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SYSTEMIC INSECTICIDES, THEIR DEGRADATION PRODUCTS AND METABOLITES IN THE ENVIRONMENT. QUANTIFICATION METHODOLOGIES IN ENVIRONMENTAL SAMPLES RELEVANT FOR TOXICOLOGICAL AND ECOTOXICOLOGICAL STUDIES

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Index

Abstract

Systemic insecticides are widely used for pests control and their success is due to their ability of protect the whole plant from roots to the upper leaf. In particular, seeds coating technique is very popular and it is applied to many crops (e.g. corn). However, the use of high amount of Active Ingredient (AI) for seeds coating is causing concern about negative effects to non-target animals. Pollinators insects are exposed to contaminated pollen and nectar, but also herbivore insects are exposed through contaminated leaf. In addition, these insecticides can leach from fields and contaminate wild plants or waterbodies. Therefore, also aquatic species are exposed to insecticides pollution and vertebrates like birds and small mammals could be exposed through coated seeds, seedling and insects.
The aim of this study was to develop an UHPLC-HRMS method for the identification of insecticides and their degradation production in corn guttation drops. Particular attention was posed to metabolites, because few information are available in the literature about their presence in relevant matrix for eco-toxicological studies. In addition, some metabolites may have greater toxicity if compared with their parent compounds. In particular, neonicotinoids imine metabolites are characterised by an inversion of selectivity between insects and mammals. Therefore, they can be more toxic for mammals if compared to the neonicotinoids AI.
Several metabolites were identify in corn guttation and an extraction procedure based on QuEChERS strategy coupled with a target UHPLC-MS^2 method was developed and validated for the quantification of these compounds in corn leaf. High concentration of neonicotinoids thiamethoxam and thiacloprid were observed in corn seedling. In addition, high concentration of the thiamethoxam metabolite clothianidn was observed. Concerning the carbamate methiocarb, the AI was observed only at low concentration, but its metabolites were present at $\mu g/g$ level. Particularly interesting was the presence of methiocarb sulfoxide, because this metabolite is more toxic of the parent compounds for some species.
In conclusion, guttation analysis with UHPLC-HRMS is a powerful technique in order to assess the presence of insecticides metabolites in plants treated with systemic AI. However, UHPLC-MS^2 still provide better performance for quantitative analysis, in particular for complex matrices as corn leaf. Therefore, HRMS and MS^2 are complementary technique useful to provide levels of contamination and exposure.

Chapter 1

Introduction

1.1 Systemic insecticides

Systemic insecticides were first developed in the 1950s, with the introduction of soluble organophosphorus (OP). Systemic carbamates followed in the 1960s with aldicarb and carbofuran. Since 1990 onwards, cartap, fipronil and neonicotinoids are replacing the old chemicals (Sánchez-Bayo et al. 2013). Systemic pesticides are compounds able to penetrate into the plant tissue and to move upward and downward in the plant's xylem and phloem. Usually they are compounds with a relative high water solubility and their translocation into the xylem sap is influenced by the lipophilicity of the compounds (Sicbaldi et al. 1997). The term systemic underline the ability of these compounds of using the plant vascular system in order to reach distant location of the plant and the systemic distribution can be achieved by foliar application as well as by roots uptake. Therefore, systemic pesticides can be applied with many different technique as: spray, seeds coating and granular for soil. Irrespective of their mode of application, neonicotinoids become distributed throughout the plant, including the apices of new vegetation growth, making them particularly effective against sucking pests (Simon-Delso et al. 2014). They are also valuable in controlling burrowing larvae in many crops (Sánchez-Bayo et al. 2013). They are not only able to protect the plant from direct damage by herbivorous (mainly sap feeding) insects, but also indirectly from damage by plant viruses that are transmitted by these insects (Simon-Delso et al. 2014). It is the systemic nature of these insecticides that has made them so successful and the technique of seed coating is probably the most used for using this products. It consists in the application of the pesticide directly to the plant seed previously of sowing and it is a very successful strategy of pesticides application. One great advantage of this technique is that the plant will be protected in the early stage of growing, because the Active Ingredient (AI) will be dissolved into the soil and it will protect plant roots from pest. The AI is released in soil and it will penetrate in the seedling from the roots. Furthermore, the systemic insecticides will protect plant seedling also from sucking pests. In addition, through seed coatings and granular applications, systemic insecticides pose minimal risk of pesticide drift or worker exposure in agricultural, nurseries and urban settings (Sánchez-Bayo et al. 2013).

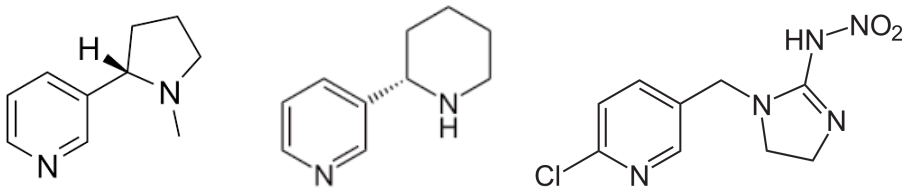
However, the drawback of this application strategy is that the treatment is prophylactic and it does not take into account if an effective pest attack is occurred. Therefore, for every sowing an high amount of AI is released in the field (around 1 mg/seed, depending on the crop and the insecticide used). Seeds coating was supposed to reduce the environmental spread of AI typical of the spray application, but only a small portion of the applied AI actually reach the treated plant (Alford and Krupke 2017). Instead, the bulk remain in soil and from there it can leach and contaminate wild plant and water bodies close to the field (Goulson 2014). Several studies reported insecticides leaching from fields and the relative high water solubility of systemic insecticides is a key factor in this process (Anderson et al. 2015; Bonmatin et al. 2015). The systemic insecticides neonicotinoids were introduced in the watch list for the emerging pollutants in the surface water by the European Union (EU) (Carvalho et al. 2015) and the situation is particularly critical in Holland, where high amount of insecticides were used for horticultural flowers production (Van Dijk et al. 2013). In addition, recently neonicotinoids insecticides were detected also in the tap water in USA (Klarich et al. 2017). Therefore, the seeds coating technique seems not to respect the Good agricultural practice (GAP), because it requires higher amount of AI to ensure crops protection and it causes high environmental contamination. Seeds coating utility is now under question (Krupke et al. 2017; Sgolastra et al. 2017b). In addition, due to their systemic distribution, systemic insecticides contaminate also pollen and nectar of treated plants (Bonmatin et al. 2005). For this reason their effects to pollinator insects were intensively studied in these years (section 1.4).

1.2 Active Ingredient studied

1.2.1 Neonicotinoids

Neonicotinoids insecticides are a particular class of systemic insecticides and they were introduced in the 1990s as AI for pests control. Nicotine is a natural insecticide extracted from tobacco plant and it is used since 1690. Neonicotinoids are related to nicotine for their structure (Figure 1.1) and their name indicate a new class of insecticides, different from the former nicotinoid (Jeschke and Nauen 2008). Nithiazin can be considered the first neonicotinoid synthesised, but it was not developed commercially, in part, because of its photo-instability. Structure modification of nithiazin led to imidacloprid, and related nitroimines, all of which possessed enhanced photostability (Matsuda et al. 2001). Seven commercial neonicotinoids are now on the market: the five-membered neonicotinoids imidacloprid and thiacloprid (Bayer CropScience), the six-membered thiamethoxam (Syngenta) and four open-chain compounds nitenpyram (Sumitomo Chemical Takeda Agro Company), acetamiprid (Nippon Soda), clothianidin (Sumitomo Chemical Takeda Agro Company/Bayer CropScience) and dinotefuran (Mitsui Chemicals). In these years, neonicotinoids have substituted other insecticides like carbamate, organophosphate and pyretroids. In the 2010 they reached the 27 % of the global insecticides market with a value of 2.63 $ billions. Imidacloprid was the most sold insecticide in the 2008, but later it was partially replaced by

thiamethoxam and clothianidin (Simon-Delso et al. 2014). Now, they are one of the most used insecticides classes and they are the leader products for the seeds coating technique for which the 93 % in weight is relative to neonicotinoids AI (Elbert et al. 2008).
However, between 2006 and 2008, there was high concern about poisoning incidents of honeybees colonies and these compounds were identify as a possible cause. Therefore, in many European countries the neonicotinoids most toxic to honeybees were banned for seeds coating. In Italy they were banned in 2008 (d.p.r. No 290, 2008) and they are banned in all Europe since 2013 (Commission Implementing Regulation (EU) No 485/2013). Therefore, in these countries they were partially replaced by thiacloprid and methiocarb. The first one is a a neonicotinoid with low toxicity for honeybees, the latter is a carbamate insecticide.
The neonicotinoid were presented as having several key attributes that led to their rapid adoption in both agricultural and urban environments. These included the following: lower binding efficiency to vertebrate compared to invertebrate receptors, indicating selective toxicity to arthropods, high persistence, systemic nature, versatility in application (especially as seed treatments), high water solubility, and assumed lower impacts on fish and other vertebrates. Neonicotinoids are also relatively persistent, offering the potential for long-term crop protection activity. The half-lives of these compounds in aerobic soil conditions can vary widely, but they are measured in months or longer (Simon-Delso et al. 2014).
Neonicotinoids act as nicotinic acetylcholine receptor (nAChR) agonists, therefore they interact with the acetylcholine receptors of the neurons system causing paralysis and death of the insects. The specific interaction between acetylcholine receptors and neonicotinoids is well studied (Ihara et al. 2015). They are highly toxic for honeybees, but cyano substituted neonicotinoids like thiacloprid are less toxic compared to the nitro substituted thiamethoxam and clothianidin (Iwasa et al. 2004). Their use rapidly grow due to excellent selectivity in toxicity for insect compared to mammals. In fact, they are currently used also for veterinary purpose. However, change in their structure can lead to an inversion of selectivity with toxic effects for mammals (Tomizawa and Casida 2000, 2003).

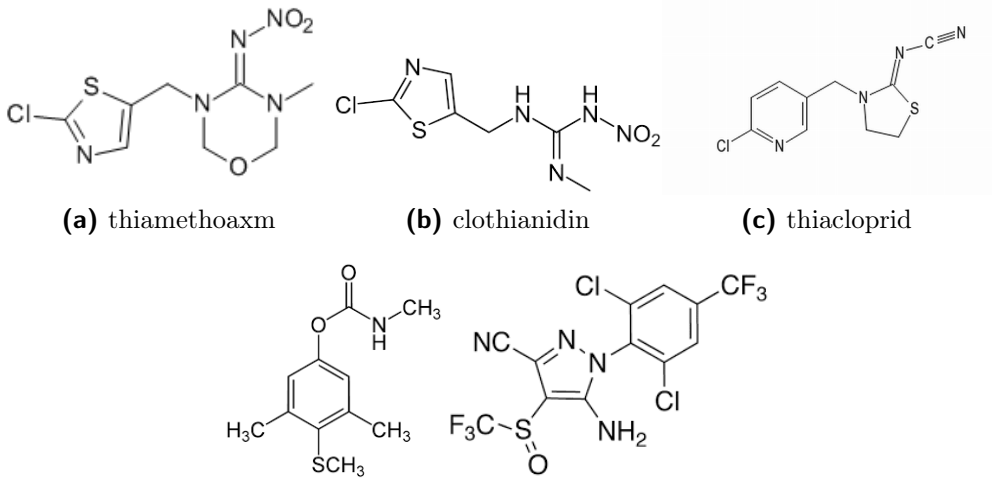


(a) Nicotine **(b)** Neonicotine **(c)** Imidacloprid

Figure 1.1: structures of nicotine, neonicotine and imidacloprid

1.2.2 Methiocarb

Methiocarb is a carbamate pesticide known since 1964 as a bird repellent (Guarino 1972). Now, it is used as a wide spectrum AI against insects, mites and mollusc and, since the neonicotinoids ban, it was used as a coating product for corn seeds in



(a) thiamethoaxm **(b)** clothianidin **(c)** thiacloprid

(d) methiocarb **(e)** fipronil

Figure 1.2: structures of the studied Active Ingredient.

Table 1.1: structures and identification numbers of the studied Active Ingredient.

Active Ingredient	CAS number	MW (g/mol)	raw structure
Thiamethoaxm	153719-23-4	291.71	$C_8H_{10}ClN_5O_3S$
Clothianidin	210880-92-5	249, 70	$C_6H_8ClN_5O_2S$
Thiacloprid	111988-49-9	252, 72	$C_{10}H_9ClN_4S$
Methiocarb	2032-65-7	225.31	$C_{11}H_{15}NO_2S$
Fipronil	120068-37-3	437, 15	$C_{12}H_4Cl_2F_6N_4OS$

Italy. Recently, it was introduced in the watch list for potential pollutants present in surface water together to neonicotinoids (Carvalho et al. 2015). However, the uses of this active substance was restricted by withdrawing the authorisation for its use as a molluscicide (Commission Implementing Regulation (EU) No 187/2014) . Methiocarb was still included in the Watch List to gather information on the post-banning environmental concentration, in order to assess whether the banning as a molluscicide has been effective in eliminating the risk from this substance (Carvalho et al. 2015).

This insecticides is substantially different from neonicotinoids for physical chemical proprieties (Table 1.2). It does not act as a systemic compounds and it is also rapidly degraded in the environment. Its toxicity is due to the interference with the acetylcholine mechanism of the nervous system like the neonicotinoids. However, it is not an agonist of the nAChR, but it blocks reversibly the enzyme responsible for acetylcholine degradation (Buronfosse et al. 1995). Methiocarb toxicity profile is completely different from neonicotinoids. It is not highly selective for insecticides and its toxicity for mammals is relevant. Therefore, it poses risk also for worker operating with this insecticide. It is also classified as highly toxic for birds,

Table 1.2: physical-chemical proprieties of the studied Active Ingredient (AI).

Active Principle	melting point (℃)	density (g/mL)	solubility in water at 20 ℃ (mg/L)	K_{ow} at pH 7 and 20 ℃	vapour pressure at 25 ℃ (mPa)	Henry constant at 25 ℃ ($Pa \cdot m^3 \cdot mol^{-1}$)
Thiamethoxam	139.1	1.57	4100	0.741	6.60×10^{-6}	4.70×10^{-10}
Clothianidin	176.8	1.61	340	8.04	2.8×10^{-8}	2.90×10^{-11}
Thiacloprid	136.0	1.33	184	18.2	3.0×10^{-7}	5.00×10^{-10}
Methiocarb	118.5	1.25	27	1.51×10^{3}	0.015	1.2×10^{-4}
Fipronil	203	1.71	3.74	5.62×10^{3}	0.002	2.31×10^{-4}

acquatic organisms and insects. Even if it is characterised by a lower toxicity for honeybees compared to neonicotinoids, it is considered highly toxic for honeybees (EPA 1987). Therefore, the neonicotinoids ban indirectly cause the reintroduction of an AI less safe for humans and with an eco-toxicological profile that may be worst of the banned neonicotinoids.

1.2.3 Fipronil

Fipronil is an insecticide of the phenylpyrazole class. It is often group with neonicotinoids during environmental assessment, however its systemic proprieties are still debated (Mortensen et al. 2015). Like neonicotinoids it is characterised by high toxic selectivity for insects compared to mammals. However, fipronil act in a different way from neonicotinoids. Its toxicity is due to the interaction with the gamma-aminobutyric acid (GABA) receptors. Furthermore, fipronil block the glutamate-activated chloride channels (GluCls), which are present in insects cockroaches but not in mammals. GluCls play a crucial role in selectivity of fipronil to insects over mammals (Narahashi et al. 2010). Fipronil was used for seeds coating until it was ban in Europe together with neonicotinoids due to its high toxicity for honeybees (Commission Implementing Regulation (EU) No 781/2013).

1.3 Insecticides and pollinator insects

1.3.1 Decline of honeybees population

In 2006 and 2007 sever honeybees death was recorded by beekeepers in the USA and almost one third of the entire honeybees population was lost. These events were characterised by total lack of dead bees in the colony or apiary and the phenomena was called Colony Collapse Disorder (CCD) (vanEngelsdorp et al. 2008). Also in Europe, starting from 2002, many honeybees colonies were lost, but some differences were observed from the CCD and a high number of adult honeybees were found dead close to the hives. The worst episodes were between 2006 and 2008, when acute intoxication by insecticides was observed during spring, that caused the loss of entire colonies (Pistorius et al. 2010; vanEngelsdorp and

Meixner 2010). These incidents were caused by the release in the environment of dust produced from maize seeds treated with insecticide during the sowing process (section 1.3.2). In Italy, in 2007 and 2008 poisoning incidents were recorded during spring and high over winter mortality was observed (Mutinelli et al. 2010). Therefore, Italy banned insecticides particularly toxic for honeybees and used for corn seeds coating (imidacloprid, thiamethoxam, clothianidin and fipronil; d.p.r. No 290, 2008). Other European countries (France, Germany, Swiss and Slovenia) took actions in order to ban these insecticides. Furthermore, in 2013 the same AIs were banned in all Europe for seeds coating treatment (Commission Implementing Regulation (EU) No 485/2013).
The attention posed by the honeybees death triggered the beginning of monitoring program and scientific research focused on honeybees health and its relation with pesticides. However, the number of honeybees colonies in USA and Europe was already falling before 2006. In the USA from 1947 to 2008 the number of colonies passes from 5.9 millions to 2.3 (Figure 1.3) and in Europe since '70 a decline was observed and it was intensified after 1990s leading from 21 to 15.5 millions of colonies in the 2008 (Kluser and Peduzzi 2007; vanEngelsdorp and Meixner 2010). There are many factors involved in the honeybees population decline. Other then the already cited pesticides, honeybees are threatened by many disease. *Varroa jacobsoni* and *Varroa destructor* are very common parasites in the honeybees colonies and they can cause the colony death in 2-3 years if no treatments were applied (Rosenkranz et al. 2010). Other relevant parasites are *Nosema apis* and *Nosema ceranae*. Also, virus can affect honeybees colonies and particularly relevant are Deformed Wing Virus (DWV) and Acute Bee Paralysis Virus (ABPV). Furthermore, the Israeli Acute Paralysis Virus (IAPV) was correlated to the CCD (Cox-Foster et al. 2007). In addition, other factors can impair bee health as the lack of bee forage due to the changed agricultural practices as well as increased urbanisation. Increased world trade in non-bee products can also inadvertently introduce new bee pests and diseases. Also hives renting for pollination could impair the colonies health with negative effects due to the frequent movements of the colonies (vanEngelsdorp and Meixner 2010).
Honeybees are economically important for honey and other bee products production. However, they are widely more important for the pollination of crops, because 52 of the 115 principals crops are dependent from honeybees pollination (vanEngelsdorp and Meixner 2010). Globally the calculate value due to pollination is 212 billions of dollars equal to the 9.5 % of the total world agricultural market (vanEngelsdorp and Meixner 2010). Honeybees are not the only pollinator insects, but they are the most important specially in case of extensive mono-culture where wild species are rare. However, also wild pollinators are important to provide the eco-system service of pollination. They not only sustain the crop, but also provide a vital service for wild plants. Therefore, the decline of pollinator insects is a threat for the whole eco-system.

1.3.2 Insecticides exposure routes for pollinator insects

Information about environmental contamination by systemic pesticides are well summarized in Bonmatin et al. 2015 and Giorio et al. 2017. Table 1.3, 1.4, 1.5 and

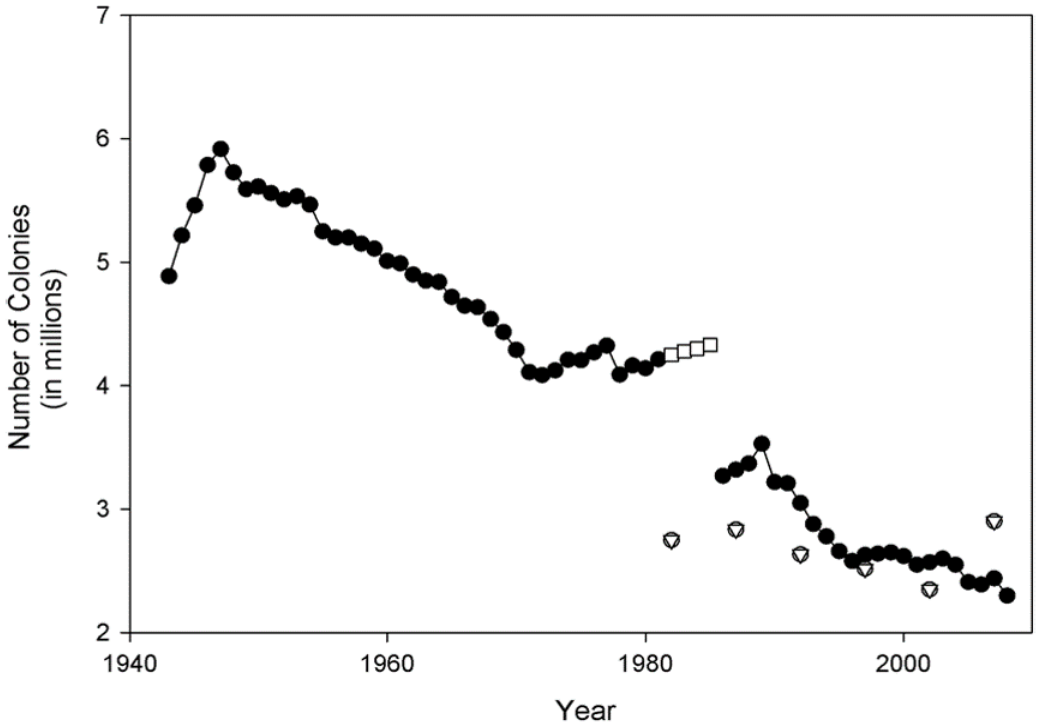


Figure 1.3: fall of honeybees population in the USA (vanEngelsdorp and Meixner 2010).

1.6 are adapted from Giorio et al. 2017 and they summarize the information available in the literature about concentrations of these compounds in environmental samples, products and non-target animals.

Pollinators insects can be exposed to insecticides in many different ways, but the most studied exposure route is the collection of contaminated pollen and nectar. Because systemic pesticides are able to penetrate and diffuse into the plant tissues, also pollen and nectar of treated crops are contaminated independently from the method used for pesticides application. Neonicotinoids residues were detected in pollen collected from corn plants grown from coated seed (Bonmatin et al. 2005). Further studies have underlined that not only treated crops contain pesticides residue, but also wild plant growing close the a treated field could have contaminated pollen and nectar (Botías et al. 2016, 2015; Long and Krupke 2016). This imply a longer exposure due to different blooming periods. Furthermore, insecticides concentration in wild plants pollen and nectar resulted to be more variable compared to treated plants and sometimes higher concentration were detected. Analysis of bees and bee collected pollen revealed that pollinators insects are highly exposed to pesticides residue both in agricultural and urban landscapes (Botias et al. 2017; David et al. 2016; Tosi et al. 2018). As a consequence, neonicotinoids are routinely identified in honeybees, honey, bee-bread and beeswax samples (Mitchell et al. 2017; Porrini et al. 2016; Table 1.6). The concentration usually detected in pollen and nectar are very low (few ng/g, Table 1.5) and no acute toxic effects were observed. However, honeybees store this contaminated material inside the hive where a complex situation of simultaneous long time exposure to several toxic chemicals could be realised. Therefore, many studies are

focused on the sub-lethal effects of pesticides to honeybees, that are discussed in more detail in the next section.
However, pollinators insects can also be exposed to high amount of insecticide causing lethal effects. One example is the exposure through guttation drops. Guttation are sap drops emerging from leaf edge and they are naturally produced, usually overnight or in early morning. Many plants are known to produce guttation (rice, wheat, barley, oats, corn, sorghum, tobacco, tomato, strawberry etc.) and many factors influence their production (wind, soil moisture, air relative humidity, etc.; Singh and Singh 2013). Guttation drops collected from corn plants grown from seeds coated with neonicotinoids contain high concentration of the AI (mg/L) and their ingestion by honeybees can kill them in few minutes (Girolami et al. 2009). However, guttation drops are not usually collected by honeybees and specific experiments showed that they are collected only if any other water source is absent (Frommberger et al. 2012). Therefore, guttation drops cannot be considered a relevant exposure route for honeybees. However, nothing is known about exposure of wild bees or other beneficial arthropods. Therefore, more studies about exposure through guttation drops are needed in order to obtain a proper risk assessment for wild insects.
As said above, another issue is the particulate matter produced during sowing operation. Coated seeds are abraded by seeds movement with the consequent formation of a powder containing the AI applied with coating. The pneumatic drilling machines used during sowing operation release in the environment part of this powder and a relevant amount of the applied AI (Krupke et al. 2012). The particulate matter produced is characterised by coarse particles that contaminate the surrounding vegetation (Greatti et al. 2006, 2003). This poses a serious risk for the pollinators insects that collect food from these vegetation. Furthermore, honeybees flying close the field during sowing operation can collect high amount of insecticides (Tapparo et al. 2012) and mortality tests have shown that particulate matter collected during flying can cause honeybees death (Girolami et al. 2013, 2012). It is now widely accepted by the scientific community that the high honeybees mortality observed in Europe during spring in the 2000s was caused by the exposure to these toxic powders (Porrini et al. 2016; Sgolastra et al. 2012).

