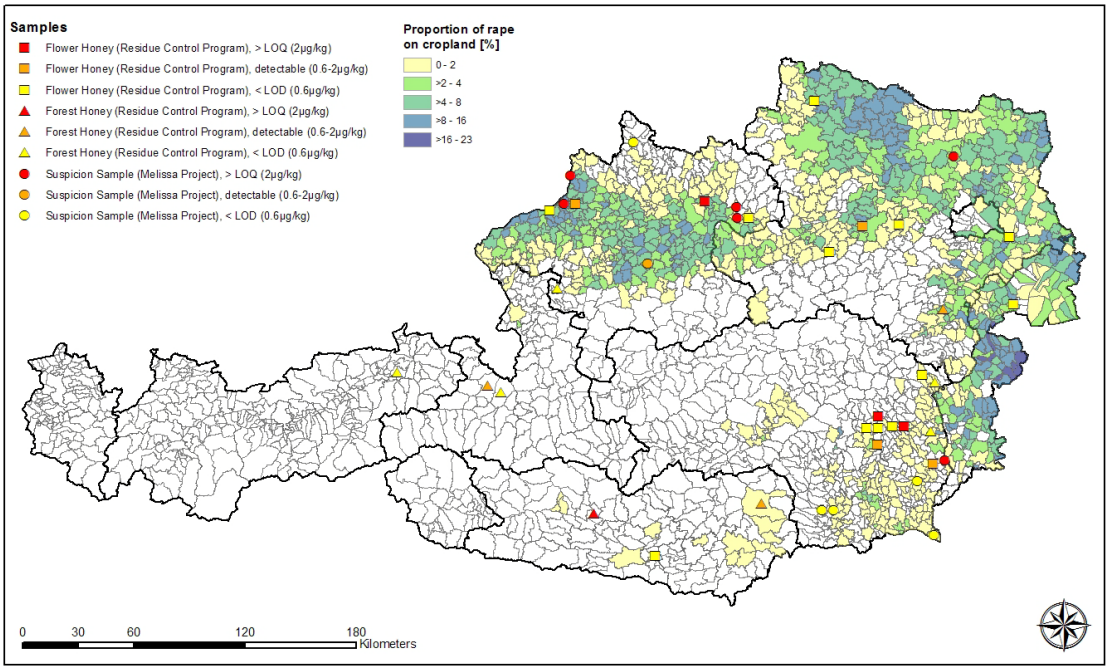

Figure 34: Map of Austria showing rape cultivation areas, origins of the analysed samples and concentration levels of thiacloprid in the samples

Most of the rape cultivation is concentrated in Central Upper Austria, Northern Lower Austria and Burgenland.

The presence of thiacloprid in samples from Styria, Carinthia and Salzburg is most likely not linked to local rape cultivation. Further, the comparison of the distribution of positive and negative samples with the proportion of rape cultivation on cropland in provinces with rape cultivation does not reveal conclusive evidence.

There are several possible reasons for the presence of thiacloprid in samples from locations with no or little cultivation of maize or rape. The honeybees that collected and produced positive samples in areas with no or little cultivation of maize or rape may have come in touch with thiacloprid applied to other agricultural crops such as barley, rye or wheat. Since the intensity of maize or rape cultivation is expressed as the proportion of maize or rape cultivation on total cropland per municipality there is no information on the geographic

proximity of honeybees of a certain beehive to treated maize or rape fields and thus their effective exposure. It is therefore possible that even in municipalities with a small proportion of maize or rape cultivation a beehive is located next to maize or rape fields. Another potential factor is the information on the geographical origin of the honey samples. The information on the origin of the suspicion honey samples is provided by the beekeepers and thus should not be flawed. However, the origin of the honey samples from the residue control program refers to the place of residence of the beekeeper which is not necessarily equivalent to the location of the beehive. Consequently, the exact geographical origin is not exactly identifiable for most of the analysed samples, even though one can assume that beekeepers favour to keep their beehives near to their domicile. A further explanation might be the fact that honeybees collect in the circumference of several kilometres and possibly pass municipal borders. Thus the positive or negative samples may result from nectar collection by honeybees in a neighbouring municipality with a larger or smaller proportion of maize or rape compared to the municipality of the beehive.