1.4 Insecticides impact on non-target insects

The usual concentration of systemic pesticides observed in pollen and nectar (1-10 $\mathrm{ng\,g^{-1}}$) is insufficient to cause acute toxicity. However, the constant exposure of pollinators to low concentration of systemic pesticides present in pollen and nectar bring to study about the effects of this chronic exposure to low concentration of toxic compounds. The term sub-lethal has been used to describe all the negative effects caused by insecticide that cause a modification of pollinators behaviour or reproduction ability without death for acute toxicity (Desneux et al. 2007). These effects can impair colony ability to collect food leading to a lower over winter survival probability. Until now several different disturbs were observed in honeybees or bumblebees exposed to low concentration of neonicotinoids: reduced orientation ability (Henry et al. 2012), lower queen production (Whitehorn et al.

Table 1.3: residues of neonicotinoids and fipronil in dust and soil samples. Values indicate the range of concentrations (ng/g) and the frequency of detection (%).

Matrix	Clothianidin	Imidacloprid	Thiacloprid	Thiamethoxam	Fipronil	Reference
Dust (ng/g)						
Maize planting (Italy)	0.4 – 905[a]	11.9 – 2704[a]		3.0 – 940[a]	1.6 – 115[a]	Biocca et al. 2017
Urban dust (California, USA)					1 – 6188[b]	Richards et al. 2016
Maize planting (Canada)				0.05 – 8.41[b]		Xue et al. 2015
Corn fields (Canada)	17.8 – 42.3			10.2 – 65.0		Limay-Rios et al. 2016
Soil& sediment (ng/g d.w.)						
Canola fields (Midwest US)	4.4 – 21.4					Xu et al. 2016
Cocoa plantation (Ghana)	9.8 – 23.1 (10%)	4.3 – 251 (54%)				Dankyi et al. 2014
Corn field (Midwest USA)	2.0 – 11.2					de Perre et al. 2015
Corn fields (Canada)	0.16 – 0.2			4 ± 1.1		Schaafsma et al. 2015
Corn fields (Midwest US)	6.4 – 20.3					Xu et al. 2016
Cotton fields (China)					40 – 650	Wu et al. 2017
Maize fields (Canada)	2.9 – 5.1 (100%)			0.3 – 1.8 (86%)		Limay-Rios et al. 2016
Oilseed rape (UK)	5.1 – 28.6 (100%)	0.7 – 7.9 (100%)	<0.01 – 0.2 (43%)	0.5 – 9.7 (100%)		Botías et al. 2015
Rice fields (China)	17 – 600					Li et al. 2014
Rice fields (Japan)		50 – 280			10 – 90	Boulange et al. 2016
River sediment (China)		141 (87.5%)				Chen et al. 2015
Several crops (Canada)				5.6 ± 0.9		Schaafsma et al. 2016
Several crops (Central Europe)				72 – 98		Hilton et al. 2016
Wheat field margins (UK)	0.4 – 19.1 (100%)	<0.07 – 6.3 (75%)	<0.01 – 0.1 (25%)	<0.04 – 0.5 (50%)		Botías et al. 2015

[a]units: $\mu g\ m^{-3}$
[b]total parent + metabolite

Table 1.4: residues of neonicotinoids and fipronil in water samples. Values indicate the range of concentrations (ng/L) and the frequency of detection (%).

Matrix	Clothianidin	Imidacloprid	Thiamethoxam	Fipronil	Reference
Arade river (Portugal)		2.5 – 8.0 (100%)			Gonzalez-Rey et al. 2015
Corn fields (Canada)	2.28 – 43.6 (100%)		1.12 – 16.5 (98%)		Schaafsma et al. 2015
Drinking water (Iowa, USA)	3.9 – 57.3 (100%)	1.12 – 39.5 (100%)	0.2 – 4.1 (100%)		Klarich et al. 2017
Ebro river (Spain)		1.1 – 15.0 (45%)			Ccanccapa et al. 2016
Forest streams (N Carolina, USA)		29 – 379 (70%)			Benton et al. 2016
Groundwater (Wisconsin, USA)	210 – 3340 (20%)	260 – 3340 (24%)	200 – 8930 (55%)		Huseth and Groves 2014
Infiltration water (Midwest USA)	10 – 203				de Perre et al. 2015
Llobregat river (Spain)		2.1 – 66.5 (78%)			Masiá et al. 2015
Mekong river (Vietnam)			630 – 950 (4%)	170 – 410 (83%)	Chau et al. 2015
Pothole wetlands (Canada)	310 – 3500 (98%)	40 – 120 (48%)	290 – 6900 (54%)		Evelsizer and Skopec 2016
Reservoir (Brazil)		<0.7 – 3.0 (31%)		1.1 – 2.0 (91%)	López-Doval et al. 2017
Rice fields (China)	9.6 – 166				Li et al. 2014
Rice fields (Japan)		5.0 – 30		1.3 – 2.5	Boulange et al. 2016
Rice fields (Vietnam)		53 – 83			La et al. 2015
Rivers (California, USA)				30 – 13800 (100%)	Sengupta et al. 2014
Runoff water (Midwest USA)	<LOD – 850				de Perre et al. 2015
Rural streams (Germany)		2 – 20 (32%)	20 – 44 (10%)		Münze et al. 2015
Rural streams (Iowa, USA)	8.2 – 257 (75%)	<2 – 42.7 (23%)	<2 – 185 (47%)		Hladik et al. 2014
San Francisco Bay (USA)		13.5 – 1462 (80%)		1.1– 27.4 (81%)	Weston et al. 2015
Soybean crops (Canada)	3.0 – 40 (100%)		3.0 – 1090 (100%)		Chrétien et al. 2017
Stream (Brazil)			1230 – 1580 (100%)		Rocha et al. 2015
Streams (Indiana, USA)	6 – 671 (96%)	2 – 177 (90%)	15 – 2568 (98%)		Miles et al. 2017
Streams (USA)	34 – 64 (24%)	5.7 – 143 (37%)	7 – 190 (21%)	0.1 – 10 (84%)	Bradley et al. 2017
Streams (USA)	1.7 – 62 (56%)	2.1 – 65.9 (87%)	5.6 – 35.9 (44%)		Hladik and Kolpin 2015
Sugarbeet crops (Switzerland)		1290	2830		Wettstein et al. 2016
Wetlands (Canada)	59.7 – 3110 (76%)	7.1 – 256 (12%)	40.3 – 1490 (52%)		Main et al. 2015
WTP effluent (N Carolina, USA)				10 – 500 (100%)	McMahen et al. 2016

Table 1.5: residues of neonicotinoids and fipronil in plant samples. Values indicate the range of concentrations (ng/g) and the frequency of detection (%).

Matrix	Clothianidin	Imidacloprid	Thiacloprid	Thiamethoxam	Fipronil	Reference
Cotton seedlings					48 - 646	Wu et al. 2017
Foliage (oilseed rape)	1.3 - 8.7 (100%)	<0.2 - 3.1 (2%)		<0.1 - 2.6 (100%)		Botías et al. 2016
Guttation fluid (turfgrass)		23 - 88				Larson et al. 2015
Guttation fluid (oilseed rape)	10 - 132			3.2 - 12.9		Reetz et al. 2015
Nectar (canola)	0.3 - 2.4					Xu et al. 2016
Nectar (clover) mowed	6.2 - 18	8.4 - 26				Larson et al. 2015
Nectar (clover) sprayed	2882 - 2992	5493 - 6588				Larson et al. 2015
Nectar (oilseed rape)	<0.17 - 13.2 (31%)		<0.03 - 1.2 (54%)	<0.1 - 13.3 (54%)		Botías et al. 2015
Nectar (oilseed rape)	6.7 - 16					Rundlöf et al. 2015
Nectar (oilseed rape)	0.7 - 0.8					Rolke et al. 2016
Pollen (beans)				0.2 ± 0.3		David et al. 2015
Pollen (corn)	1.2 - 5.7					Xu et al. 2016
Pollen (oilseed rape)	6.6 - 23					Rundlöf et al. 2015
Pollen (oilseed rape)	0.5 - 0.97					Rolke et al. 2016
Pollen (oilseed rape)	<0.12 - 14.5 (90%)		<0.04 - 7.3 (86%)	1.0 - 11.1 (100%)		Botías et al. 2015
Pollen (oilseed rape)	<0.72 - 11 (73%)		<0.22 - 78 (100%)	2.4 - 11 (100%)		David et al. 2016
Pollen (raspberries)	6.0 ± 5.9		9.4 ± 2.1	23 ± 38		David et al. 2015
Pollen (strawberries)	8.9 ± 1.3	3.1 ± 5.4	5.9 ± 0.7	1.5 ± 0.3		David et al. 2015
Pollen (wildflowers)		<0.36 - 1.1 (13%)	<0.07 - 4 (63%)	<0.12 - 21 (50%)		David et al. 2016
Pollen collected by honey bees	<0.72 (8%)	<0.36 - 3.5 (12%)	<0.07 - 10 (48%)	<0.12 - 1.6 (64%)		David et al. 2016
Pollen in apiaries - treated maize fields	0.64 - 9.37 (22%)		0.25 (3%)	0.07 - 0.95 (22%)		Long and Krupke 2016
Pollen in apiaries - non treated land	4.7 (3%)	0.9 - 1.1 (7%)		0.5 - 1.7 (10%)		Long and Krupke 2016

Table 1.6: residues of neonicotinoids and fipronil in agricultural products and animal samples. Values (in ng/g) indicate average and maximum (in brackets) residues and their frequency of detection (%), unless a range is specified.

Matrix	Clothianidin	Imidacloprid	Thiacloprid	Thiamethoxam	Fipronil	Reference
Products						
Beebread	7.2 (18.4)					Pistorius et al. 2015
Beebread	12	0.5 (1.5)	0.2 (1.8) 29.3%	1.7		Vázquez et al. 2015
Beebread	<5%	25%	(177) 96%	25%		Daniele et al. 2017
Beebread	5.2 (15.7) 58%	4.2, 5%	<0.1	28.7 (62.5) 21%		Codling et al. 2016
Beeswax		<1, 2.5%	(3.4) 26%	(106.5) 3%		Daniele et al. 2017
Beeswax		3.0 – 5.1, 5%	4.0 – 10.4, 3%		1.0, 1%	López et al. 2016
Honey	13.7 (192.8)					Gbylik-Sikorska et al. 2015
Honey	0.25 (0.82) 72%	<0.1		0.27 (0.79) 68%		Jones and Turnbull 2016
Honey	6.7 (20) 68%	1.1 (6.2) 32%	14.4, 4%	19.4 (41.1) 75%		Codling et al. 2016
Honey	1.35					Rolke et al. 2016
Cabbage	74 (724)					Li et al. 2014
Honeysuckle leaves			22 (4400)	17 (3200)		Fang et al. 2017
Mango fruit		80 (3710)				Bhattacherjee and Dikshit 2016
Oilseed rape plants	<LOD – 6.5					Rundlöf et al. 2015
Rice grain (bran)				131 (244)		Teló et al. 2015
Rice grain (hull)				143 (225)		Teló et al. 2015
Rice grain (polished)				1.2 (4.0)		Teló et al. 2015
Winter melon		10 (210)				Huang et al. 2015
Animals						
Amphipods			0.1 (0.39)			Inostroza et al. 2016
Bumblebees	<0.48 – 1.4, 0.7%	<0.7 – 10, 7%	<0.02 – 1.17, 2%	<0.3 – 2.3, 6%		Botias et al. 2017
Eels					4.0 – 20[a]	Michel et al. 2016
Honey bees	2.50%	(1.7) 9%	(1.6) 13%	8%		Daniele et al. 2017
Honey bees	6.5 - 33					Pistorius et al. 2015
Honey bees	5.3 – 76.2	3.3 – 174	21.9 – 28.8	588	232 – 590	Kiljanek et al. 2016
Honey bees	2.5 – 7.1	0.1 – 11.1[a]				Codling et al. 2016
Honey bees	4 – 13.1	4.5 – 27[a]				Gbylik-Sikorska et al. 2015
Honey bees				0.3 – 0.95		Reetz et al. 2015

[a]Total residues of parent compound and metabolites

2012), impaired olfactory behaviour (Yang et al. 2012) and flight abilities (Tosi et al. 2017) are some examples. However, these experiments were criticised, because honeybees were exposed to pesticides during laboratory experiments in non-realistic scenario (Carreck and Ratnieks 2014). In addition, during field experiments, with colonies placed close to treated crop fields, often no effects were observed (Balfour et al. 2017; Pilling et al. 2013; Rolke et al. 2016). However, recently negative effects on colonies exposed in field realistic condition were observed (Ellis et al. 2017; Rundlöf et al. 2015; Tsvetkov et al. 2017). The high number of studies published in high rated journals reveal that sub-lethal effects of pesticides is an important topic now, but it is still debated and contradictory results were obtained.

Honeybees are exposed to a complex mixture of toxic chemicals and many others stress factors simultaneously. Many studies tried to assess the presence of synergy between different stress factors. A first example is the ability of fungicides to increase neonicotinoids toxicity acting on the detoxification mechanism (Iwasa et al. 2004; Sgolastra et al. 2017a). Furthermore, the effects of insecticides are studied also in combination with other different stress factors like parasite and lack of flowers (Goulson et al. 2015). The quality of pollen available was observed to impair performance of bumblebee colonies in combination with thiamethoxam exposure at sub-lethal concentration (Dance et al. 2017). Also an increase of the parasite *Nosema* spp. was observed when honeybees are exposed to sub-lethal concentration of neonicotinoids (Pettis et al. 2012). Neonicotinoids does not only act at level of the nervous system, but they can also impair the immune system of pollinators. As a consequence, these insects are more exposed to attack by virus and other disease (Brandt et al. 2017; Pamminger et al. 2017; Prisco et al. 2013; Simmons and Angelini 2017).

Honeybees are the most studied pollinators insects for negative effects of pesticides. However, also wild bees have an important ecological value due to the pollination of plants. Less studies were published for wild bees and the most studied are bumblebees (Heard et al. 2017). However, these studies proved that the information obtained from experiments with honeybees cannot be used to assess pesticides effects to wild pollinators, because different toxicity mechanism could be observed (Moffat et al. 2016). In addition, an experiment conducted in Sweden proved that honeybees could be not affected by the presence of crops treated with insecticides, but negative effects were still observed for wild pollinators (Rundlöf et al. 2015).

Other invertebrates species affected by pesticides are predator insects, useful to control crops pests (Wanumen et al. 2016). For example, non-target insects like *Harmonia axyridis* can be negatively affected by feeding on seedling grown from seeds treated with systemic insecticide (Moser and Obrycki 2009). In addition, the diffuse neonicotinoids pollution observed in surface water (Table 1.4), due to their use in agricultural production, caused the exposure of aquatic species to relevant concentration of systemic insecticides and negative effects were observed (Sánchez-Bayo et al. 2016). Aquatic insects resulted to be very sensitive to neonicotinoids (Finnegan et al. 2017) and a reduction of their abundance was observed when concentrations of imidacloprid in water are above 1 or $2\,\mu g\,L^{-1}$ (Colombo et al. 2013; Hayasaka et al. 2012).

1.5 Insecticide impact on vertebrates

A cause of great concern is that the negative effects of neonicotinoids environmental pollution could now be observed also on vertebrates. Birds are particularly exposed to the risk posed by seeds coating, because they can eat treated seeds that contain high amount of AI (around 1 mg; Prosser and Hart 2005). Concerning the acute toxicity clothianidin ranges from moderate to practically non-toxic for both birds and mammals, but methiocarb and fipronil are highly toxic for some birds species (EPA 1987; Gibbons et al. 2015). In addition, indirect effects are possible for insect-eating birds, because the use of insecticides reduce their prey population. In Netherlands a correlation was found between surface-water concentrations of imidacloprid and birds population decline (Hallmann et al. 2014). This results underline how environmental pollution can have negative effects in a wide range of non-target animals.

As said earlier, neonicotinoids have an excellent selectivity for insects compared to mammals. They are agonistic of acetylcholine receptors and because of structural differences between the insects and mammalian receptors, they have higher affinity for the first one (Tomizawa and Casida 2003). However, their metabolites can have an inversion of selectivity with higher toxicity for mammals (Tomizawa and Casida 2005). In particular, neonicotinoids imine derivative presents similar proprieties with nicotinic compounds with higher toxicity to mammals (Figure 1.4). From this point of view, the availability of quantitative data about the presence of neonicotinoids metabolites in the environment is a key factor in order to assess the effects of insecticides treatment to non-target animals.

Even if neonicotinoids toxicity is very low for mammals, several studies observed potential chronic negative effects of these insecticides to non-target animals (Gibbons et al. 2015). New studies showed that neonicotinoids may act also to other target site of the nervous system other than the acetylcholine receptors and clothianidin administration to mice at No Observed Adverse Effect Level (NOAEL) concentration induced anxiety-like behaviour (Hirano et al. 2017). In addition, clothianidin and acetamiprid were observed to be partial agonists of mammalian neuronal $\alpha 7$ nicotinic receptors (Cartereau et al. 2017).

Concerning the potential effects as carcinogens, a study of chronic exposure of mice to thiamethoxam concluded that it is hepatotoxic and hepatocarcinogenic as a result of its metabolism to desmethyl-thiamethoxam (Green et al. 2005; Swenson and Casida 2013). In addition, both thiamethoxam and thiacloprid are rated as likely human carcinogens (Tomizawa and Casida 2005). Due to their relevant use in agriculture and urban environment, in combination with the new evidence of multiple potential toxic mechanisms, new studies are required to properly assess the impact of neonicotinoids to human health (Cimino et al. 2017; Prisco et al. 2017; Seltenrich 2017).

1.6 Analytical method for exposure assessment

Many analytical methods were developed in the last years in order to quantify pesticides in environmental matrices. Liquid chromatography coupled to mass

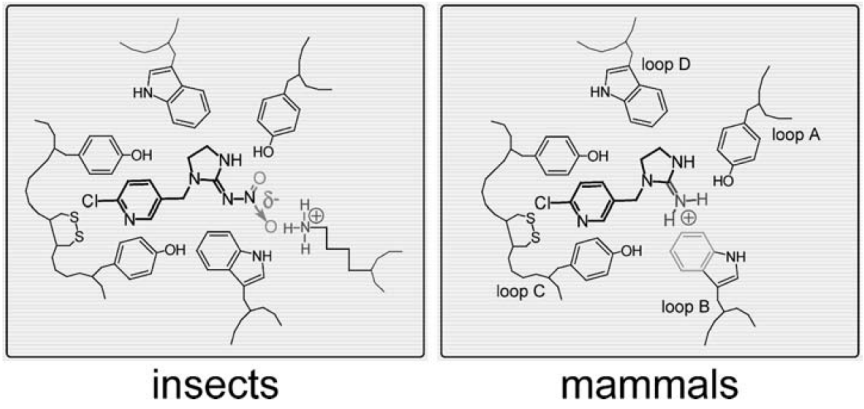


Figure 1.4: different interaction of imidacloprid and its desnitro metabolite with the acetylcholine receptors of insects and mammals (Tomizawa and Casida 2005).

spectrometry is widely used for analysis of pesticides in environmental matrices (Pérez-Fernández et al. 2017). Plant material is a complex analytical matrix, because it required physical treatment for homogenization and after extraction often a clean-up step is necessary (Matamoros et al. 2012). Homogenization can be performed by mechanical grinding, lyophilization or grinding with liquid nitrogen and it improves the extraction from the solid matrix. After homogenization, usually a solid-liquid extraction is performed followed by a clean-up like Solid Phase Extraction (SPE), dispersive Solid Phase Extraction (d-SPE), Liquid Liquid Extraction (LLE) and others.

In the last years the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) procedure, developed by Anastassiades et al. 2003, was widely used for pesticides analysis in many different commodities. This procedure was initially developed for the determination of pesticide residues in fruits and vegetables and during years it has been modify for analysis of many pesticides classes in many different samples (Bruzzoniti et al. 2014). It involves an initial single-phase extraction with an organic solvent (usually acetonitrile), followed by liquid-liquid partitioning formed by addition of a salts mix. Then a clean-up of the organic phase is performed by using a rapid procedure called d-SPE, in which a sorbent phase is simply mixed with the acetonitrile extract. Finally the cleaned extract can be evaporated and the sample reconstituted with a new solvent. The extraction step can be optimised tuning the organic solvent and the salt mix used. In addition, the dispersive clean-up is a key step in order to obtain effective matrix removal and, at the same time, good analytes recoveries. Many different sorbent phase are now available for this purpose. The Primary Secondary Ammine (PSA) effectively removes many polar matrix components, such as organic acids, certain polar pigments and sugars, to some extent. Graphitized Carbon Black (GCB) phase is known to be very effective in pigments removal from leafy matrix (Han et al. 2015), but it may retain more hydrophobic analaytes, in particular if they are planar. Z-Sep+ is a mixture of two sorbents: C18 and silica coated with zirconium dioxide (ZrO_2). ZrO_2 act as Lewis acid to remove lipids and some pigments and this is a new sorbent phase indicated for commodities containing lipids in some extent (Rajski et al. 2013). The main advantages of QuEChERS are that it is very simple and

fast if compared to other methods like SPE. Furthermore, because this method was developed for cover a wide range of pesticides it can be easily tuned in order to obtain good recovery factors. However, with such a general method is difficult to obtain at the same time a proper clean-up and high recoveries for analytes with different proprieties.
Liquid and gas chromatography can be used both for pesticides analysis depending on their physical-chemical proprieties. However, since the 80s, Liquid Chromatography (LC) is grown in popularity as technique for environmental control. LC offers a series of advantages compared to GC: elimination of the derivatization step of non-volatile and heat-labile compounds, increase of the number of analysable chemicals and reduction of the total analysis time (Pérez-Fernández et al. 2017). In addition, the development of Electrospray Ionization (ESI) make possible to couple LC instrument with mass spectrometry detector. Compared to other classical detector, mass spectrometry allow to achieve the best analytical performance, because of its selectivity and sensitivity. In addition, High Resolution Mass Spectrometry (HRMS) allow to measure accurate molecular mass in order to identify compounds present in samples and combined with MS^2 experiments is possible to confirm the structure of suspect pollutants. In annex II the acquisition mode available with the instrument used for this research (Q Exactive™ Hybrid Quadrupole-Orbitrap) are discussed in details.

Chapter 2

Aim of the work

The high amount of pesticides used in modern agriculture is the principal cause of environmental pollution and these toxic chemicals spread in the environment can negatively affect several non-target animals. Many analytical methods were developed in order to be able of quantifying pesticides in several kind of environmental samples (leaf, pollen, nectar, etc) and a lot of information are available about the presence of these compounds in the environment. Instead, few information are available about the presence of their degradation products and rarely these compounds are included in the available analytical methods. Therefore, their effects to non-target animals are difficult to assess. Even if many analytical methods were developed in the last years for pesticides quantification in pollen, bee-products, fruits and vegetable; less work was done with leaf and vegetative material. Leaf is a complex matrix due to the presents of many interfering compounds like pigments.
The first goal of this research project was to use LC chromatography coupled with High Resolution Mass Spectrometry (HRMS) in order to identify degradation products of systemic insecticides applied to corn seeds. A suspect screening approach was used starting from the metabolic pathways available in the literature (Ford and Casida 2006a,b; Kuhr 1970; Pei et al. 2004; Simon-Delso et al. 2014). Instead of analyse corn leaf samples, guttation samples were chosen for metabolite identification, because they do not required heavy samples preparation and usually systemic insecticides are present at high concentration in this matrix. Once the insecticides metabolites were identified, a method for an accurate quantification was validated and further guttation samples were analysed in order to assess the concentration of insecticides and their degradation products.
During the second step of the research, a Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method for the corn leaf extraction and analysis in UHPLC-MS^2 was developed and validated for thiamethoxam, clothianidin, thiacloprid, methiocarb, fipronil and their metabolites previously identify in guttation drops. The method was later used for analyse corn samples treated with these insecticides in order to assess the exposure of non-target animals to insecticides and their metabolites. Unlike guttation drops, corn seedling are a potential exposure route for birds and other small vertebrates.
Furthermore, guttation and leaf samples from an ornamental plant treated with a solution containing thiamethoxam were analysed in order to collect further information about systemic insecticide uptake. The information about the presence

of systemic insecticides and their degradation products both in guttation and leaf are used to assess the utility of guttation drops as an innovative tool for the identification of insecticides metabolites in plants treated with systemic compounds.

Chapter 3

Materials and methods

3.1 Chemicals and reagents

Pure compounds for the preparation of standard solutions of thiamethoxam, N-desmethyl thiamethoxam, thiamethoxam-d3, clothianidin, clothianidin-d3, thiacloprid, thiacloprid-d4, thiacloprid amide, methiocarb, methiocarb-d3, methiocarb sulfoxide, methiocarb sulfone and fipronil were obtained from Pestanal® Sigma-Aldrich (Milan, Italy). All pesticides were > 99 % compound purity (except fipronil that was 97.5 % pure) and deuterated compounds were > 97 % isotopic pure. Individual standard pesticide stock solutions ($100\,\mathrm{mg\,l^{-1}}$) were prepared in methanol using volumetric flask. Standard solutions for instrumental calibration were prepared weekly from stock solutions in H_2O/MeOH (80:20). All solutions were stored at $-20\,^{\circ}\mathrm{C}$ in the dark.
Methanol (HiPerSolv Chromanorm VWR, $\geq 99,8$ %) and acetonitrile (LiChrosolv®, $\geq 99,9$ %) were of HPLC grade and water was purified using Millipore Milli-Q (Vimodrone, Milan, Italy) equipment.
Analytical grade magnesium sulfate anhydrous (99 %; VWR—AnalaR NORMAPUR, Milan, Italy), sodium acetate trihydrate (99.0 %, Fluka, Milan, Italy) and sodium chloride (> 99 %; VWR—AnalaR NORMAPUR, Milan, Italy) were used in the sample preparation step. Amberlite XAD-2 resin (Restek Ultraclean, Bellefonte, PA, USA), Supel™ QuE Z-Sep+, Supel™ QuE PSA/C18/ENVI-Carb, PSA sorbents were obtained from Supelco® analytical (Sigma-Aldrich, Milan, Italy). 2-chloro-5-(chloromethyl)pyridine, 2-chloroethylamine HCl, triethylamine and KSCN used for thiacloprid imine synthesis, were obtained from Aldrich® chemistry and Fluka® analytical.

3.2 Synthesis of non-commercial standards

Some insecticides degradation products standard are not commercially available. Therefore, thiacloprid imine, methiocarb phenol, methiocarb sulfoxide phenol and methiocarb sulfone phenol were synthetized in order to obtain the analytical standard. Thiacloprid imine was synthesised using a two-step procedure available in literature (Latli et al. 1999). Briefly, 2-chloro-5-(chloromethyl)pyridine, 2-chloroethylamine HCl and triethylamine were mixed in acetonitrile for 40 h at

29 °C. The intermediate 1-[(6-chloro-3-pyridinyl)methyl]-2-chloroethyl-amine was isolated from the crude reaction product and it was mixed with KSCN for 3 h at 95 °C in a H_2O/ACN (50:50) solution. Finally, preparative chromatography was used to purify the synthesised thiacloprid imine.
Methiocarb phenol, methiocarb sulfoxide phenol and methiocarb sulfone phenol are methiocarb degradation products and they were synthesised according to a procedure reported in Tian et al. 2013. Briefly, 5 mg of methiocarb, methiocarb sulfoxide and methiocarb sulfone pure standards were dissolved in 5 ml of methanol in separated volumetric flasks. 1 ml of NH_3 1 M was added and the solution was place in ultrasonic bath at 50 °C for 30 min. Finally, 200 µl of a formic acid 5 M were added to neutralize the base.
The products obtained were characterized with ^{1}H-NMR (Bruker 300, only thiacloprid imine) and HRMS (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer). Their purity was determined using a Shimadzu Prominence UFLC-XR chromatograph (SIL 20AC-XR autosampler; CTO-20A column oven; SPD-M20A UV–vis diode-array detector, set at $\lambda = 202\ nm$) equipped with a Kinetex Biphenyl column (2.6 µm, 100 mm × 2.1 mm).

3.3 Sample collection and preparation

3.3.1 Corn seeds used

Corn coated seeds from "Pioner Italia" were used. They were treated with different pesticides for coating: Cruiser® 350 FS (thiamethoxam 1.0 mg/seed, Syngenta, Basel, Switzerland), Regent® (fipronil 0.5 mg/seed, BASF SE, Ludwigshafen, Germany), Sonido® (Thiacloprid, 1.0 mg/seed, Bayer Cropscience AG, Leverkusen, Germany) and Mesurol® (methiocarb, 1.25 mg/seed, Bayer Cropscience). All seeds were coated also with the fungicide Celest XL® (Fludioxonil 2.4% and Metalaxyl-M 0.93%; Syngenta, Basel, Switzerland). The seeds coated with thiamethoxam and fipronil were supplied by A.I.S. (Italian seed association) courtesy of MiPAAF (Ministry of Agriculture, Food and Forestry) for the research project Apenet. Instead, seeds coated with methiocarb and thiacloprid were purchased.

3.3.2 Corn plants

In order to obtain samples from corn plants treated with insecticides, seven corn seeds coated with methiocarb, thiamethoxam, thiacloprid or fipronil were sowed in pots (Ø 12 cm, h 12 cm). Three pots were prepared for every AI studied. Leaf samples from the same pot were collected in consecutive days (between 19 and 42 days after sowing for thiamethoxam, thiacloprid and fipronil; between 9 and 16 days after sowing for methiocarb) and different pots were used to obtain samples replicates. Instead plants from the same pot were not distinguished. Corn leaf samples consisted in one corn seedling manually gathered and stored at −20 °C. Prior to extraction, leaves were ground with liquid nitrogen followed by manual homogenisation using a micro-spatula.
Guttation samples were collected after few days from the plants emergence for

two weeks (between 17 and 21 days after sowing for thiamethoxam, thiacloprid and fipronil; between 7 and 15 days after sowing for methiocarb). Micropipetts was used in order to collect guttation drops twice a day from the same plants used for leaf samples. In addition, for corn plants grown from seeds coated with methiocarb, guttation samples were collected also from plants sowed in field. In this case, five different plants were sampled in the morning (between 9:00 and 11:00 a.m.) for three non-consecutive days. For all the experiments, guttation samples were stored at -20 °C. Prior to LC injection, samples were filtered (0.22 µm), in order to remove eventual soil particles, diluted 1:1 with methanol spiked with internal standards (final IS concentration was $100\,\mathrm{ng\,ml^{-1}}$).

3.3.3 Ornamental plants

The ornamental plant *Aglaonema commutatum* was chosen for its high production of guttation drops. One potted plant received three treatment with 100 ml of a thiamethoxam water solution of $3.5\,\mathrm{mg\,l^{-1}}$. A week passed between every insecticide applications. This treatment corresponds to $200\,\mathrm{g\,ha^{-1}}$ as suggested for soil treatment of ornamental plants (*ACTARA® 25 WG, Syngenta Crop Protection* 2016). Leaf samples consisted in one leaf (around 10 g) manually gathered. Guttation samples were collected in the morning using micropipetts from the same leaf used for leaf samples. All samples were stored at $-20\,^{\circ}\mathrm{C}$ until analysis. The sample preparation applied to ornamental plant leaf and guttation is the same used for corn samples and it is already reported in section 3.3.2.

3.3.4 Leaf extraction procedure

Several experiments were performed in order to obtain satisfactory recoveries of the analytes ($70 - 120$ %). Every specific step of the extraction procedure was separately tested in order to obtain the best condition. Particular attention was posed on the salt mix used, the d-SPE step and the time needed for solvent evaporation. The optimisation experiments were carried out using an UHPLC-DAD instrument, using the same setting described in section 3.2.

The optimized procedure consisted in the extraction for 1 minute of 100 ± 5 mg ground leaf with 500 µL of acetonitrile with acetic acid 1 %. Then 400 µL of water and 250 mg of a salt mix (magnesium sulphate and sodium acetate; 4:1) were added. The solution was shaken for 30 seconds and then it was placed in ultrasonic bath for 10 minutes. After centrifugation, the upper organic phase was removed into an eppendorf containing 30 mg of the d-SPE phase (PSA). The samples were extracted again with other 500 µL of solvent and the combined extract was mixed and placed in ultrasonic bath for 5 minutes. After centrifugation, the supernatant was removed and it was evaporated with a nitrogen stream at 30°C. Finally, the extract was reconstituted with 300 µL of a H_2O/MeOH solution (80:20). It was spin filtered (0.22 µm) and diluted 1:1 with H_2O/MeOH (80:20) before injection.

Table 3.1: LC columns tested for insecticides and metabolites analysis.

Name	brand	phase	particles size (μm)	diameter (mm)	length (mm)
XR-ODS III	Shimadzu	C18	1.6	2.0	75
PFP	Kintex	pentafluorophenyl	1.7	2.1	100
Luna® Omega	Phenomenex	polar C18	1.6	2.1	100

Table 3.2: acquisition settings of the different methods used in the HRMS analysis with Q Exactive™ Hybrid Quadrupole-Orbitrap (/=not congruent).

	full-scan	dd-MS^2	PRM
Resolution	35000	17500	17500
AGC target	3×10^6	1×10^5	2×10^5
Max injection time (ms)	100	50	50
Scan range (m/z)	60 - 750	/	/
Isolation window (m/z)	/	4.0	3.0
Isolation offset	/	0.0	1.0

3.4 Chromatographic methods

The LC chromatography condition were optimized to achieve good chromatographic efficiency and the best ESI ionization. Several eluent additives were tested (formic acid 5 mM, ammonia 5 mM, ammonium formiate 5 mM and ammonium acetate 5 mM). In addition, several analytical column were tested for the analysis of the pesticides mix in order to select the most efficient one (Table 3.1). All the optimisation experiments were carried out using the UHPLC-DAD instrument described in section 3.2.

In order to identify the insecticides degradation products present in corn plants, a suspect screening analysis of guttation drops samples was performed. UHPLC-HRMS was used in order to identify the compounds. The analysis were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC system coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer. Samples were separated using a reverse phase Luna® Omega C18 polar column (1.6 µm, 2.1 mm × 100 mm, Phenomenex), mainteined at 30 °C. Injection volume was 20 µl and mobile phase solvents were water, ammonium acetate 5 mM (A) and acetonitrile, ammonium acetate 5 mM (B). The initial ratio (A/B) was 100 : 0 and the flow rate was 0.2 ml min^{-1} with the following gradient: 100% A for 3 min, from 100 : 0 to 30 : 70 at 20 min, from 30 : 70 to 0 : 100 at 22 min and held for 3 min prior to return to initial condition and equilibration for 3 min. The acquisition method was a fullscan-data dependent MS^2 in both polarities and the setting used is reported in Table 3.2.

Once the AI degradation products were identified, a list of target compounds

were built and a UHPLC-MS^2 target analyses were carried out using the same instrumentation and LC parameters reported above. MS^2 was performed in the Parallel Reaction Monitoring (PRM) mode using ESI in the positive and negative ionization (Table 3.2). The masses of the precursor ions were isolated by the quadrupole and the multiplexed MS (MSX) option was used in order to increase the scan rate (Table 3.3). This feature allowed to trap several target ions in the instrument C-trap at the same time and then their were fragmented and analysed in the orbitrap simultaneously. The Normalized Collision Energy (NCE, 10 - 40 eV) was optimised for each analyte. Other parameters were optimised as follows: spray voltage 3.3 kV for positive and 2.8 kV for negative, capillary temperature 320 °C, probe heather temperature 340 °C, sheath gas 40 psi, aux gas 20 a.u. and S-lens RF 60 V. Data were acquired using Thermo Xcalibur 3.0.63 and the quantification was carried out by calculating the response factor of pesticides to their respective IS. Concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio (analyte to IS).

3.5 Method validation

Method recoveries and precision were evaluated by spiking control leaves, and the method performance acceptability criteria from EU guidelines were used for assessment (SANTE/11945/2015). Leaf samples (100 ± 5 mg) were used for the recovery experiments and to prepare matrix-matched standard solutions for calibration. For recovery experiments, leaves samples (four replicates) were spiked at two concentration levels of the analytes (60 and 600 $\mathrm{ng\,g^{-1}}$). After extraction of the analytes from the spiked samples, 15 ng of the IS mix were added. Matrix matched calibration solutions were prepared using non-spiked leaf extracts and spiked after extraction with standard solutions. The calibration curve consisted of six points for each tested analyte equivalent to 30, 60, 300, 600, 1500 and 3000 $\mathrm{ng\,g^{-1}}$ together with 150 $\mathrm{ng\,g^{-1}}$ of IS mixture. The repeatability of the method was determined as the intra-day relative standard deviation (RSD %) of repeated extractions (n= 4) of a matrix extract spiked at the two concentrations used in recovery studies. The sensitivity of the method was calculated in terms of method detection and quantification limits (MDL and MQL, respectively) and their determination was based on the calibration curve (Hubaux and Vos 1970). The equation 3.1 and 3.2 were used for this purpose, where $S_{y,x}$ represent the standard deviation of the analytical signal (peaks area) associated to the calibration curve and b is its slope (Evard et al. 2016). A specific calibration curve in the concentration range of $0.5 - 50\,\mathrm{ng\,g^{-1}}$ for each analyte was used for this purpose. Linearity was evaluated both in solvent and matrix, using matrix-matched calibration curves prepared as described above. A F-test between linear and polynomial regression model applied to the calibration functions obtained was used to assess their linearity (Brüggemann et al. 2006). The effect of the matrix was evaluated by comparison of the slopes of the calibration curves in solvent only (H_2O/MeOH; 80:20) and in the matrix. The percent increase or decrease of the matrix-matched calibration curve was measured in relation to the solvent-only curve as described in other studies (Walorczyk 2014).

Table 3.3: retention times and optimized parameters for insecticides and metabolites quantification by UHPLC-MS^2.

Analyte[a]	tR (min)	MSX ID	ionization	NCE (eV)	Q1 precursor ion (m/z)[b]	Q3 quantification ion (m/z)
thiacloprid immina (a)	9.8	1	+	30	228.03567	126.00966
methiocarb solfossido idrossido[c]	10.4	1	+	30	258.07946	185.0626
clothianidin urea[c]	10.49	1	+	30	206.01494	131.96687
desnitro thiamethoxam[c]	10.67	1	+	30	247.04149	131.96907
thiamethoxam nitroso[c]	10.85	1	+	30	276.03165	131.9673
methiocarb solfossido fenolo (a)	11.09	2	+	40	185.06308	170.03819
thiacloprid ammide ossido[c]	11.16	2	+	40	287.0364	126.00977
thiamethoxam (a)	11.34	2	+	20	292.02656	211.06308
methiocarb solfossido (a)	12.00	3	+	15	242.08454	185.06322
thiacloprid ammide (b)	12.44	3	+	15	271.04149	254.01416
thiamethoxam urea[c]	12.51	3	+	15	265.05205	176.9699
clothianidin (b)	12.65	3	+	15	250.01600	169.05421
methiocarb sulfone fenolo (b)	13.19	4	-	35	199.04344	184.02113
thiamethoxam desmethyl (b)	13.29	4	+	10	278.01091	131.96596
thiacloprid S->O[c]	13.35	4	+	35	237.05377	237.0538
thiacloprid ossido/idrossido[c]	13.51	4	+	35	269.02584	126.01079
methiocarb sulfone (c)	14.12	5	+	10	258.07946	122.0719
thiacloprid (c)	14.61	5	+	35	253.03092	126.00977
methiocarb fenolo (d)	18.29	6	-	10	167.05361	152.03069
methiocarb (d)	18.75	7	+	10	226.08963	169.06700
fipronil (d)	21.77	8	-	15	434.93143	329.96289
thiamethoxam-d3 (a)	11.34	2	+	20	295.04539	214.08183
clothianidin-d3 (b)	12.65	3	+	15	253.03483	172.07280
thiacloprid-d4 (c)	14.61	9	+	35	257.05603	126.00965
methiocarb-d3 (d)	18.75	10	+	10	229.10846	169.06662

[a]a, b, c and d indicate which internal standard has been used for the analyte.
[b]thiamethoxam urea was detected as the ammonium adduct, all the other compounds were detected as $[M+H]^+$ in positive or $[M-H]^-$ in negative mode.
[c]analytical standard not available. The quantification is based on the calibration curve of the closest analyte for which the standard was available.

For guttation samples recovery experiments were not performed because guttation samples were not extracted. Matrix matched calibration curves were prepared at 0.5, 1, 5, 10, 25, 50 and 100 $\mathrm{ng\,g^{-1}}$ together with 2.5 $\mathrm{ng\,g^{-1}}$ of IS mixture. MDL and MQL values were calculated using the same calibration curved used for leaf samples.

$$MDL = 3.3 \times \frac{S_{y,x}}{b} \tag{3.1}$$

$$MQL = 10 \times \frac{S_{y,x}}{b} \tag{3.2}$$

$$S_{y,x} = \sqrt{\frac{\sum (y_i - \hat{y}_i)^2}{n-2}} \tag{3.3}$$

Chapter 4

Results

4.1 Choice of analytes

Four insecticides were chosen for this study because of their use for seeds coating. Thiacloprid and methiocarb were chosen because they are currently used for corn seeds coating in most EU countries. Thiamethoxam was also included even if it is now banned for coating in Europe, because it is the most used neonicotinoid AI and its toxicity proprieties are drastically different if compared to thiacloprid. Furthermore, AI with different characteristic were chosen in order to make possible to compare different systemic proprieties of pesticides. Fipronil was chosen because its systemic proprieties are under question and therefore was interesting to study them.
In order to evaluate the presence of insecticide metabolite and to assess their potential impact to non-target animals, also the degradation products were included in the study. Many degradation products of the studied AI were identified in guttation samples using High Resolution Mass Spectrometry (HRMS), through the acquisition of the accurate mass values and studying the fragmentation patter. Detail of the analysis of corn guttation samples are reported in section 4.4. Most of their analytical standards were commercially available, but for some others the analytical standards were prepared by synthesis. In particular, the standards of methiocarb phenol, methiocarb sulfoxide phenol, methiocarb sulfone phenol and thiacloprid imine were not commercially available, but they were syntetized in order to make possible the validation of the quantitative method of analysis. Unfortunately, it was not possible to synthesise the standard of all the degradation products not commercially available. Therefore, for some of them a semi-quantitative analysis was performed based on the calibration curve of the closest analyte in terms of retention time.

4.2 Synthesis of non-commercial standards

The thiacloprid imine standard was successfully synthetized using the procedure reported in Latli et al. 1999. This procedure is summarised in section 3.2 The product obtained was characterized using HRMS, the mass fragmentation spectrum and ^{1}H-NMR (Figure 4.1). The UHPLC-DAD analysis was used in order to

confirm the high purity of the standard obtained and it is > 99 % based on the measurement of the peaks area. (Figure 4.2).
The synthesis of methiocarb phenol degradation products was developed starting from the procedure used in Tian et al. 2013. However, the use of HCl and NaOH is not suitable with the ESI source used for LC-MS analysis, because during evaporation salts deposition could impair the source performance clogging the ESI capillary. Therefore, the procedure was slightly modified in order to use volatile acid and base (formic acid and ammonia). The optimized procedure is reported in section 3.2 and it need longer time and higher temperature in order to obtain good yield. In conclusion, the methiocarb phenolic standards were obtained and their purity was > 99 % based on the peaks area detected by UHPLC-DAD analysis (Figure 4.3). Instead, their structures were confirmed by HRMS analysis and fragmentation spectra (Figure 4.4).

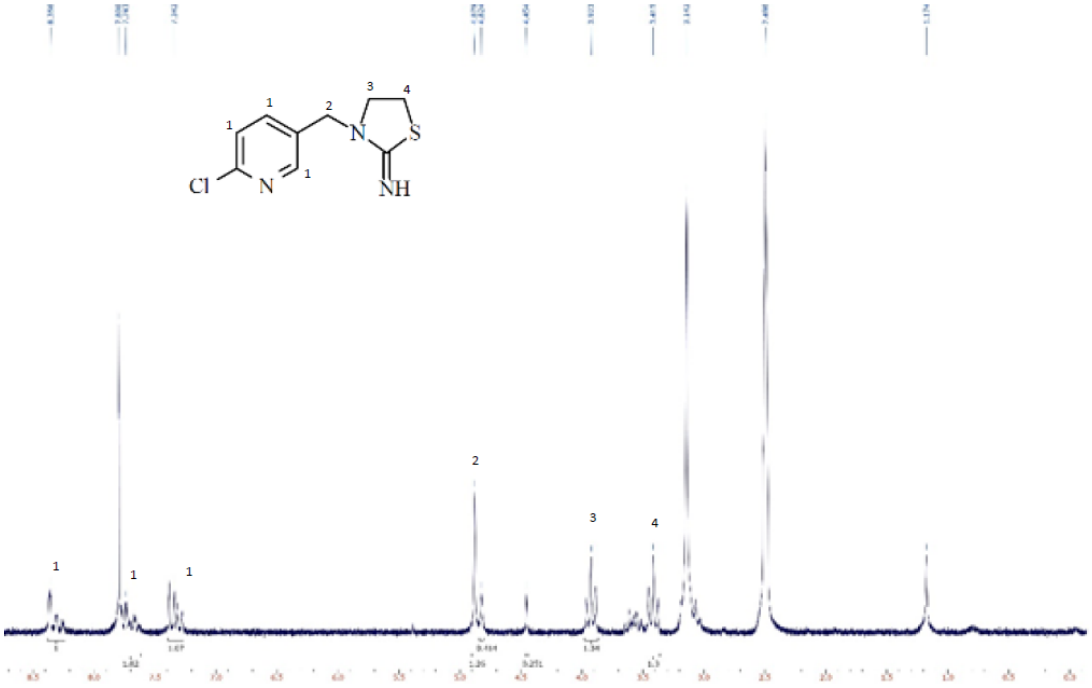
4.3 Method development

4.3.1 Chromatography condition optimization

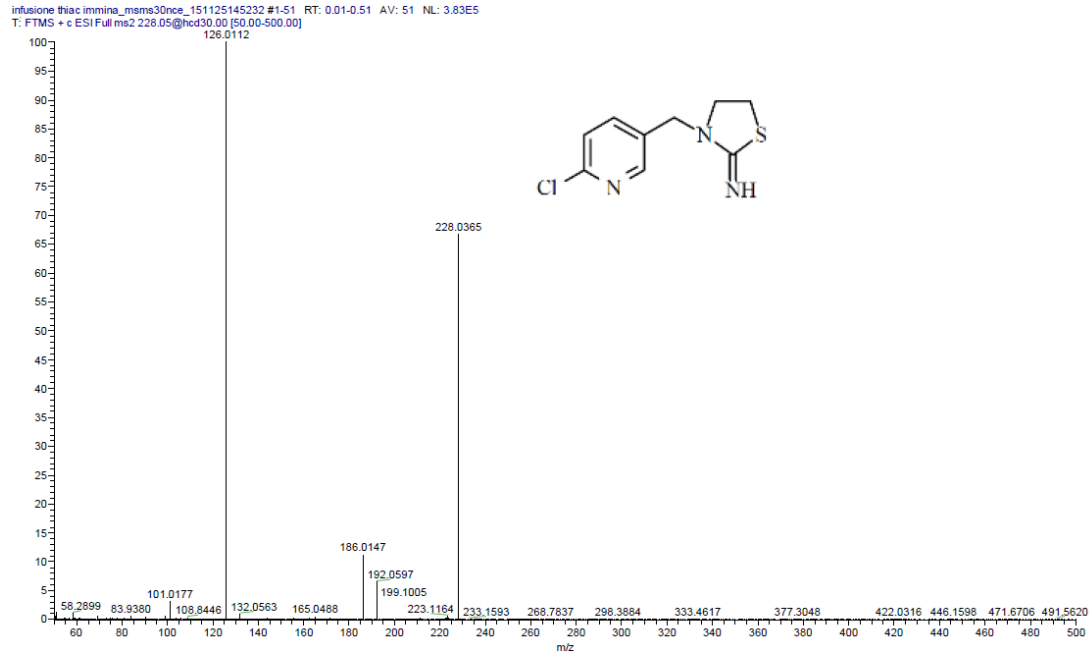
Several eluent additives were tested in order to obtain good chromatographic condition and the the best ESI ionization. Concerning the ionization efficiency, the aim was to work with positive and negative polarity both. Therefore, it was inconvenient to use acid or basic eluent adding formic acid or ammonia, because a signal suppression was always detected for negative ions with formic acid and for positive ions with ammonia. Furthermore, the peak shape of thiacloprid imine resulted to be heavily influenced by the presence of additives. Without additives a broad peak unsuitable for quantitative purpose was obtained (Figure 4.5). Instead, using formic acid or ammonium acetate in the eluent, good peak shape was obtained. Also the methiocarb phenol gave poor results in terms of chromatographic efficiency without any additives. The best results were obtained adding ammonium acetate (NH_4Ac) to water and ACN. It gave better results in terms of chromatographic efficiency and also ESI ionization is generally improved compared to other additives. Therefore, water and ACN with ammonium acetate 5 mM was used as eluent for further experiments (Figure 4.6).
In addition, many different analytical column were tested (Table 3.1) and the Luna® Omega column was chosen as the most efficient one. Its stationary phase is recommended for the balanced retention of polar and non-polar analytes, and solely for the enhanced retention of highly polar compounds. It gave the narrower peaks, compared to the other tested column, in particular in the first part of the chromatogram (Figure 4.7).