Beside positive honey samples from areas with no or little cultivation of maize or rape the phenomenon of negative samples from areas with intensive maize or rape cultivation was observed as well. The same reasons as mentioned above might be responsible for these findings. Furthermore, the honey may have been produced outside of the application period of thiacloprid.

Finally the limited number of investigated honey samples added to the difficulty of linking the analytical findings of thiacloprid to maize or rape cultivation regarding geographical distribution. A higher number of samples as well as an even geographical distribution of the samples might provide a better basis for establishing such a correlation on a firm basis.

The quantities of the neonicotinoid residues found in the investigated honey and nectar samples did not exceed the maximum residue limit in any case. Thus, all honey samples could be sold legally on the market. Even though no maximum residue limit of the investigated neonicotinoid insecticides was exceeded in the positive samples, the contamination of honey with neonicotinoid insecticides

raises the question whether these substances might represent a potential danger for consumers or not. Taking into account their particular target-selectivity neonicotinoid insecticides can basically be described as safe to mammals (Matsuda et al. 2009). Additionally, the exposure of human-beings to neonicotinoid compounds in honey can be seen as rather moderate because of the quantitatively marginal intake of honey (1.2 kg/year per capita intake in Austria in 2008) as well as the low levels of contamination. Thus, negative impacts of the detected residues of neonicotinoid insecticides in the honey samples on the health of the consumers are not to be expected. This conclusion concerning the health risk for consumers is in agreement with that from Bogdanov (Bogdanov 2006b).

Nevertheless, the presence of neonicotinoid residues in honey indicates the need for ongoing analyses. Depending on the future application of neonicotinoid insecticides on Austrian agricultural crops a regular monitoring of neonicotinoid residues in honey samples may be reasonable.

5 Conclusions

The investigations of neonicotinoid insecticides in the guttation liquid of maize plants grown from dressed seeds showed the presence of notable concentrations of neonicotinoids. These findings are in good agreement with similar analyses at the Universities of Padua (Girolami et al. 2009) and Hohenheim (Wallner 2009b) as well as at Bayer CropScience (Nikolakis 2009). The transfer of neonicotinoid insecticides from seed dressings into the guttation liquid of maize plants grown from dressed seeds can therefore be considered as firmly established. The detected concentrations of neonicotinoids suggest that guttation liquid can be a possible route of exposure of honeybees and other non-target insects to neonicotinoids and may represent a potential threat for these organisms. However, the importance of guttation liquid as water source for honeybees and its ensuing impact on the loss of bee colonies is not yet clear (Wallner 2009b). Further investigations regarding the attractiveness of guttation liquid as water source for honeybees may thus provide important information for risk assessment and management. The detection of deficiencies in the seed dressings of the utilized maize seeds suggests that a regular monitoring of seed dressing quality would be useful.

The LC-MS/MS method developed for the analysis of neonicotinoid residues in honey proved to be rapid, sensitive and reliable. It encompassed all analytes contained in the EU residue definitions for honey for all eight neonicotinoid insecticides developed to date. The detection of three neonicotinoids (acetamiprid, thiacloprid, thiamethoxam) in honey and nectar samples confirms the actual occurrence of a transfer of neonicotinoid insecticides from honeybees into honey and indicates the need for further analyses and the usefulness of a regular monitoring of honey. The residue concentration levels of neonicotinoids in the analysed honey and nectar samples in the lower ppb-range were in good accordance with analyses of neonicotinoids in honey samples carried out by the CVUA Stuttgart in 2008 (Chemisches und Veterinäruntersuchungsamt Stuttgart 2008a&b).

Despite the fact that none of the positive samples exceeded a maximum residue limit, the least possible contamination of honey with pesticides is desirable, especially considering the high consumer expectations regarding the purity of honey. Simply the message of the presence of neonicotinoid residues in honey might be a threat for its good image. However, a more serious problem than possible residues in honey might be the potential danger of the neonicotinoid insecticides for honeybee health if one considers the major share of honeybees in the pollination of agricultural crops.