4.3.2 Extraction procedure optimization

The developed extraction method is based on the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) strategies described for the first time in Anastassiades et al. 2003. In order to optimise the experimental setup for the extraction of the target analytes from corn leaf, several recovery experiments were performed



(a)



(b)

Figure 4.1: a) ^{1}H-NMR spectrum of 1-[(6-chloro-3-pyridinyl)methyl]-2-iminothiazolidine (thiacloprid imine). The solvent used was a mixture of CCl_3D ($\delta = 7.8$ ppm) and DMSO ($\delta = 3.1$ ppm). DMSO was necessary to dissolve completely the compound. The high peak at $\delta = 2.5$ ppm is due to acetonitrile used as reaction solvent (^{1}H-NMR in $CDCl_3$, δ: 8.32 (d, J=2.1 Hz, 1H), 7.69 (dd, J=2.1, 8.2 HZ, 1H), 7.31 (d, J=8.2 Hz, 1H), 4.57 (s, 2H), 3.51 (t, J=6.8 Hz, 2H), 3.17 (t, J=6.8 Hz, 2H)). b) mass fragmentation spectrum of thiacloprid imine. The 228.0365 m/z peak correspond to the $[M+H]^+$ ion (theoretical mass 228.0632 m/z, $\Delta m = -3.9$ ppm). The major peak at 126.0112 m/z correspond to fragment $[C_6H_5NCl]^+$, observed also in thiacloprid MS^2 spectrum.

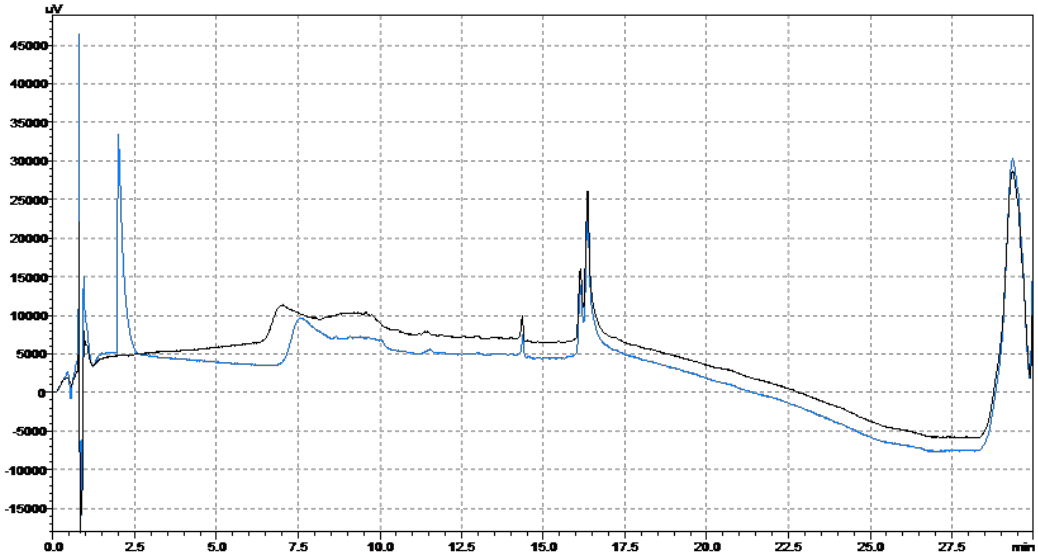


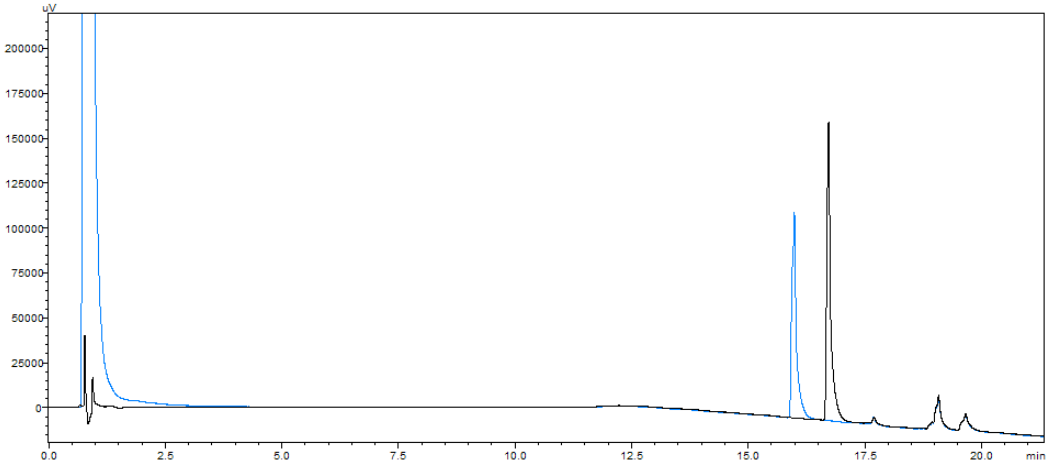
Figure 4.2: HPLC-DAD chromatogram of the synthetized thiacloprid immine standard (retention time 2.4 min). No other relevant peaks were identified in the standard (blue line) if compared to a solvent injection (black line).

and the main challenge was to obtain high recovery factors ($70 - 120$ %) with a sufficient matrix removal.

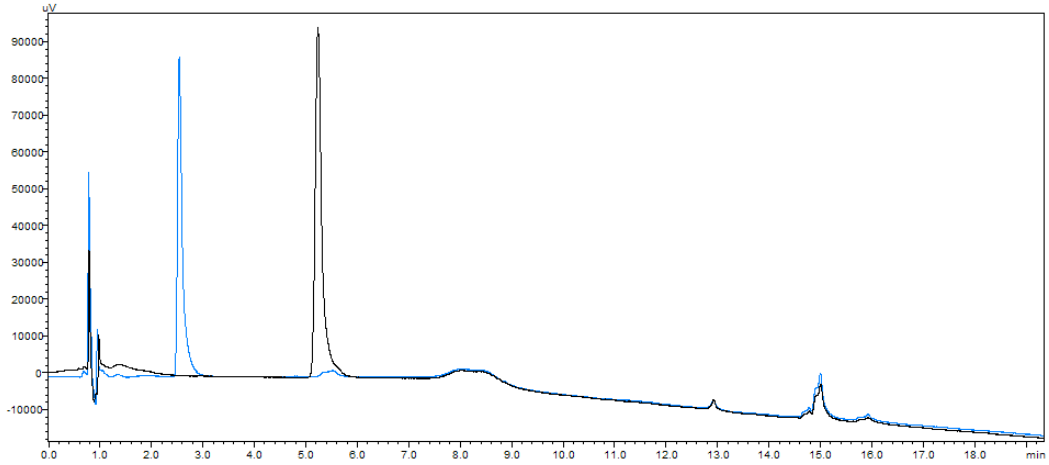
Regarding the use of different salts during the phase separation step, the best results were obtained using sodium acetate (in addition to magnesium sulphate), but combined with the addition of 1% of acetic acid in the extraction solvent (Figure 4.8). The use of acetic acid and its conjugate base, sodium acetate, allow to control the pH during the analytes extraction and it is known to improve the recoveries for pH sensitive compounds (Lehotay et al. 2005).

About the use of different d-SPE sorbent for matrix removal, their efficiency was evaluated looking at the presence and intensity of peaks of extracted leaf samples treated with different d-SPE sorbent materials. Our results showed that PSA is very effective in the matrix removal of corn leaf samples that mainly consist of pigments and organic acid (Figure 4.11). Instead, XAD and Z-Sep+ phases gave poor matrix removal and their use was discarded. In particular, the main part of matrix compounds were eluted between 6 - 10 minutes and they overlap to some compounds of interest. The mix sorbent containing PSA, C18 and a GCB phase gave good matrix removal, but higher recoveries were obtained with PSA compared to the mix sorbent (Figure 4.9). Therefore, PSA was chosen as d-SPE sorbent phase for its good balance between high recoveries and effective matrix removal. It must be noted also that vegetative matrix was dissolved by ACN during extraction, but after evaporation part of these material was not re-dissolved by the recovery solvent (H_2O/MeOH, 80:20). It remained stuck on the vial glass wall or it was filtered reducing the presence of matrix in the final sample.

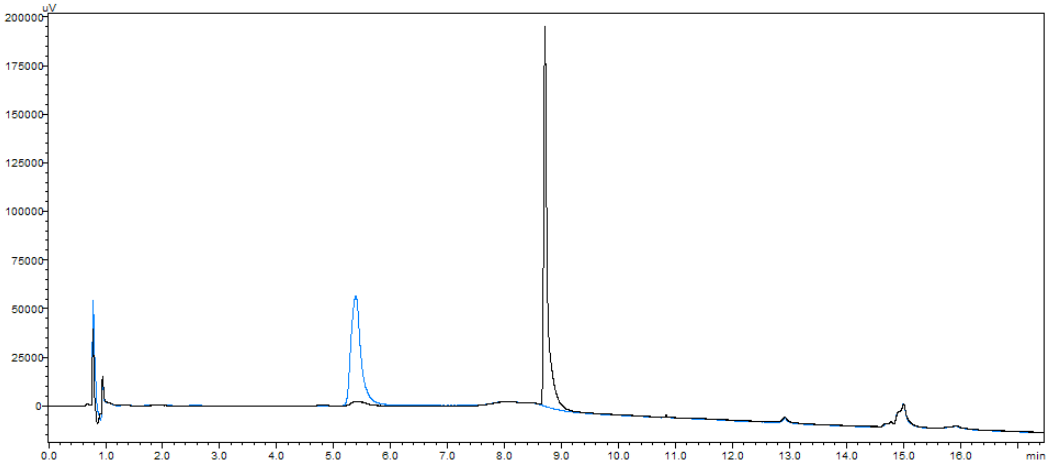
In Figure 4.10 is shown that low recoveries were obtained for methiocarb phenol when samples extracts were left to evaporate for a long time. Therefore, evaporation time for acetonitrile was carefully monitored in order to obtain higher recovery factors. The final extraction procedure, applied to leaf samples, is reported in



(a) methiocarb phenol

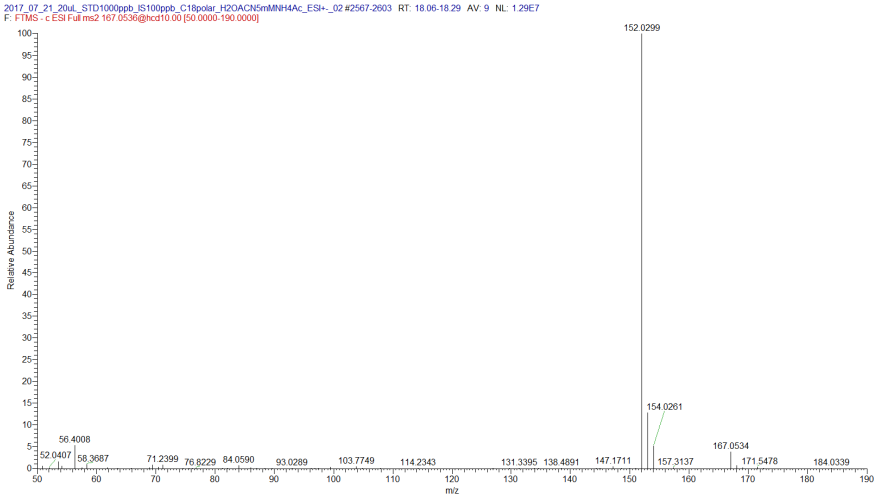


(b) methiocarb sulfoxide phenol

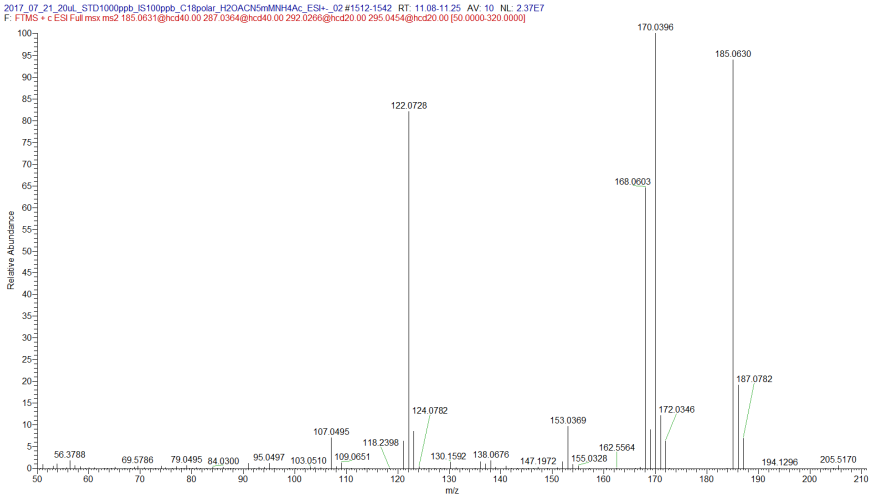


(c) methiocarb sulfone phenol

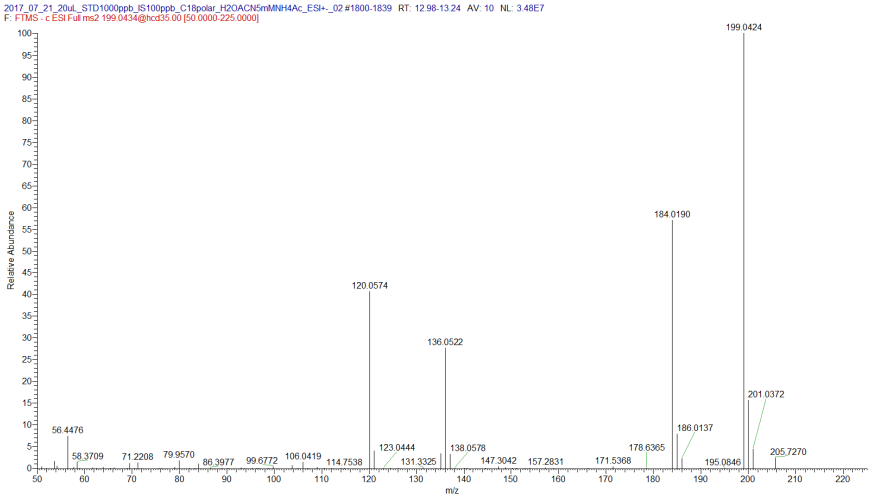
Figure 4.3: HPLC-DAD chromatograms of the synthesized methiocarb phenol standards. No relevant peaks were identified in the standard (blue line) when compared to a parent standard injection (black line).



(a) methiocarb phenol, $[M-H]^-$ = 167.05382 m/z, Δm = 1.26 ppm



(b) methiocarb sulfoxide phenol, $[M+H]^+$ = 185.06308 m/z, Δm = 0.32 ppm



(c) methiocarb sulfone phenol, $[M-H]^-$ = 199.04256 m/z, Δm = 4.42 ppm

Figure 4.4: HRMS spectra of the synthesized phenolic standards. The accurate masses measured for the parent ions are in agreement with the masses of the desired products and the fragmentation patterns confirmed the identity of the compounds.

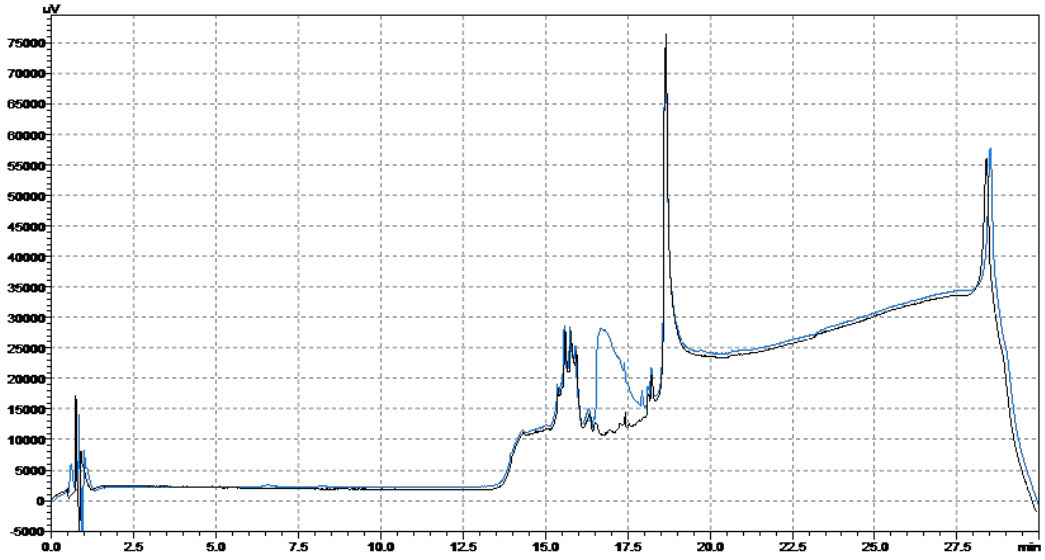


Figure 4.5: HPLC-DAD analysis using water and acetonitrile without additives for eluition. The synthesized standard (blue line) is compared to a solvent injection (black line). It is possible to observe that the thiacloprid imine gives a broad peak, that is unsuitable for quantitative purpose.

section 3.3.4.

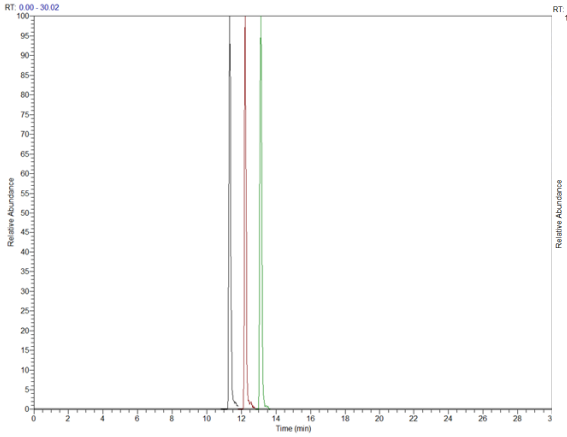
4.3.3 Method validation

Linearity

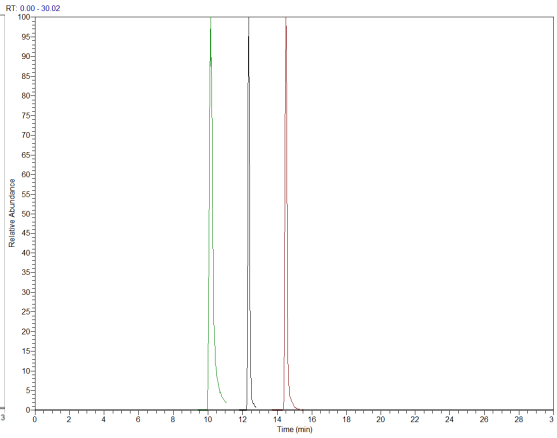
The linearity of calibration functions was assessed by a F-test between the applied linear and polynomial regression model. For all the analytes in both the matrices, no significant differences were observed between the two regression models and so the linear function was used for samples quantification ($F < 7.4$ and $p > 0.072$ for leaf, $F < 7.2$ and $p > 0.055$ for guttation). R^2 values were comprised between 0.9804 and 0.9997, except for N-desmethyl thiamethoxam in leaf it was 0.9494 (Table 4.1). Therefore, the R^2 obtained for all compounds were acceptable and the calibration allowed accurate measurement of analyte concentrations in both matrices in the studied range. However, in some samples analytes concentration exceed this range. In those cases, samples were appropriately diluted in order to obtain a concentration within the calibration range that allowed an accurate quantification.

Matrix effect

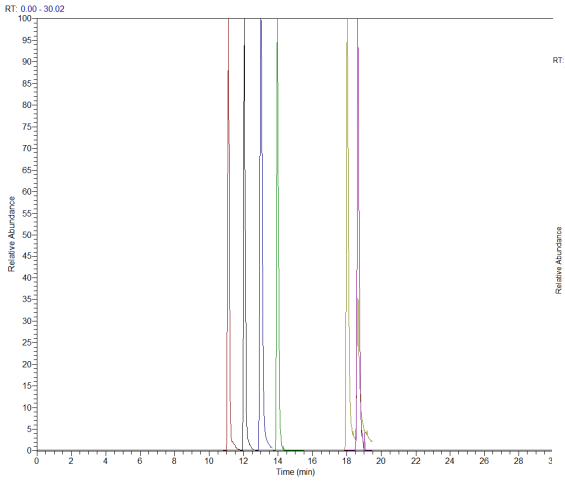
Matrix effect for guttation drops samples was comprised between -9.5 and 18% and for leaf samples it was comprised between -22 and 22% (Table 4.1). The threshold value set by the EU for the analysis of pesticide residues is 20%. Therefore, calibration with standards in solvent may be used for quantification of insecticides in corn guttation drops and leaf (SANTE/11945/2015). Considering that the sample preparation applied to guttation samples is a simple dilution, the



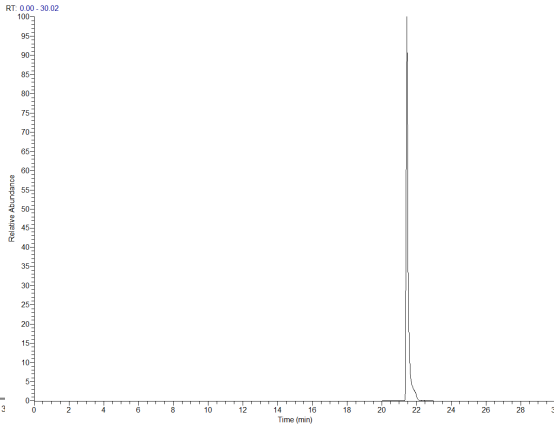
(a) from left to right: thiemthoxam, clothianidin and N-desmethyl thiamethoxam



(b) from left to right: thiacloprid imine, thiacloprid amide and thiacloprid



(c) from left to right: methiocarb sulfoxide phenol, methiocarb sulfoxide, methiocarb sulfone phenol, methiocarb sulfone, methiocarb phenol and methiocarb



(d) fipronil

Figure 4.6: UHPLC-HRMS chromatograms obtained with ammonium acetate 5 mM as additive in both the eluents.

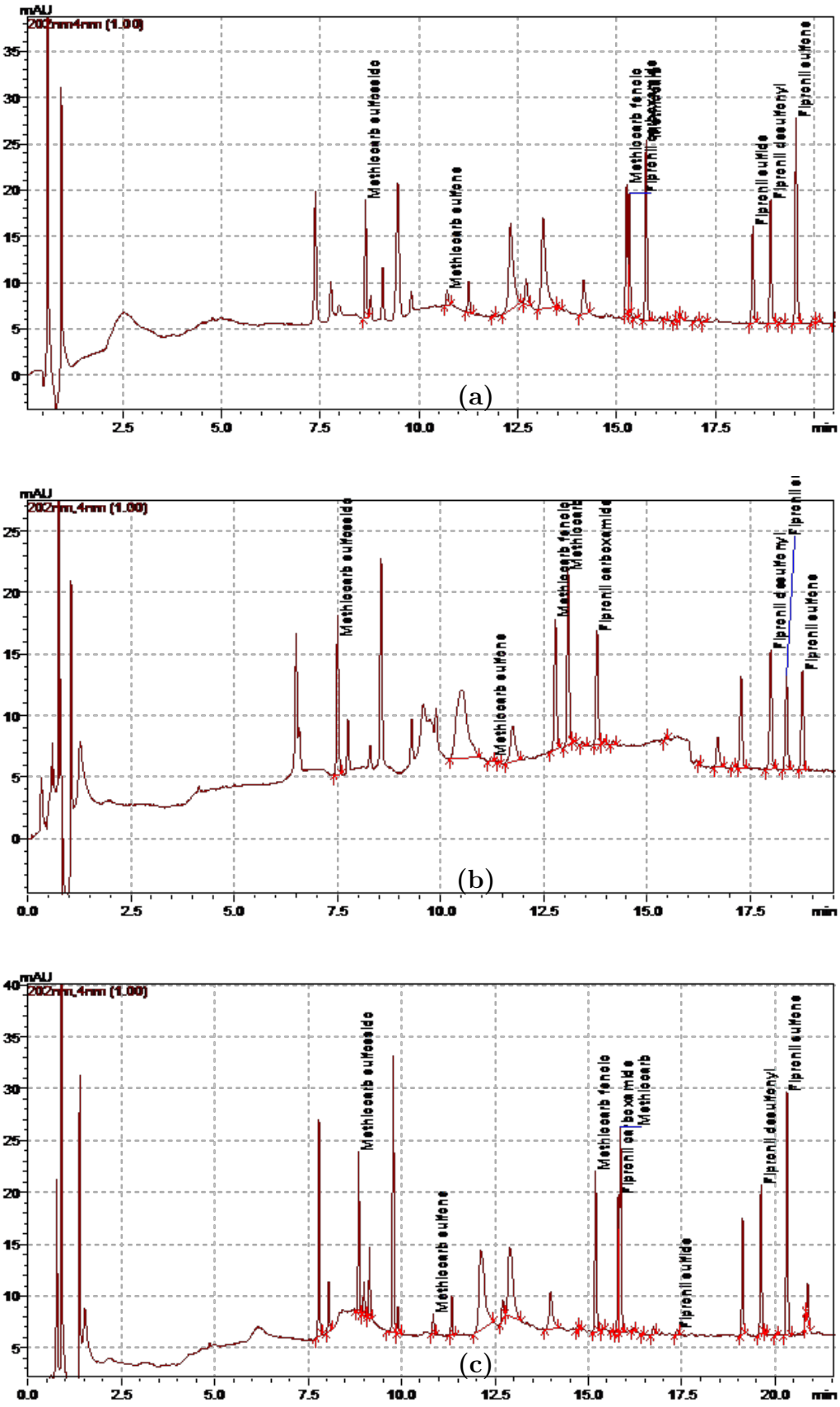


Figure 4.7: UHPLC-DAD chromatograms of standard mix obtained with different analytical columns (a: XR-ODS III; b: PFP; c: Luna® Omega).

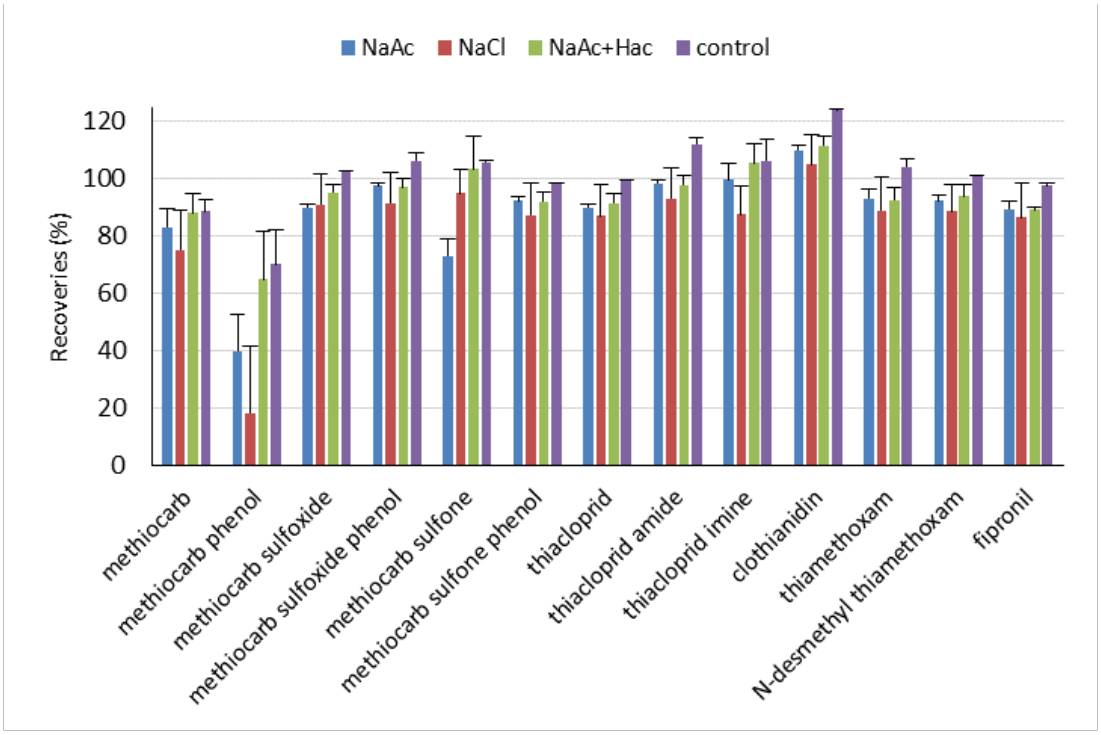


Figure 4.8: recovery factors obtained using different salts and/or additives in the acetonitrile during the extraction.

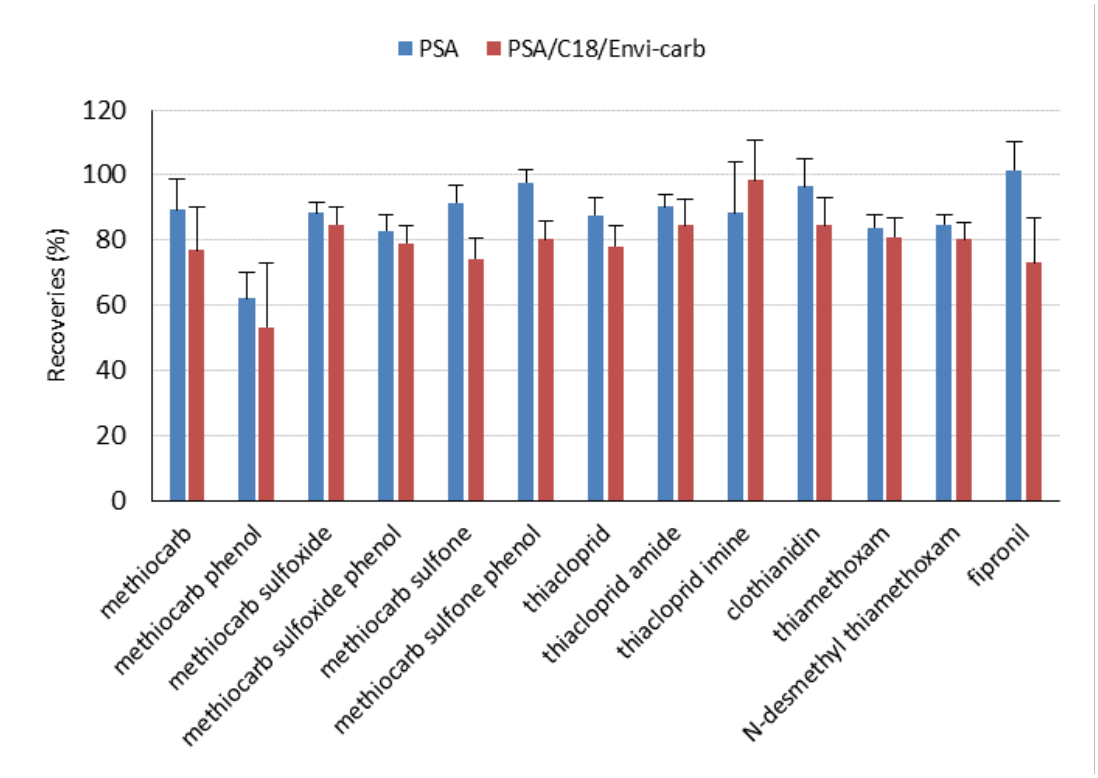


Figure 4.9: recovery factors obtained using different d-SPE phase for the clean-up step.

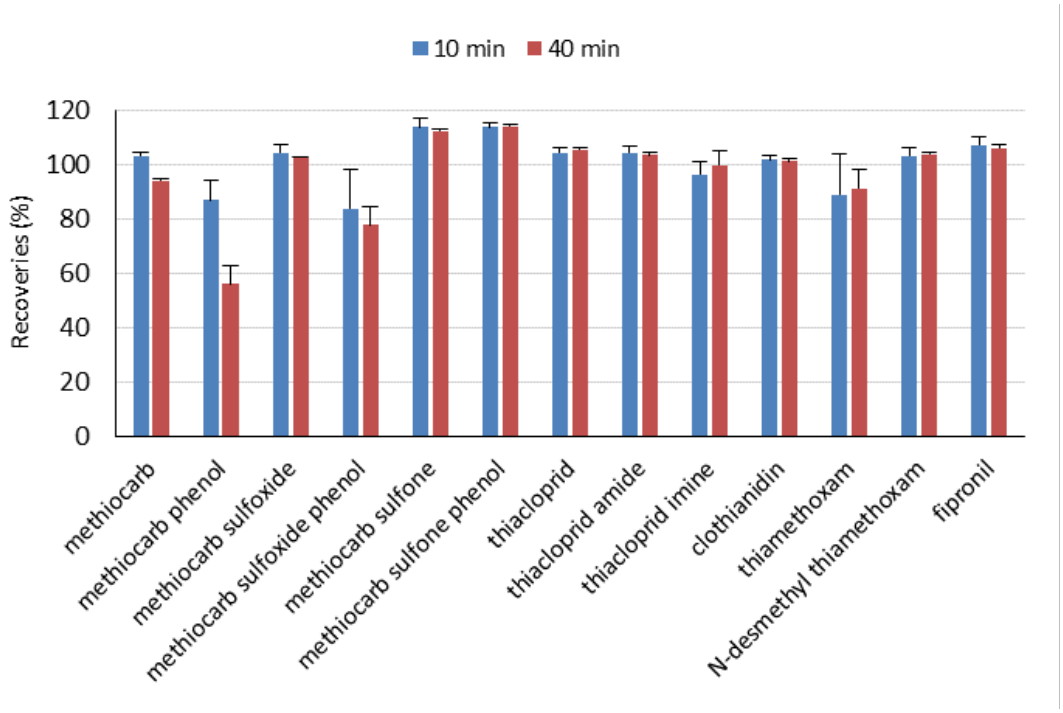


Figure 4.10: recovery factors obtained using different time for the evaporation of the extraction solvent.

only thing that can bias the results is the matrix effect. Therefore, these results proved that guttation matrix (sap) do not required clean-up step prior to injection in order to obtain a proper quantification in LC-ESI-MS.

Recoveries

The recoveries obtained applying the optimised method to the leaf samples were comprised between 69 and 126% (Table 4.1). The method precision was comprised between 2.1 and 16% and it was below the threshold value of 20% for the analysis of pesticides residues. Therefore, the extraction method fit the requirements of accuracy and precision for pesticides residues analysis.

Detection limits

MDL for guttation samples were comprise between 0.27 and 8.7 $\mathrm{ng\,ml^{-1}}$, instead MQL were between 0.81 and 26 $\mathrm{ng\,ml^{-1}}$. Considering that typical concentration of systemic insecticides in guttation drops are in the range of μg/mL the first days after sowing and few ng/mL in the following month (Girolami et al. 2009; Reetz et al. 2015), the MDL and MQL obtained fit the sensitivity requirement for the quantification of insecticides and their degradation products in guttation drops. MDL for leaf samples were comprised between 0.81 and 26 $\mathrm{ng\,g^{-1}}$ (ppb) and the MQL were between 2.4 and 79 $\mathrm{ng\,g^{-1}}$ (ppb). These values were slightly higher compared to MDL and MQL obtained for guttation samples and this is mainly due to the different sample preparation, that leads to a greater dilution of analytes in leaf samples. Their dilution was necessary in order to reduce the matrix

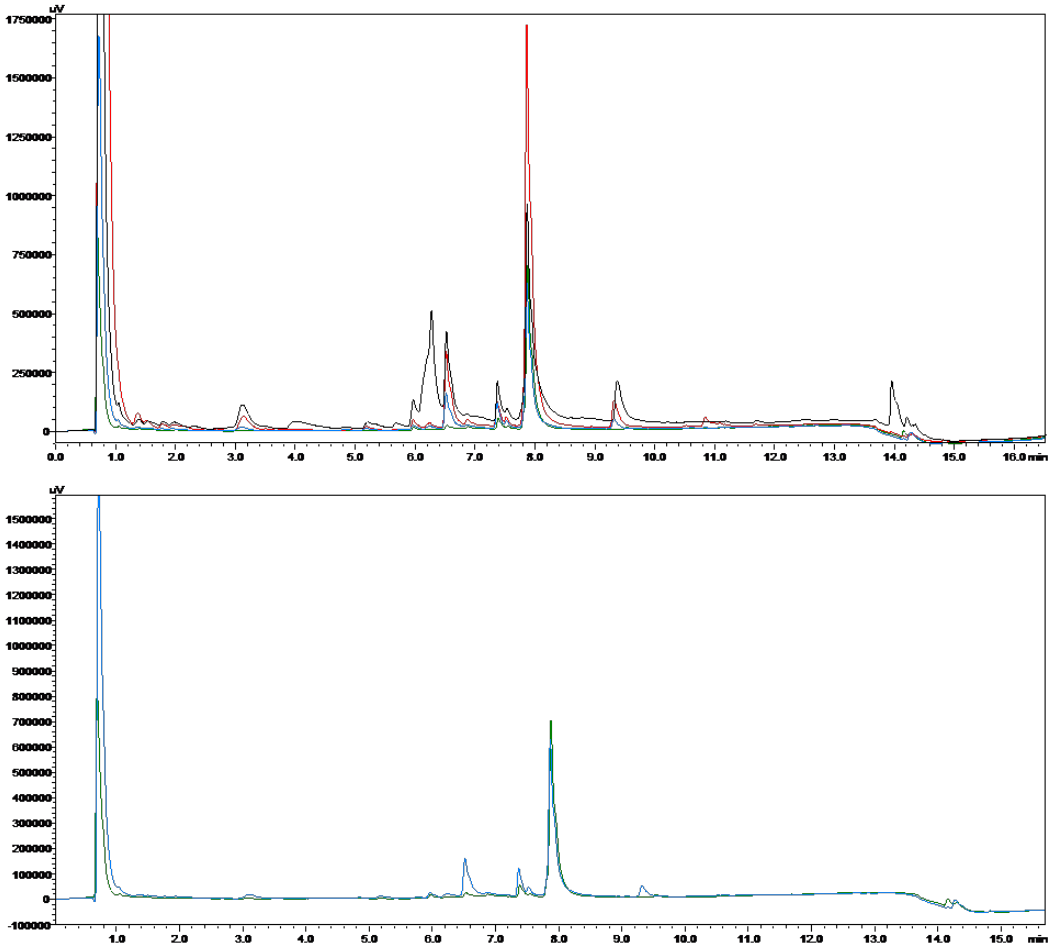


Figure 4.11: UHPLC-DAD chromatograms of corn leaf extract after clean-up with different d-SPE phase (black: XAD, blue: PSA/C18/ENVI-Carb, red: Z-Sep+, green: PSA). PSA and PSA/C18/ENVI-Carb were the most effective phase for corn leaf matrix removal.

injected into the LC-MS system, but it is also convenient in order to maintain the analyte concentration in the linear dynamic range of the instrument. In conclusion, the optimised method allow to quantify the target list of analytes both in guttation and leaf samples and it was applied to real samples in order to assess the presence of insecticides and their degradation products in leaf and guttation. The characteristic of guttation drops makes them very useful to evaluate the presence of systemic insecticides in treated crops and the validated methods is reliable for this purpose.

4.4 Corn guttation drops

Several insecticides and their degradation products were identified in corn guttation drops. The analytes identification was based on the accurate mass of the pseudo molecular ion acquired in the full-scan mode and the structure was confirmed by the MS^2 spectra. For neonicotinoids and fipronil also the characteristic isotopic pattern, due to the presence of chloride, was useful in order to identify peaks of the insecticide or its degradation products.
Seven guttation samples were collected from plants treated with thiamethoxam. The AI was always detected and its mean concentration was 5018 ± 1579 ng mL^{-1}. Also clothianidin was identified in all the analysed samples (Figure 4.12). Clothianidin is a well known thiamethoxam metabolite, but it is also a systemic insecticides used for several products. Therefore, as expected, its concentration was close to the AI with a mean value of 1330 ± 306 ng mL^{-1}. Other metabolites identified were clothianidin urea, desmethyl thiamethoxam, thiamethoxam nitroso and thiamethoxam urea (Figure 4.15, 4.16, 4.14 and 4.13). However, all these metabolite, except for thiamethoxam urea, showed a low signal and their mean concentration is reported in Table 4.2. The presence of clothianidin is relevant, because it is still a toxic compounds. In addition, other metabolites are charachetrised by the modification of the thiamethoxam nitro group. These modification of the nitro group are very important for the toxicity proprieties of the molecule (Tomizawa and Casida 2005). Therefore, their presence could have eco-toxicological implication.
For thiacloprid, four samples were analysed. Thiacloprid was detected in all the analysed samples and its mean concentration was 1024 ± 571 ng mL^{-1}. In addition, five metabolite were identified: amide, hydroxy, olefine, imine, hydroxyl amide and sulphur atom substituted by an oxygen atom (hereinafter called thiacloprid SO) (Figure 4.17, 4.20, 4.19, 4.18, 4.21 and 4.22). This neonicotinoid is charachterised by a lower toxicity for insects compared to thiamethoxam and this is attribute to the presence of the cyano group instead of nitro (Iwasa et al. 2004). Also in this case, many metabolites are characterised by a modification of the cyano group with potential effects to toxicity proprieties.
Concerning methiocarb quantification in guttation drops, its concentration was lower than MQL ($26\,ng\,mL^{-1}$) in 17 of the 21 analysed samples. In one sample its concentration was $31\,ng\,mL^{-1}$ and in three samples its concentration was between MQL and MDL ($8.7\,ng\,mL^{-1}$). However, six methiocarb metabolites were identified in guttation drops: methiocarb phenol, methiocarb sulfoxide, methiocarb sulfoxide phenol, methiocarb sulfoxide hydroxyl, methiocarb sulfone

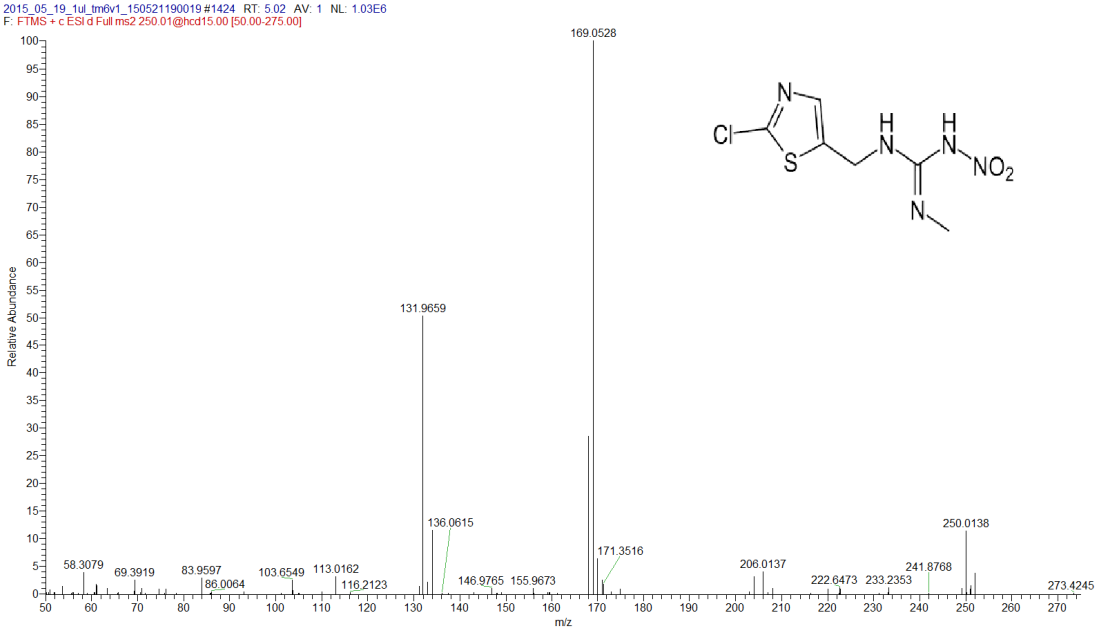


Figure 4.12: fragmentation spectra of the thiamethoxam metabolite clothianidin.

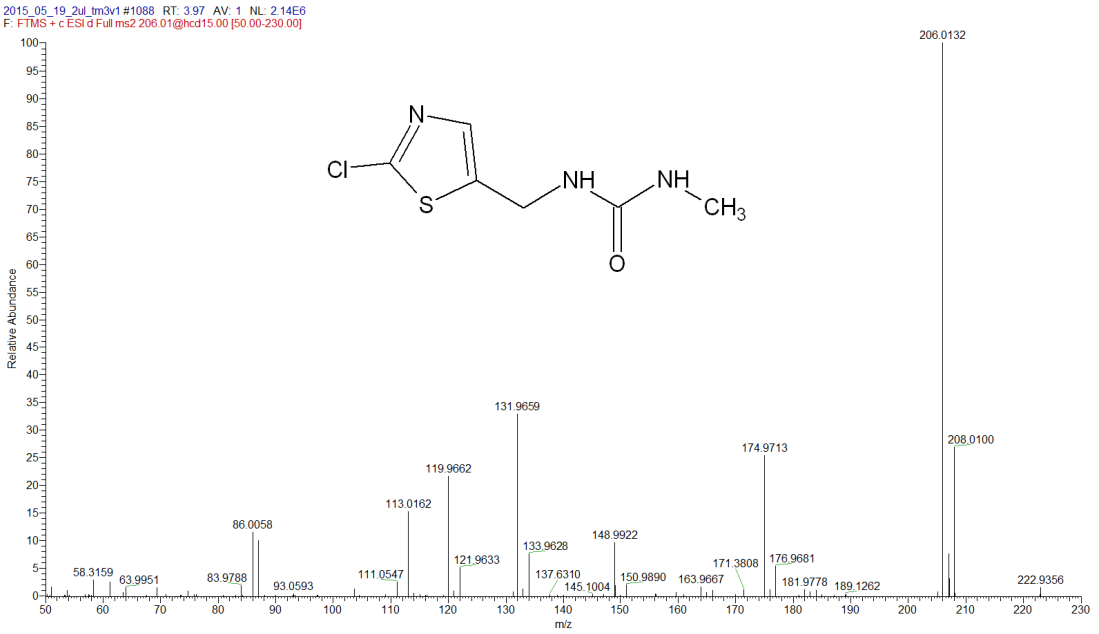


Figure 4.13: fragmentation spectra of the thiamethoxam metabolite clothianidin urea.