The obtained results of the honey and nectar samples did not indicate a geographic correlation between the origin of the samples and the proportion of maize or rape cultivation on cropland. Further investigations with a larger number of samples and an optimum distribution of the samples' origins throughout Austria might provide more conclusive evidence.

The conflict between the application of neonicotinoid insecticides as an effective measure of pest management in agriculture and the health of honeybees, considering their important pollination work and production of high quality bee products, requires a close and effective collaboration of agriculture, apiculture, science and authorities. Multidisciplinary investigations and the implementation of effective measures are necessary to ensure both optimum agricultural crop protection and maximum safety for beneficial insects such as honeybees in the future.

6 Summary

Neonicotinoid insecticides belong to the most important pesticides in the protection of agricultural crops. Honeybees may come into contact with them as a consequence of their wide-spread application. The first matter of investigation of this thesis dealt with a possible exposure route of honeybees towards neonicotinoid insecticides, focusing on the detection and quantification of neonicotinoids in the guttation liquid of maize plants cultivated from neonicotinoid-treated seeds using LC-MS/MS. The investigated neonicotinoids clothianidin, imidacloprid and thiamethoxam were detectable in the guttation liquid in considerable quantities in the ppm range. In the context of the possible exposure of honeybees to neonicotinoid insecticides these pesticides might be transferred into honey which represents a food with very high purity demands by consumers. In this context the main objective of this thesis was the development and validation of an analytical method for the simultaneous determination of neonicotinoid insecticides in honey and the subsequent analysis of Austrian honey and nectar samples. A total of eight neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, flonicamid, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) and four metabolites (IM 2-1, TFNA-AM, TZMU and TZNG) were included in the multi-residue method. The final method involved a sample preparation procedure based on acetonitrile extraction and subsequent clean-up by dispersive solid-phase extraction followed by detection and quantification using LC-MS/MS. Three neonicotinoid insecticides were detectable in the analysed honey samples: 18 out of 41 samples contained thiacloprid, two samples acetamiprid and one sample traces of thiamethoxam. Honey samples from beehives with reported losses of honeybees proved to be contaminated more often and with higher amounts of thiacloprid than standard monitoring samples. Further, flower honey samples contained on average higher thiacloprid residues than forest honey samples. Nine out of eleven nectar samples contained thiacloprid. A correlation of positive samples with areas of high proportion of maize and rape cultivation could not be established.

7 Zusammenfassung

Neonicotinoide gehören zu den wichtigsten Pestiziden, die zum Schutz von landwirtschaftlichen Kulturen eingesetzt werden. Aufgrund der weit verbreiteten Anwendung können Honigbienen mit diesen Substanzen in Berührung kommen. Ein möglicher Expositionsweg stellt das Guttationswasser von saatgutbehandelten Pflanzen dar, welches im ersten Teil der Untersuchungen dieser Diplomarbeit anhand von Proben neonicotinoid-gebeizter Maispflanzen mittels LC-MS/MS analysiert wurde. Die untersuchten Neonicotinoide Clothianidin, Imidacloprid und Thiamethoxam konnten im Guttationswasser in Konzentrationen im ppm-Bereich nachgewiesen werden. Als Folge einer möglichen Exposition von Honigbienen gegenüber Neonicotinoiden könnten diese Substanzen in den Honig gelangen, welcher vom Konsumenten als ein besonders reines Lebensmittel angesehen wird. In diesem Zusammenhang war das Hauptziel dieser Diplomarbeit die Entwicklung und Validierung einer analytischen Methode für die simultane Bestimmung von Neonicotinoiden in Honig sowie die anschließende Untersuchung von österreichischen Honig- und Nektarproben. Die Methode umfasste insgesamt acht Neonicotinoide (Acetamiprid, Clothianidin, Dinotefuran, Flonicamid, Imidacloprid, Nitenpyram, Thiacloprid und Thiamethoxam) und vier Metabolite (IM 2-1, TFNA-AM, TZMU und TZNG). Die Probenaufarbeitung basierte auf einer Extraktion mit Acetonitril und anschließender Aufreinigung mittels dispersiver Festphasenextraktion. Der Nachweis und die Quantifizierung der Analyte erfolgte mit LC-MS/MS. Drei Neonicotinoide konnten in den Honigproben nachgewiesen werden: 18 von 41 Proben enthielten Thiacloprid, zwei Proben Acetamiprid und eine Probe Spuren von Thiamethoxam. In Honigproben von Bienenvölkern, welche von Bienenverlusten betroffen waren, konnte Thiacloprid häufiger und in höheren Konzentrationen nachgewiesen werden als in normalen Monitoringproben. Weiters enthielten Blütenhonige durchschnittlich höhere Thiacloprid-Rückstände als Waldhonige. Neun von elf Nektarproben enthielten ebenfalls Thiacloprid. Eine Korrelation zwischen den positiven Proben und Gebieten mit hohem Anteil an Mais- und Rapsanbau konnte nicht festgestellt werden.