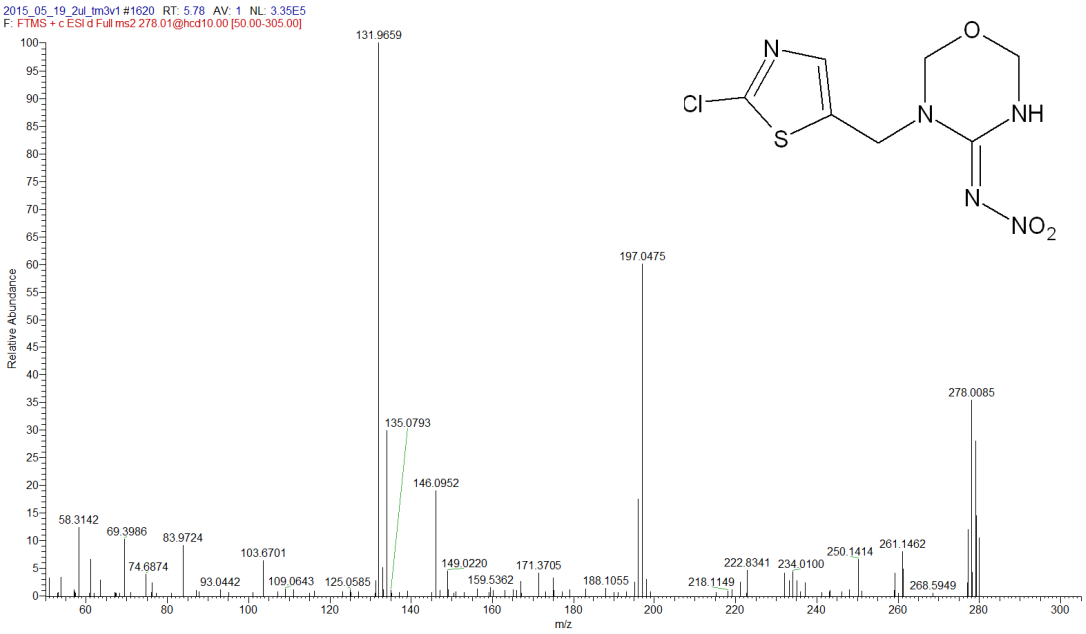


Figure 4.14: fragmentation spectra of the thiamethoxam metabolite desmethyl thiamethoxam.

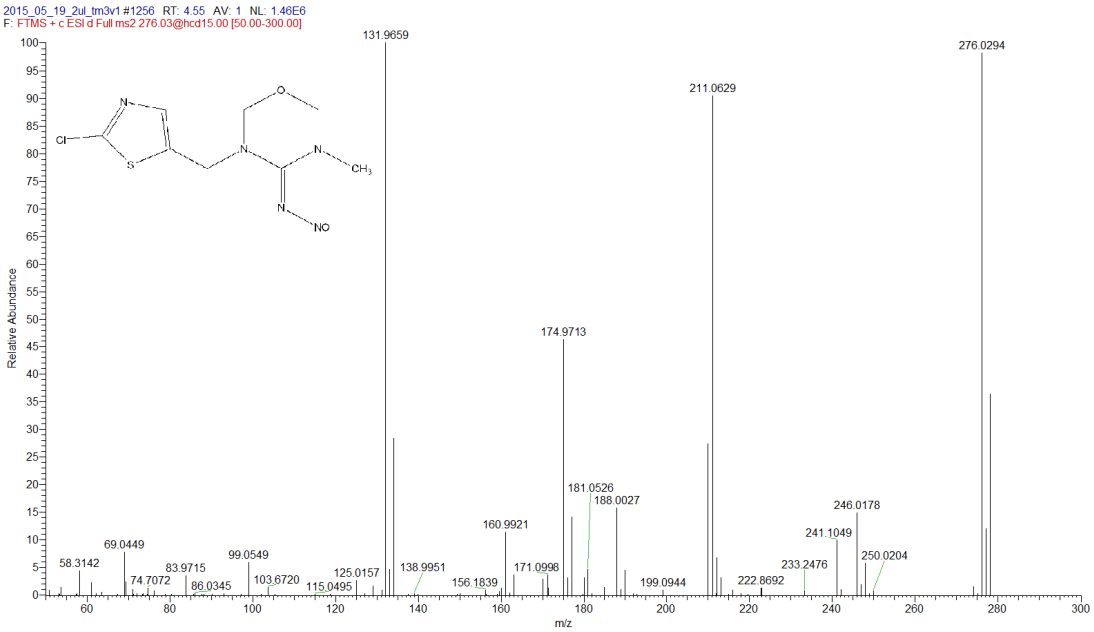


Figure 4.15: fragmentation spectra of the thiamethoxam metabolite thiamethoxam nitroso.

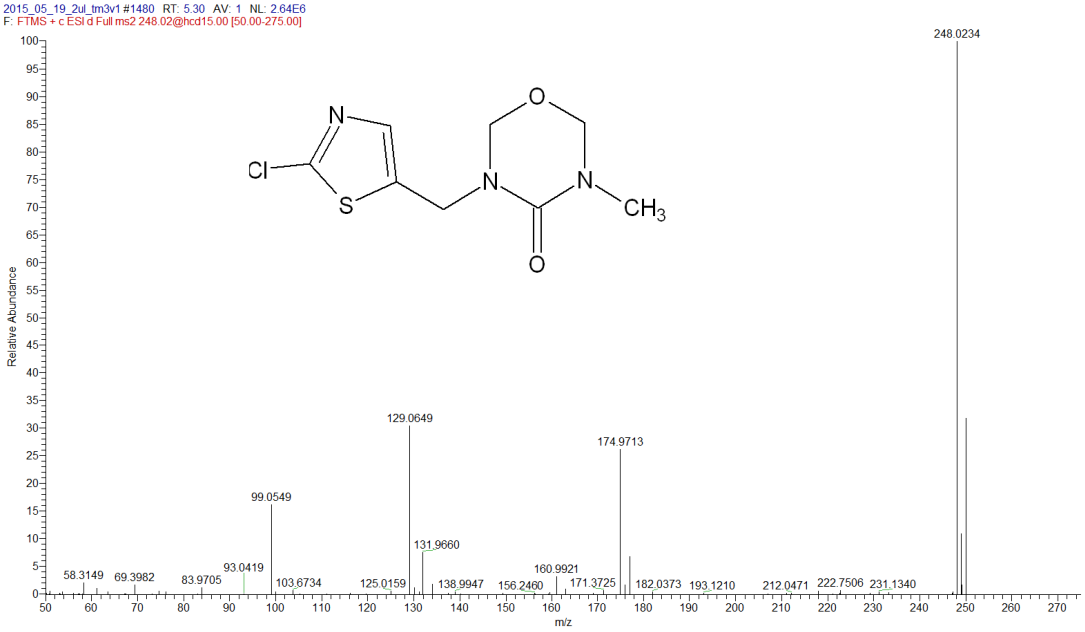


Figure 4.16: fragmentation spectra of the thiamethoxam metabolite thiamethoxam urea.

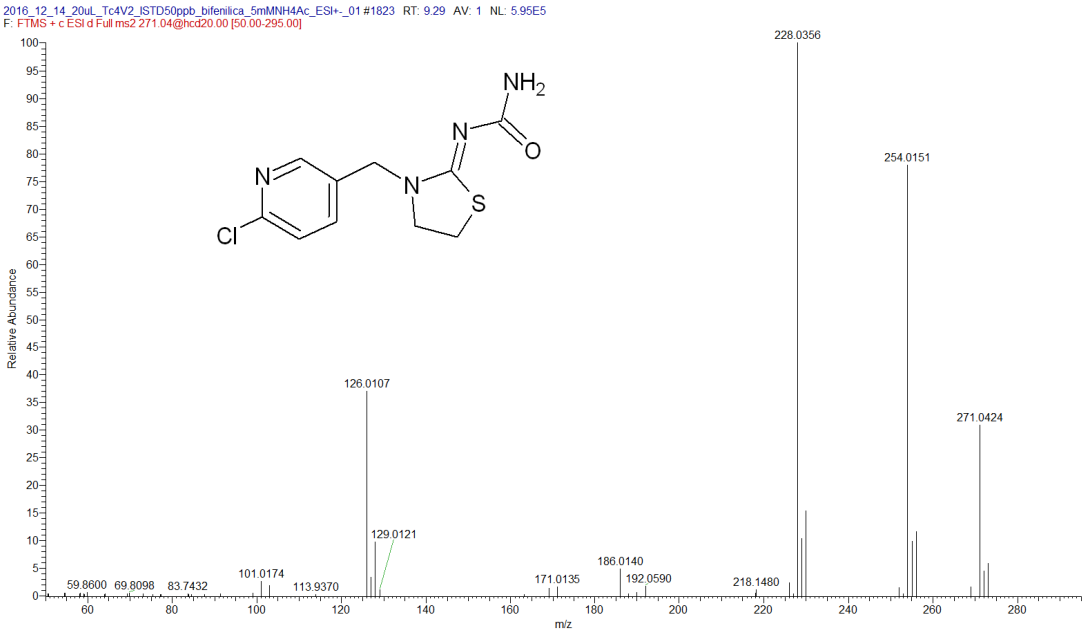


Figure 4.17: fragmentation spectra of the thiacloprid metabolite thiacloprid amide.

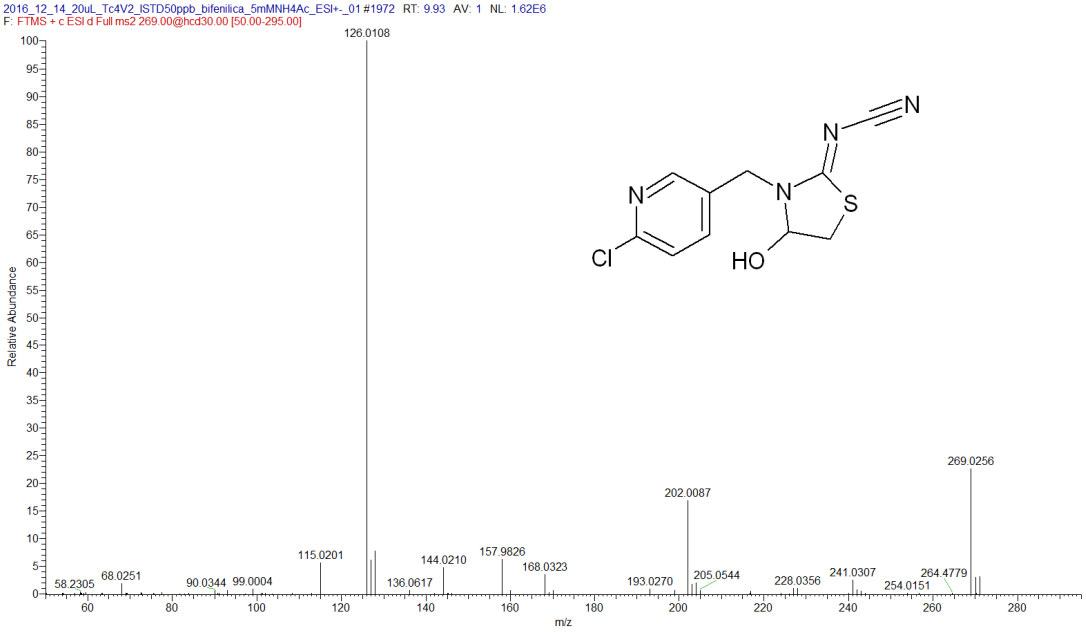


Figure 4.18: fragmentation spectra of the thiacloprid metabolite thiacloprid hydroxy.

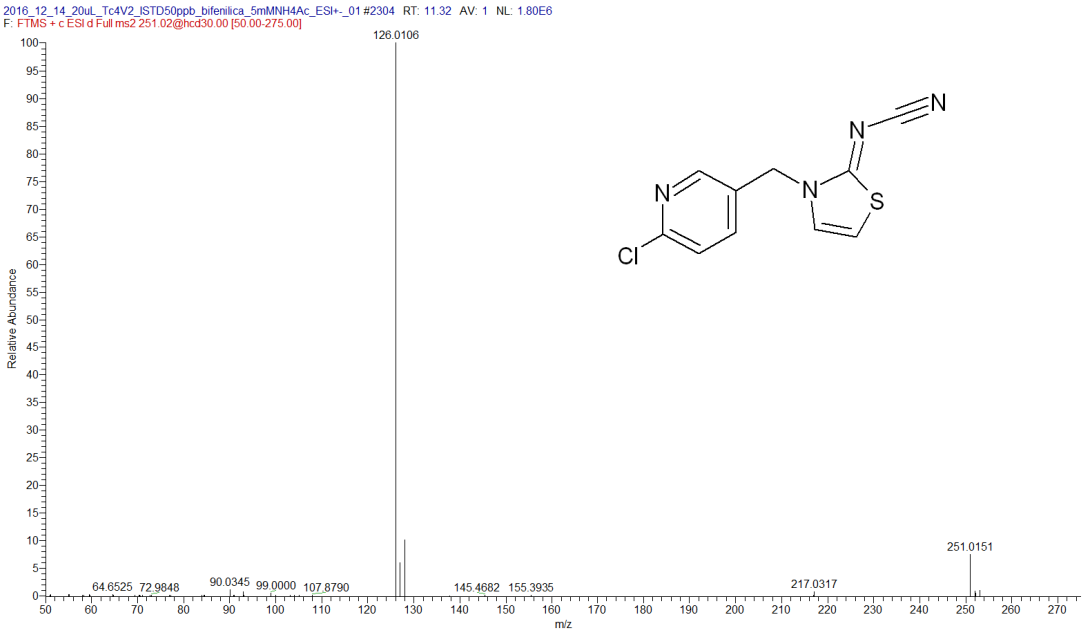


Figure 4.19: fragmentation spectra of the thiacloprid metabolite thiacloprid olefine.

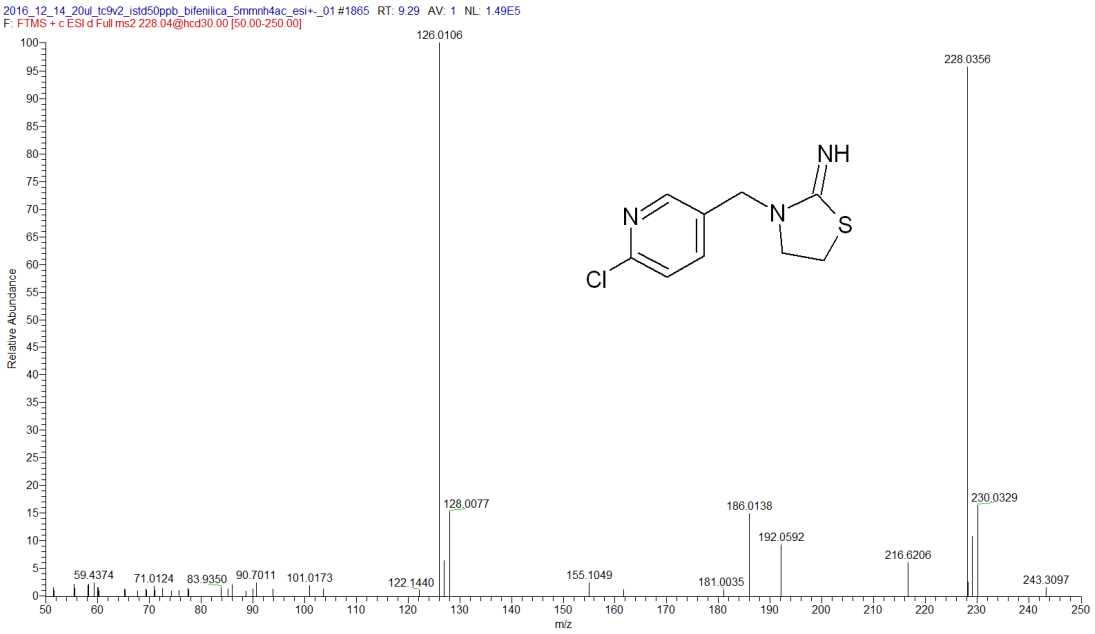


Figure 4.20: fragmentation spectra of the thiacloprid metabolite thiacloprid imine.

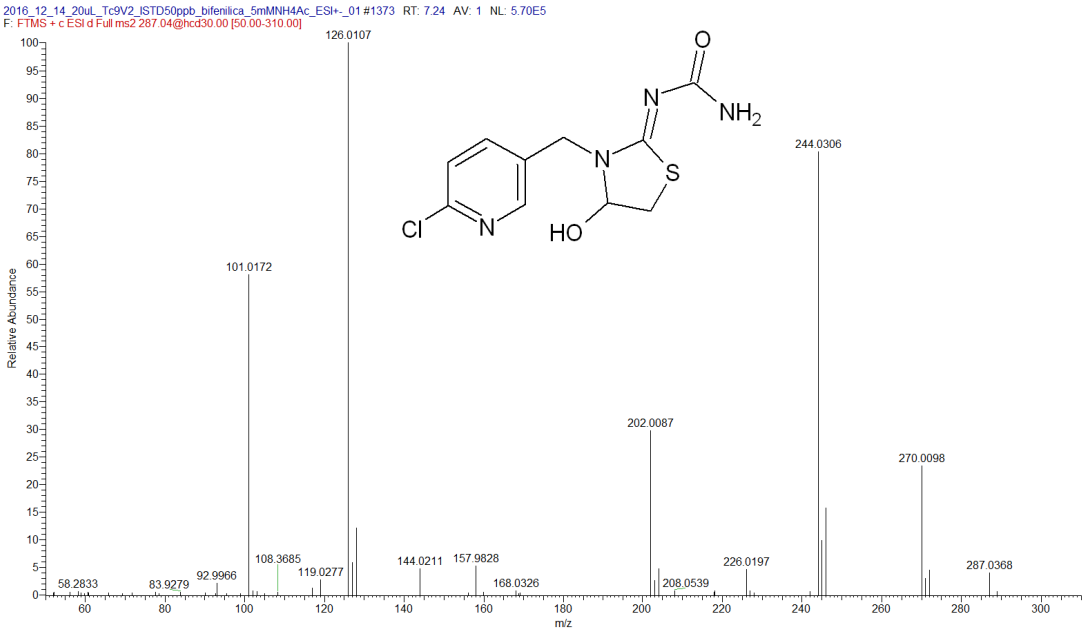


Figure 4.21: fragmentation spectra of the thiacloprid metabolite hydroxy thiacloprid amide.

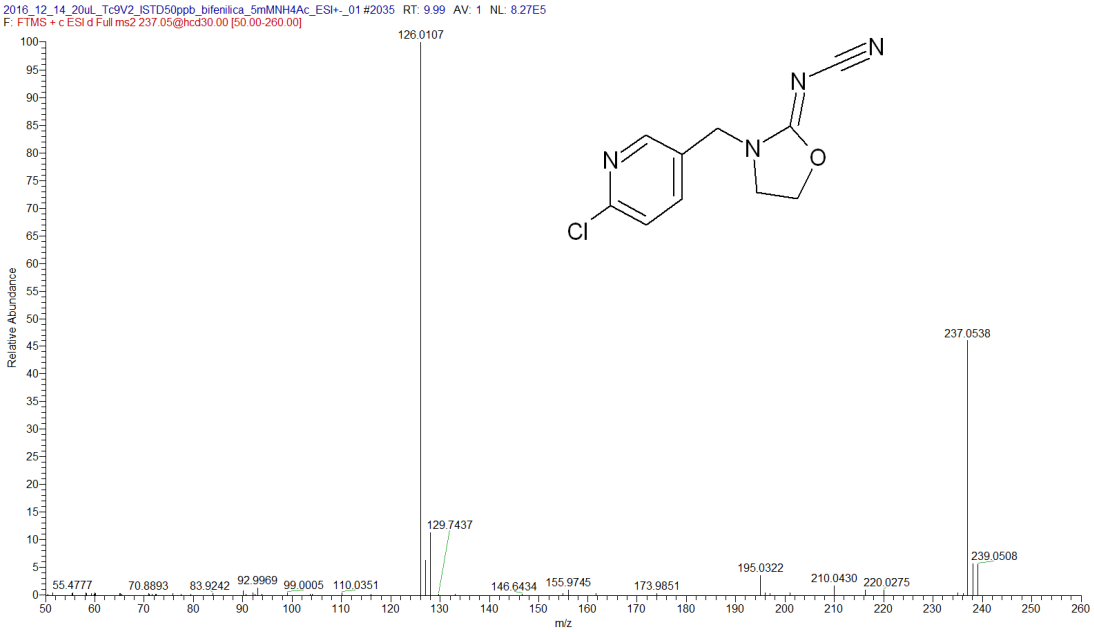
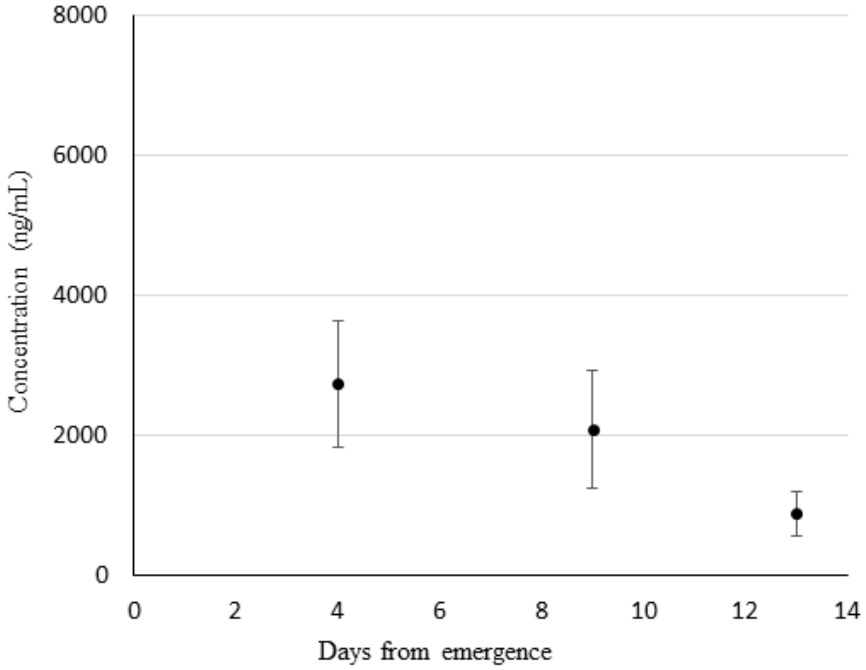


Figure 4.22: fragmentation spectra of the thiacloprid metabolite characterised by the substitution of a sulphur atom with an oxygen atom (hereinafter called thiacloprid SO).

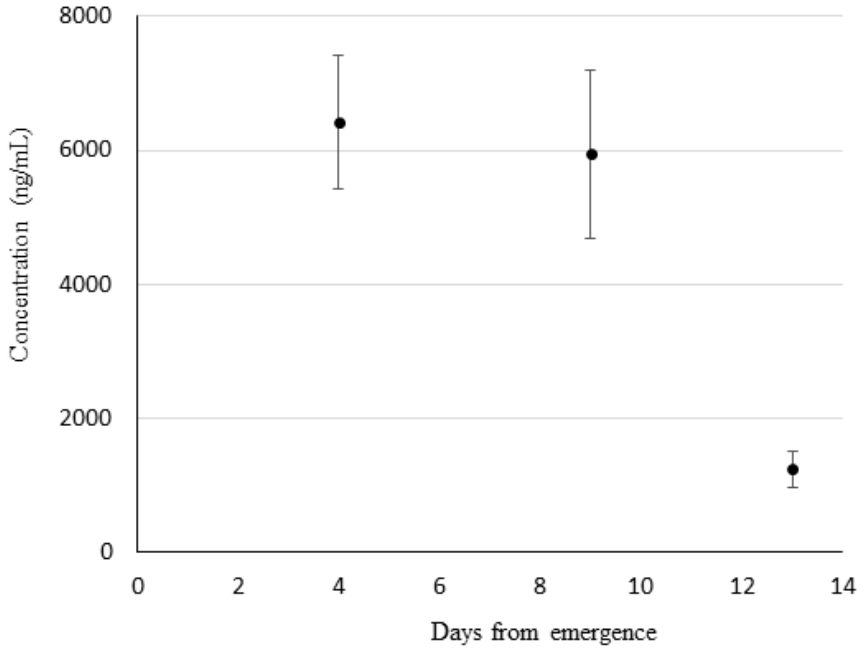
and methiocarb sulfone phenol (Figure 4.24, 4.25, 4.26, 4.27, 4.28 and 4.29). Therefore, the methiocarb modification consisted in the oxidation of the sulphur atom and the hydrolysis of the carbamate group. The concentrations of the sulfoxide, sulfoxide phenol and hydroxy sulfoxide were very high and they were close the neonicotinoids concentration (Table 4.2). This is a first indication that insecticides degradation product may have a systemic proprieties different from their parent AI. It must be noted that sulphur oxidation has relevant consequence in terms of solubility of methiocarb. In fact, methiocarb sulfoxide resulted to be more soluble in water of the parent methiocarb. Considering that guttation is a water solution, this difference can explain the great difference in terms of concentration. Furthermore, methiocarb sulfoxide maintain a relevant toxicity (Buronfosse et al. 1995). Instead, the lost of carbamate group imply a great modification of the molecules structure with a lost of toxicity proprieties.

The analysis of guttation samples collected from corn plant grown in field gave similar results. Methiocarb and methiocarb phenol concentration were below MDL (8.7 and 1.1 ng mL^{-1}). Instead high concentration of methiocarb sulfoxide and methiocarb sulfoxide phenol were observed (Figure 4.23). Also other metabolites were detected and methiocarb sulfone concentration was below MDL (5.1 ng mL^{-1}), but methiocarb sulfone phenol concentration was 163 ± 36 ng mL^{-1}. Mean hydroxy methiocarb sulfoxide concentration was 139 ± 71 ng mL^{-1}.

Four guttation samples from corn plant treated with fipronil were analysed and it was detected in all the samples but below MQL (0.81 ng mL^{-1}). Also fipronil metabolites were identified in corn guttation: fipronil sulfone and desulfonyl (Figure 4.30). However, their concentration was very low and comparable to the parent AI. Fipronil was already observed in xylem sap of sunflower, but its main transport route was individuated in phloem transport (Aajoud et al. 2008).



(a) methiocarb sulfoxide



(b) methiocarb sulfoxide phenol

Figure 4.23: concentration of methiocarb sulfoxide and methiocarb sulfoxide phenol in corn guttation samples collected from plants grown in field.

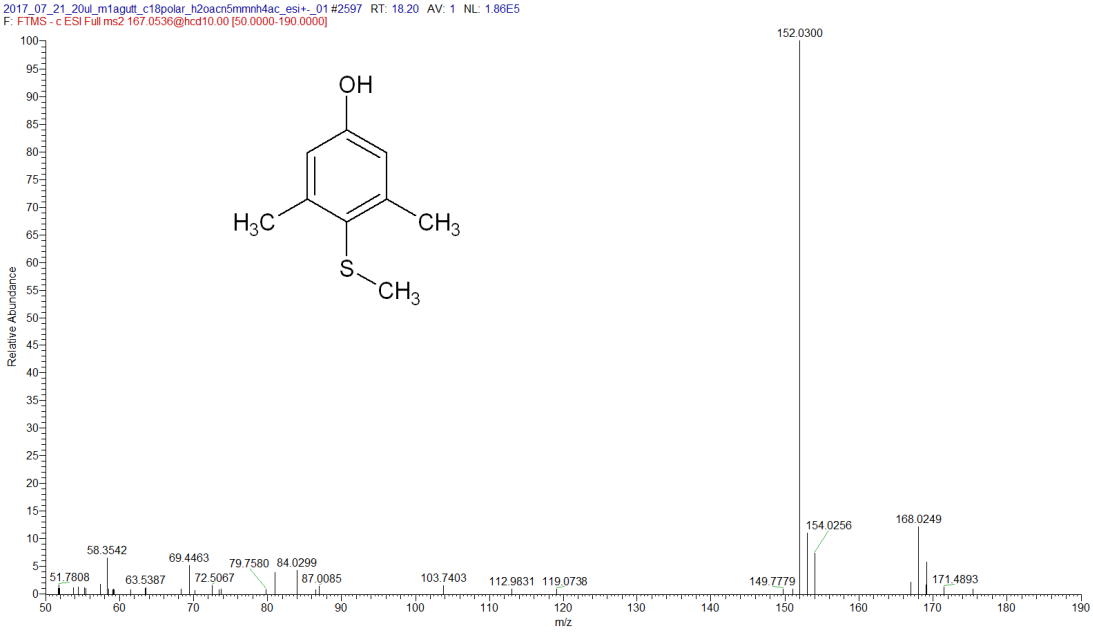


Figure 4.24: fragmentation spectra of methiocarb metabolite methiocarb phenol.

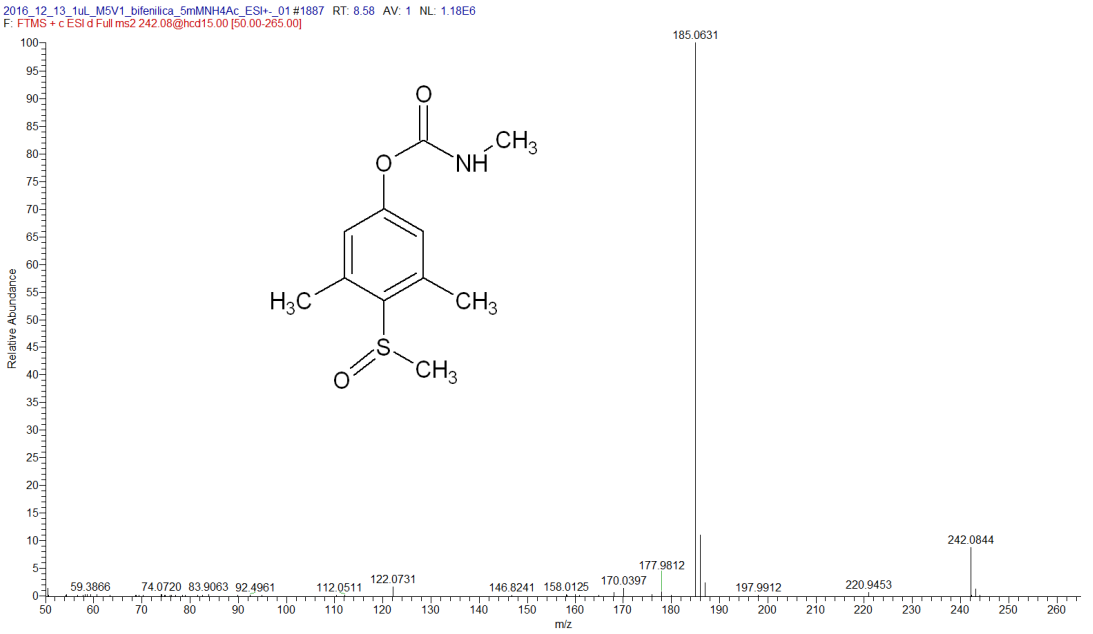


Figure 4.25: fragmentation spectra of methiocarb metabolite methiocarb sulfoxide.

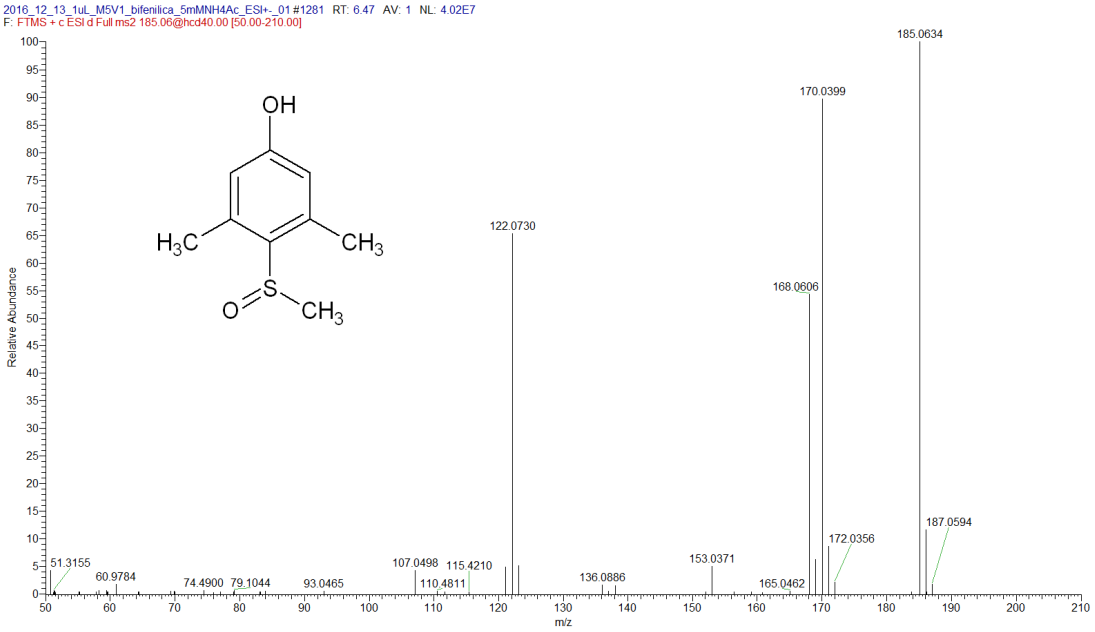


Figure 4.26: fregmentation spectra of methiocarb metabolite methiocarb sulfoxide phenol.

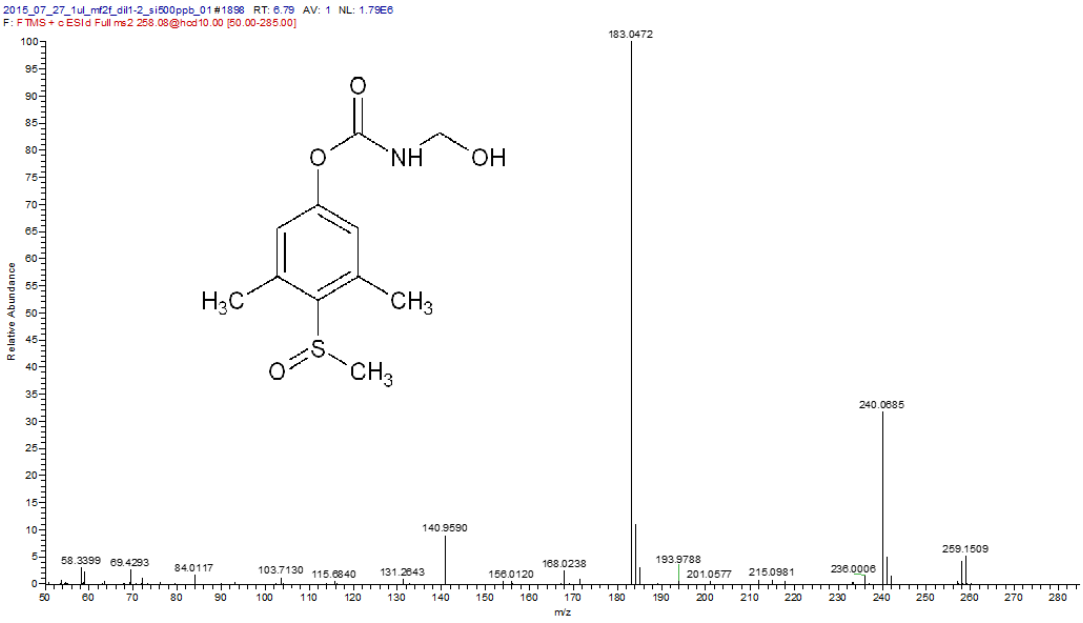


Figure 4.27: fragmentation spectra of methiocarb metabolite hydroxy methiocarb sulfoxide.

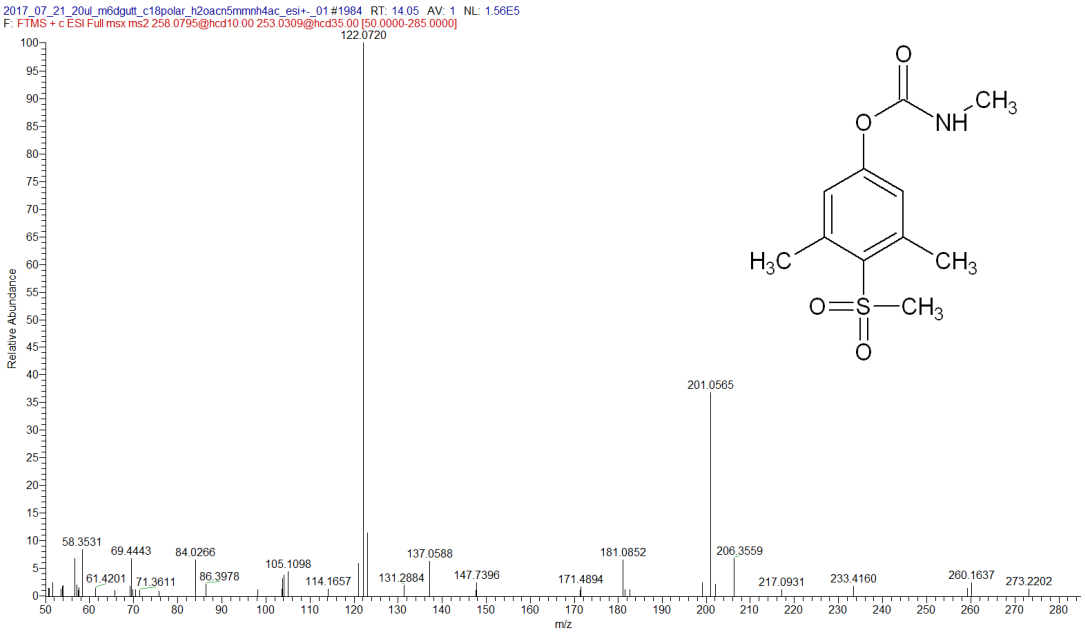


Figure 4.28: fragmentation spectra of methiocarb metabolite methiocarb sulfone.

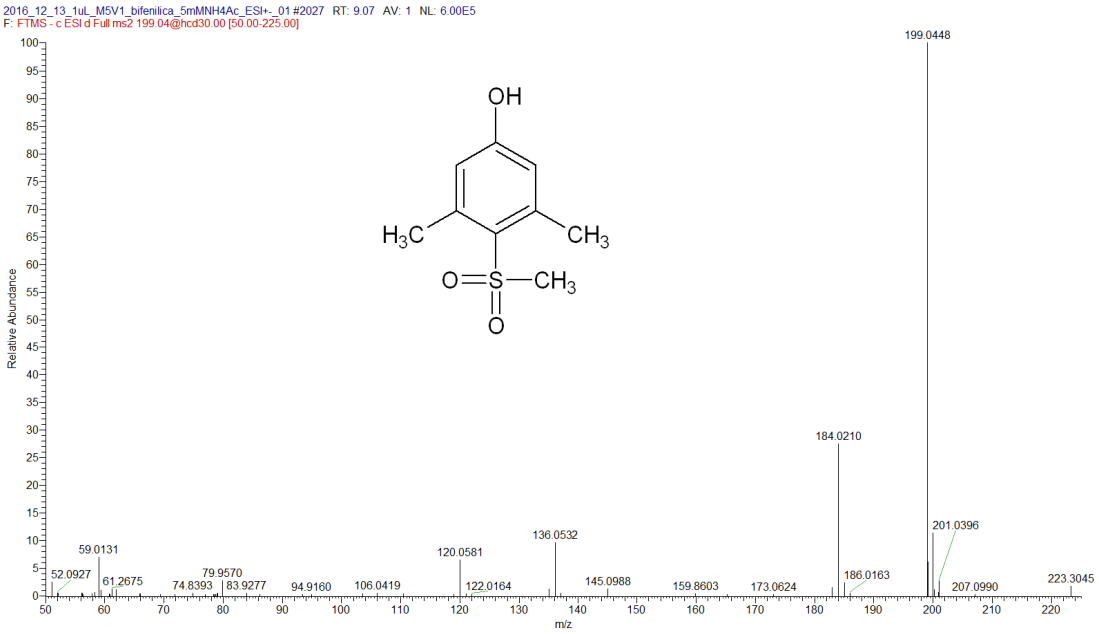
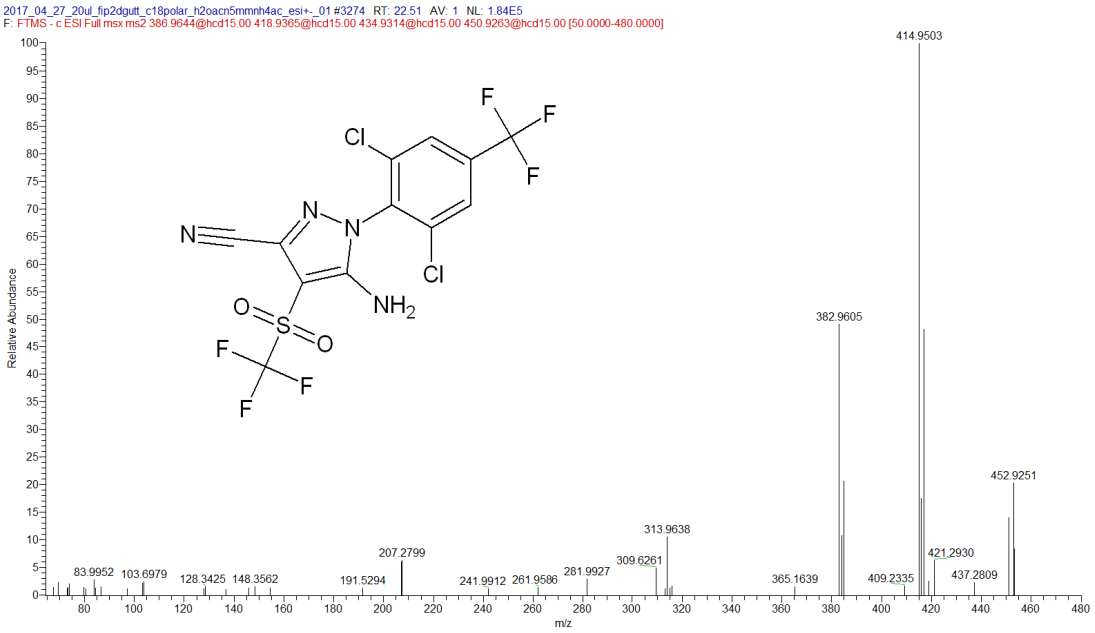
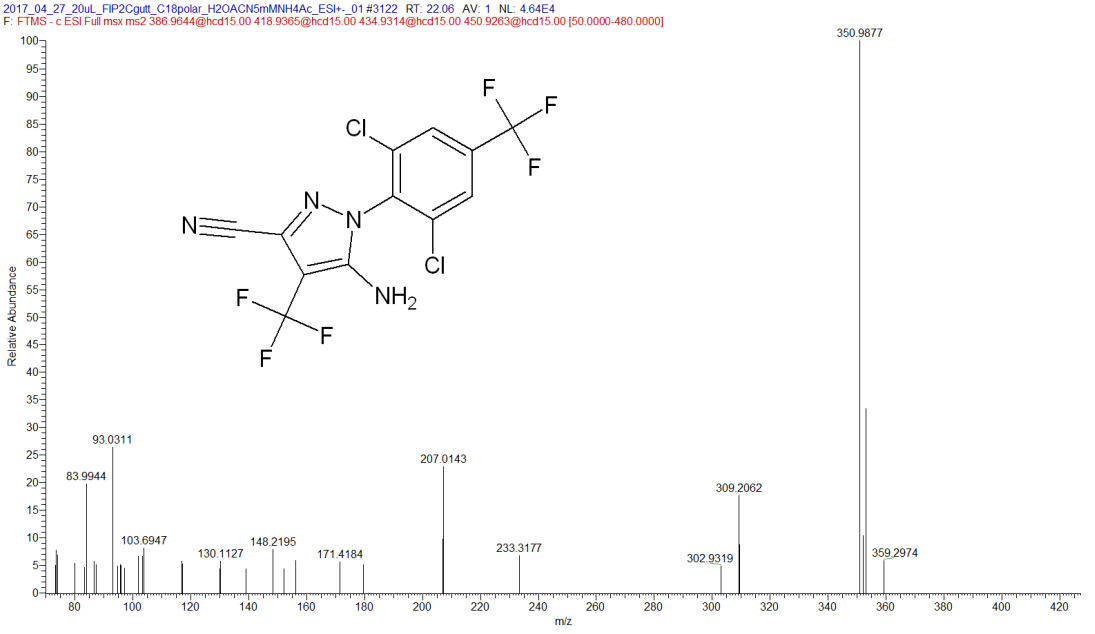


Figure 4.29: fragmentation spectra of methiocarb metabolite methiocarb sulfone phenol.



(a) fipronil sulfone



(b) fipronil desulfinyl

Figure 4.30: fragmentation spectra of fipronil metabolites identified in corn guttation samples.

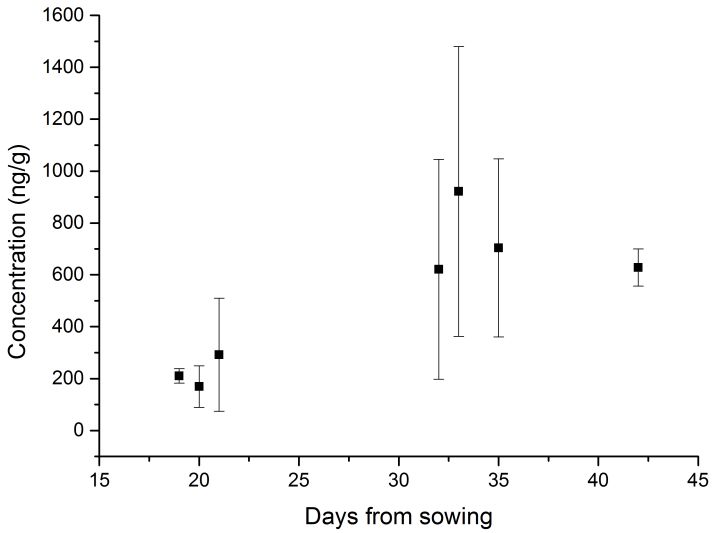


Figure 4.31: concentration of thiamethoxam urea measured in corn leaf samples collected in different days after sowing. It is possible to observe an increase in concentration and variability.

These results show that the concentration of the AI methiocarb and fipronil in guttation drop is very low, if compared to neonicotinoids AI and this seems to be somehow related to solubility in water. However, when a more soluble metabolite is formed, it can be detected in guttation drops at high concentration. These results from UHPLC-HRMS analysis of corn guttation drops allowed to built a target list of insecticides degradation products produced by corn metabolism (Table 3.3). These compounds were successively quantified in corn leaf samples.

4.5 Corn leaf

Insecticides and their degradation products were detected also in corn leaf. In plants treated with thiamethoxam, the AI was always detected and the mean concentration was 7365 ± 3504 ng mL^{-1}. Also in corn leaf many degradation products were identified and quantified. Clothianidin was highly concentrated in corn leaf and thiamethoxam urea is the second most concentrated metabolite (Table 4.3). All the other thiamethoxam metabolites previously identified in corn guttation were identified also in corn leaf: clothianidin urea, desmethyl thiamethoxam and thiamethoxam nitroso. For some of them a clear concentration trend was observed with an increase of concentration (Figure 4.31). However, the concentration in different leaf samples is highly variable, due to the high variability between different plants, therefore is difficult to obtain a clear trend of the concentration.
Also in corn plants treated with thiacloprid, its concentration was very high. In addition, several degradation products were observed. The main metabolite is thiacloprid amide (Table 4.3). Also for this metabolite a higher concentration was

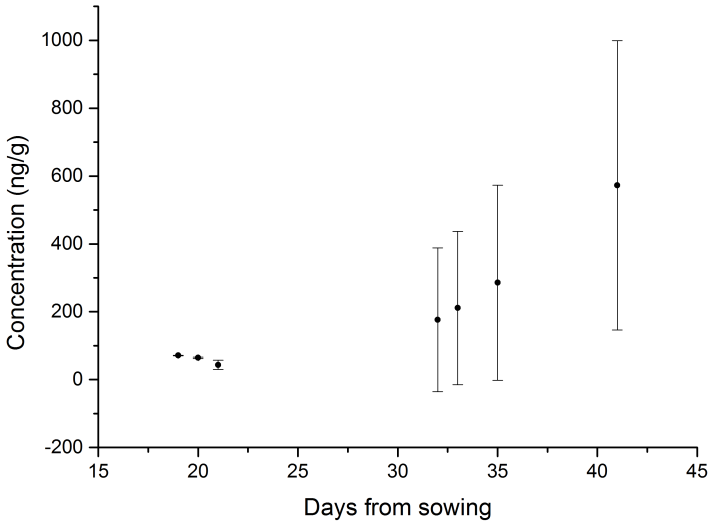


Figure 4.32: concentration of thiacloprid amide measured in corn leaf samples collected in different days after sowing. It is possible to observe an increase in concentration and variability.

observed during days (Figure 4.32).
Completely different from neonicotinoids were the results obtained from analysis of samples collected from corn plants treated with methiocarb. The AI was detected in 7 of the 30 samples analysed, but their concentration was below the MQL of $79\,\mathrm{ng\,g^{-1}}$. Instead, methiocarb metabolites were detected and some of them concentration in corn plants were very high. In particular, methiocarb sulfoxide reached a mean concentration of $4437\,\mathrm{ng\,g^{-1}}$, close to the mean concentration observed for the systemic AI. Also methiocarb sulfoxide phenol and hydroxy methiocarb sulfoxide mean concentration exceeded the value of $1000\,\mathrm{ng\,g^{-1}}$.
Fipronil was detected in 16 of the 18 corn leaf samples analysed. However, its concentration was below the MQL value of $2.4\,\mathrm{ng\,g^{-1}}$ in 12 samples. Therefore, the mean concentration calculated resulted to be lower of the MQL. Fipronil metabolites observed in guttation drops (fipronil sulfone e desulfinyl) were not detected in corn leaf. However, the recoveries obtained for these compounds were not satisfactory and their were not included in the method validation. Therefore, it is not possible to assess if they were present below the MDL or if they were not detected for the low recoveries factors. Further work is necessary to obtain accurate data about fipronil metabolites concentration in corn seedling and the extraction method has to be modify in order to achieve sufficient analytical performance.