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All photographs included in this work have either been taken by myself during the investigations in the Austrian Agency for Health and Food Safety or were courtesy of Dr. R. Moosbeckhofer from the Institute for Apiculture.

9 Publication

A poster entitled “Investigations of the effects of the application of neonicotinoid insecticides on apiculture by LC-MS/MS” was presented at the Euroanalysis 2009 conference in Innsbruck.

Abstract:

INVESTIGATIONS OF THE EFFECTS OF THE APPLICATION OF NEONICOTINOID INSECTICIDES ON APICULTURE BY LC-MS/MS

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The neonicotinoids such as clothianidin, imidacloprid and thiamethoxam, constitute a class of potent and widely used insecticides. They are used inter alia as seed treatment agents in maize crop as a measure of pest control against the corn rootworm. However, the neonicotinoid insecticides are highly toxic towards bees. Bees might come into contact with the neonicotinoids upon the sowing of treated seeds, if a substantial abrasion of the insecticide occurs. Another route might consist of plant exudates, such as guttation droplets and nectar. Consequently, the use of neonicotinoids may have negative apicultural effects in a direct way in terms of beekeeping (loss of bees) as well as in an indirect way as honey may become contaminated with residues of these pesticides. In this context investigations using LC-MS/MS were carried out. A method for the determination of neonicotinoid residues in honey and other matrices was developed. Details regarding sample preparation, measurement and data evaluation are presented and the obtained results discussed.

10 CV

Curriculum Vitae

Gina Tanner

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Personal information:

Date of birth:	7th December 1984
Nationality:	Swiss

Education:

04/2009-date:	Master Thesis „Development of a Method for the Analysis of Neonicotinoid Insecticide Residues in Honey using LC-MS/MS and Investigations of Neonicotinoid Insecticides in Matrices of Importance in Apiculture", Austrian Agency for Health and Food Safety, Vienna
2006-date:	Studies of Nutritional Sciences Vienna 2nd degree
2004-2006:	Studies of Nutritional Sciences Vienna, 1st degree, passed with distinction
1999-2003:	Secondary school Frauenfeld CH and Yverdon-les-Bains CH bilingual graduation German-French / major subject chemistry and biology
1991-1999:	Primary school Weinfelden CH

Professional Experience

01/2007-07/2008	part-time nutrition expert in an online nutrition coaching programme, Coop Switzerland/ xx-well.com Berlin
07/2008	Internship, Austrian Association for Nutrition, Vienna;
	Creation of an article about poppy seed: “Mohn zwischen Lohn und Hohn”, published in “Ernährung Aktuell”, 04/2008
	Literature research, processing of subject-specific enquiries of members, homepage updates
02-04/2007	Research internship at the Institute of Psychology, University of Basel; Swiss national project „sesam“
	Collaboration in laboratory studies about epigenetic information and metabolic profiling, preparation of preliminary nutrition studies
07-08/2007	Research internship at the Institute of Social and Preventive Medicine, University of Bern;
	Collaboration in a project about low cost product lines (organisation, literature research, data collection and evaluation)

Stays abroad

07-09/2006	Brazil, teaching activity in a social project
02/2005	Guatemala, botanical excursion
03-07/2004	England, language school

Personal Skills

Languages:	German, Mother tongue
	French, Maturité bilingue français-allemand
	English, CPE Proficiency Grade B
Computer skills:	Microsoft Office (Word, Excel, Power Point), SPSS, Analyst

Vienna, 2nd January 2010