4.6 Ornamental plants guttation and leaf

Analysis of the ornamental plant treated with a thiamethoxam solution, confirm that this systemic insecticide is uptaken by plant from roots. It was detected in all the analysed guttation samples (collected two days after treatment) and also in leaf

samples collected staring from ten days after the first treatment. Thiamethoxam concentration rise during days either in guttation and leaf samples (Figure 4.33 and 4.36a). A good precision was observed in replicates leaf samples extracted from the same leaf. This result support the good reproducibility of the extraction method for thiamethoxam. Instead, low volume of guttation samples were collected per day and therefore no replicates were analysed. Because, concentration rise during days both in guttation and leaf, a correlation was observed between these parameters (Figure 4.35). This results prove that guttation analysis can be used to assess the uptake of systemic pesticides in a treated plant. However, must be noticed that concentration in guttation is higher if compared to the leaf concentration. This is quite obvious considering that leaf is made of the same water of guttation, plus vegetative material. Furthermore, guttation concentration resulted highly variable in consecutive samples. In fact, guttation drops can roll off, evaporate or may be sucked back into the leaf (Chyi-Chuann and Yung-Reui 2005), causing variability of analytes concentration.
Also the matabolite clothianidin was detected in all the guttation samples, but its concentration was very low if compared to thiamethoxam concentration in the same samples or clothianidin concentration in corn guttation. In leaf samples, clothianidin was detected only starting from 25 days after the first treatment (Figure 4.34) and, as for guttation, its concentration was much lower compared to thiamethoxam. Instead, the concentration trend observed in guttation is very similar for thiamethoxam and clothianidin (Figure 4.36). In Figure 4.37 is shown the correlation between the concentration of the two analytes and an excellent linear correlation was observed. This result prove that the concentration measured in guttation samples was not only analyte dependent, but it was somehow related to plant physiology or drop evaporation prior to collection.
Other metabolites were identified in the ornamental plants guttation but not in the leaf. These are clothianidin urea, thiamethoxam nitroso and thiamethoxam urea. Even if the MDL is slightly higher for leaf, is possible to assume that this is due to the higher concentration of the insecticides in the guttation compared to leaf. Therefore, guttation drops seems to be an excellent matrix to study the presence of systemic pesticides metabolites.

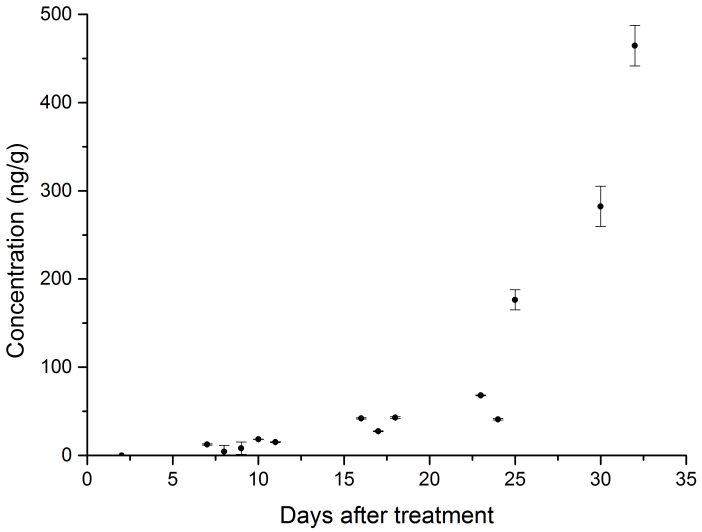


Figure 4.33: thiamethoxam concentration trend in leaf samples collected from an ornamental plant treated with a thiamethoxam solution.

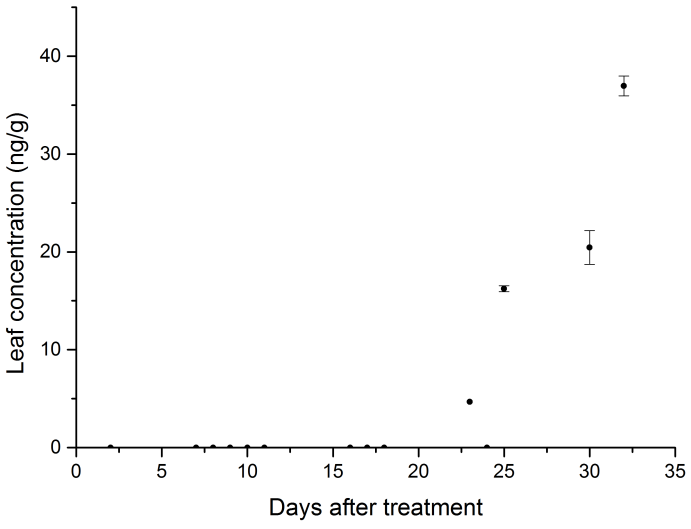


Figure 4.34: clothianidin concentration trend in leaf samples collected from an ornamental plant treated with a thiamethoxam solution.

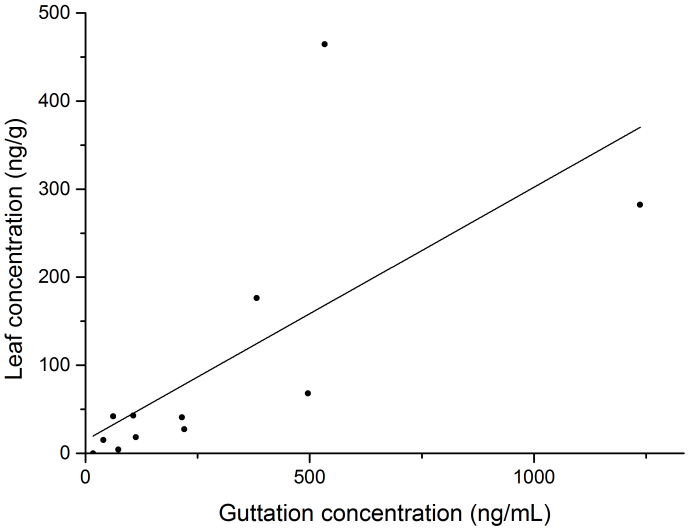


Figure 4.35: a significant correlation was observed between thiamethoxam concentration in leaf and in guttation samples collected from a treated ornamental plant (Pearson correlation, $r(12) = 0.701$, $P = 0.011$).

Table 4.1: performance of the validated methods for the quantification of insecticides and their degradation products in guttation drop and leaf samples.

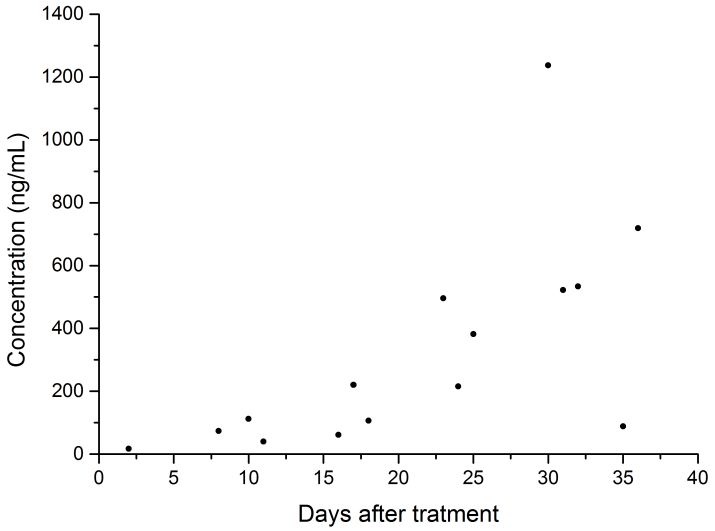
Analyte	guttation				leaf							
									accuracy (%)			
									60 ng/g		600 ng/g	
	R^2	matrix effect (%)	MDL (ng/mL)	MQL (ng/mL)	R^2	matrix effect (%)	MDL (ng/g)	MQL (ng/g)	mean	RSD	mean	RSD
Methiocarb	0.9997	18	8.7	26	0.9975	11	26	79	110	8.6	122	5.3
Methiocarb phenol	0.9984	13	1.1	3.4	0.9907	22	3.4	10	95	6.8	82	10
Methiocarb sulfoxide	0.9814	6.5	2.9	8.6	0.9932	−21	8.6	26	98	8.9	98	3.2
Methiocarb sulfoxide phenol	0.9930	−9.5	2.6	8.0	0.9966	14	7.9	24	86	3.3	101	4.5
Methiocarb sulfone	0.9901	1.6	5.1	16	0.9915	−10	15	47	71	2.1	93	5.5
Methiocarb sulfone phenol	0.9952	−14	1.3	4.0	0.9931	−21	4.0	12	126	4.2	108	4.2
Thiacloprid	0.9933	5.2	2.4	7.3	0.9895	−7	7.2	22	101	3.0	109	6.0
Thiacloprid amide	0.9880	17	1.9	5.7	0.9943	−19	5.7	17	97	2.6	69	16
Thiacloprid imine	0.9963	−4.2	0.49	1.5	0.9932	−22	1.5	4.5	113	10	84	3.0
Clothianidin	0.9998	1.9	3.4	10	0.9895	13	10	31	79	2.4	91	4.7
Thiamethoxam	0.9804	4.3	1.0	3.1	0.9977	6	3.1	9.3	71	3.3	113	5.5
N-desmethyl thiamethoxam	0.9980	18	1.0	3.1	0.9494	−9	21	64	110	4.6	69	4.8
Fipronil	0.9966	4.5	0.27	0.81	0.9851	−13	0.81	2.4	110	9.7	77	16

Table 4.2: Mean concentration of insecticides AI and their degradation products detected in corn guttation. For calculations, when concentration was below MDL it was considered zero and when it was below MQL the MDL value was assigned.

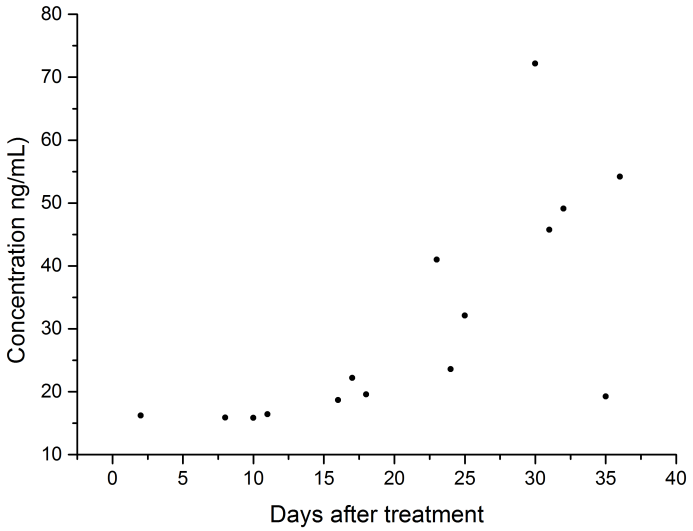
Analyte	mean (ng/mL)	SD (ng/mL)	median (ng/mL)	1st quartile (ng/mL)	3rd quartile (ng/mL)
thiamethoxam	5018	1579	4568	4395	5533
clothiandin	1330	306	1316	1255	1393
clothianidin urea	36	18	31	23	43
thiamethoxam urea	1495	534	1802	1076	1903
desmethyl thiamethoxam	14.0	7.4	16.8	7.7	19.1
thiamethoxam nitroso	44.7	21.9	43.7	31.8	59.
thiacloprid	1024	571	928	750	1202
thiacloprid amide	< 5.7	/	/	/	/
thiacloprid imine	2.54	0.11	2.49	2.46	2.57
thiacloprid amide hydroxy	2.52	0.94	2.45	2.12	2.84
thiacloprid SO	4.6	3.3	3.8	2.6	5.8
thiacloprid hydroxy	18	12	18	12	24
thiacloprid olefin	117	79	124	85	157
methiocarb	< 8.7	/	/	/	/
methiocarb phenol	< 3.4	/	/	/	/
methiocarb sulfone	< 5.1	/	/	/	/
methiocarb sulfone phenol	18	25	6.7	1.3	19
methiocarb sulfoxide	611	1124	356	191	461
methiocarb sulfoxide phenol	539	425	354	246	748
methiocarb sulfoxide hydroxy	68	138	22	16	56
fipronil	< 0.81	/	/	/	/

Table 4.3: mean concentration of insecticides AI and their degradation products detected in corn leaf. For calculations, when concentration was below MDL it was considered zero and when it was below MQL the MDL value was assigned.

Analyte	mean (ng/g)	SD (ng/g)	median (ng/g)	1st quartile (ng/g)	3rd quartile (ng/g)
thiamethoxam	7365	3504	7273	5504	7994
clothiandin	2007	1021	1767	1385	2579
clothianidin urea	55	37	42	30	70
thiamethoxam urea	489	374	440	200	669
desmethyl thiamethoxam	59	53	21	11	110
thiamethoxam nitroso	19	9.2	19	13	23
thiacloprid	4736	3792	3349	2397	6030
thiacloprid amide	233	281	106	66	261
thiacloprid imine	33	29	29	17	36
thiacloprid amide hydroxy	39	33	30	12	60
thiacloprid SO	4.7	2.2	4.1	3.3	5.6
thiacloprid hydroxy	20	17	13	7.3	27
thiacloprid olefin	8.4	7.7	6.2	4.5	9.4
methiocarb	< 79	/	/	/	/
methiocarb fenolo	13	27	3	0	12
methiocarb sulfone	93	54	93	64	131
methiocarb sulfone phenol	285	151	260	192	335
methiocarb sulfoxide	4437	2139	4334	2953	5362
methiocarb sulfoxide phenol	1432	1026	1196	1008	1586
methiocarb sulfoxide hydroxy	2998	1646	2716	1931	3571
fipronil	1.83	2.50	0.81	0.81	0.81



(a) thiamethoxam (AI)



(b) clothianidin

Figure 4.36: concentration trend of two different analytes in the same guttation samples collected from a treated ornamental plants.

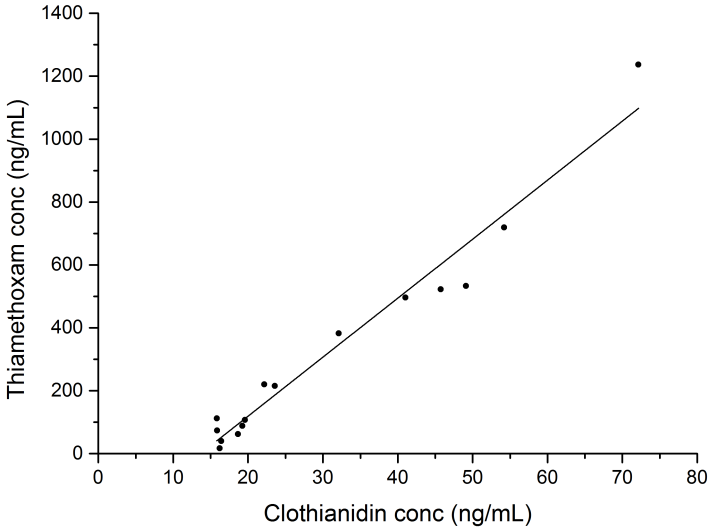


Figure 4.37: a highly significant correlation was observed between thiamethoxam and clothianidin concentration in guttation drop samples collected from an ornamental plant treated with a thiamethoxam solution (Pearson correlation, $r(15) = 0.981$, $P < 0.01$).

Chapter 5

Discussion

5.1 Systemic compounds in guttation drop

Since the first evidence that systemic insecticides concentration in corn guttation drops were sufficient to cause honeybees death (Girolami et al. 2009), many studies investigated the actual honeybees exposure through guttation (Frommberger et al. 2012; Pistorius et al. 2012; Reetz et al. 2015; Reetz et al. 2011). These studies observed that guttation are not a significant exposure route for honeybees, that regularly use other water sources (Nikolakis et al. 2015). However, the exposure of wild insects was never studied and guttation may have a negative impact to those insects living in the corn field, so they can still have an eco-toxicological relevance. Neonicotinoids persistence in soils, waterbodies, and wild plants is variable but can be prolonged (neonicotinoids half-lives in soils can exceed 1000 days), so they can accumulate when used repeatedly (Bonmatin et al. 2015). Systemic pesticides can contaminate also non-treated plants due to leaching in soil and surface water contamination. Neonicotinoids were detected in guttation liquid of plants emerged from non-coated maize seeds in Mörtl et al. 2017. Therefore, guttation drops analysis can be used to assess leaching of systemic insecticides from soil and the contamination of non-target plants. Furthermore, when exposure model are used to estimate the concentrations in water bodies of insecticides applied to corn, the addition of guttation drop in the model improved its prediction (Hartz et al. 2017). Therefore, even if guttation drops are not a significant exposure route for honeybees, their analysis can provide useful information about environmental contamination by systemic insecticides.

In this study, corn guttation drops were successfully used to identify insecticides degradation products present in corn plants grown from coated seeds. UHPLC coupled with HRMS analysis is a powerful technique for this purpose and the adopted methods was very effective for the identification of many compounds in a single chromatographic run with a minimal samples preparation. The instrument setting used allowed to collect high resolution full-scan and fragmentation spectra in the same chromatographic run in both polarities. Another advantage of this method was the possibility of a retrospective analysis to identify eventually new suspect metabolites, because fragmentation spectra were registered automatically for the most intense ions observed in full-scan without the implementation of

a target list (Annex II). If compared with analysis of leaf or other vegetative tissues, guttation drops analysis has many advantages. First of all, no extraction is required, because guttation matrix is relative clean. Therefore a dilute and inject approach was successfully used. However, the dilution was necessary also to add the Internal Standard, because usually guttation drop volume is lower than 50 μL. In our experiment a 1:1 dilution was sufficient to not observe matrix effect and, because systemic insecticides resulted to be highly concentrated in guttation drops, the dilution did not reduce the possibility of detecting systemic compounds relevant for eco-toxicological studies. One other advantage is that guttation drops are easy to collect and their sampling do not affect the plant. Therefore, several sampling from the same plant are possible for a long period. However, the main problems with guttation is to obtain a regular production by the plant and the great variability in the insecticides concentration. In order to improve these aspects, when possible is convenient to cover the plant to obtain air humidity saturation. This improve the production of guttation and reduce the evaporation of the drops, that could be a reason of variability.

Concerning the presence of the neonicotinoids active ingredients used for seeds coating, thiametoxam and thiacloprid were detected in guttations at high concentrations (mg/L level, as previously observed for neonicotinoids in Tapparo et al. 2011). In addition, many metabolites were identified and some of them had a modification of the nitro group for thiamethoxam or cyano group for thiacloprid. This may lead to important modification of their toxicity proprieties, because these groups are fundamental for toxicity (Ford and Casida 2006b). In particular, the imine metabolite is characterised by an inversion of selectivity between insects and mammals (Tomizawa and Casida 2003). Therefore, the presence of thiacloprid imine in corn plant is particular interesting.

The most interesting results were obtained for methiocarb, because its metabolites were detected at high concentration even if the AI was present with a mean concentration below the MQL of $8.7\,\mathrm{ng\,ml^{-1}}$. In particular, methiocarb sulfoxide and the relative phenol are present in high concentrations. As for methiocarb, this is the first study in which the absence of the insecticide in the guttations is evidenced together with the significant presence of its metabolites. In other terms, methiocarb and the relative phenol are present in corn guttations at concentration of ng/mL, where there are many other metabolites. Methiocarb metabolite consist mainly in the oxidation of the sulphur atom, that lead to an higher water solubility of the compound. These modification seems to be highly relevant for the systemic proprieties of the compounds. Furthermore, methiocarb sulfoxide is still a toxic compound (EPA 1987). Therefore, its presence in corn guttation indicate that a toxic methiocarb metabolite is able to penetrate into the plant vascular system. In this way, even if the AI methiocarb has not systemic proprieties, it may have a systemic effect through the formation and diffusion into the plant tissue of methiocarb sulfoxide.

5.2 Insecticides and metabolites in corn seedling

Real samples of leaf collected from corn plants grown from seeds coated with insecticides were analysed with the developed method. Several AI and their degradation product present in guttation drops were identified also in corn leaf. Corn seedlings are a potential exposure route to systemic insecticides for non target insects, birds and small herbivore mammals that may eat this young plants. Also un-buried coated seeds are dangerous from this point of view in particular for birds (Gibbons et al. 2015).

The results obtained from corn leaf analysis revealed that neonicotinoids AI were present in corn seedling at high concentration (µg/g level). This is due to the high amount of AI applied to the seeds and to the low weight of the whole plant during the first weeks after sowing. Concerning plants coated with thiamethoxam, clothianidin and thiamethoxam urea were the main degradation products identify. In particular, clothianidin is a well known metabolite of thiamethoxam, but it is also an AI by itself. Therefore, it is still highly toxic for insects. In addition, clothianidin is considered moderately toxic for some bird species (e.g. clothianidin LD_{50} for bird Japanese quail, *Coturnix japonica*, is 423 $\mu g/g$; Gibbons et al. 2015). These results suggests that negative effects observed in vertebrate wild life in presence of neonicotinoids use in crops treatment, could be due to exposure through seedling.

Also thiacloprid concentration reached the $\mu g/g$ in corn seedling, but it was significant lower compared to thiamethoxam concentration (t-test, $t = 2.49$, $p = 0.017$). The main degradation product of thiacloprid was thiacloprid amide, but its concentration is much lower if compared to the AI (Table 4.3). Few data are available about metabolites toxicity and so it is difficult to assess their effects to non-target animals. However, thiacloprid amide is reported to have 15.6 times lower mortality than the parent AI against the pest *Aphis craccivora* (Liu et al. 2011).

The carbamate insecticide methiocarb gave completely different results. The mean concentration of the AI was very low, but three metabolite (methiocarb sulfoxide, sulfoxide phenol and hydroxyl) were present at high concentration. In particular, methiocarb sulfoxide concentration was not significantly different from the thiacloprid concentration (t-test, $t = 0.37$, $p = 0.71$). This results proved that this metabolite has systemic proprieties comparable to neonicotinoid insecticides. Furthermore, methiocarb sulfoxide is a toxic compound (Buronfosse et al. 1995), therefore its presence in corn seedling may indicate a systemic action of methiocarb through the presence of this metabolite. Furthermore, methiocarb sulfoxide is known to be more toxic than the parent AI for mice (Oonnithan and Casida 1968) and so its impact against non-target small mammals is particularly dangerous. It is worth noting that methiocarb sulfoxide is highly toxic also for aquatic invertebrates with important consequences to the environment. However, both methiocarb and its metabolite methiocarb sulfoxide are not persistent in the environment.

Few data were obtained also for the insecticides fipronil. Its average concentration in corn leaf collected from plant grown from coated seeds was very low (< 2.4 ng/g) if compared to the concentration of the systemic neonicotinoids. Even if

the systemic proprieties of this AI are still debated (Mortensen et al. 2015), our result suggest that it systemic proprieties are different form neonicotinoids and it must not be group with them for environmental risk assessment.
For some metabolites a change in concentration over time was observed (Figure 4.31 and 4.32). This may prolong the exposure of non-target animals to toxic compounds. However, it must be underline that for neonicotinoids insecticides the concentration of the AI was far too high compared to metabolites. Therefore, they play the main role for the toxic action.

5.3 Systemic insecticide uptake in ornamental plant

From analysis of ornamental plants purchased from local garden centres in the Sussex area (UK), pesticides residues of different chemical classes were found in leaf, pollen and nectar samples (Annex III). In addition, in the literature are available analysis of pollen collected from bumblebees that observed pollinator insects exposure to insecticides also in urban environment (Botias et al. 2017; David et al. 2016). Therefore, an experiment was performed in order to evaluate the uptake of the systemic insecticide thiamethoxam when applied to the soil of a potted ornamental plant. The results from guttation and leaf analysis revealed that thiamethoxam was immediately present in guttation drops, instead it was later identified in leaf. Furthermore, the main difference compared to experiment with potted corn was observed in clothianidin concentration. In corn seedling this metabolite was observed at high concentration, but in ornamental plant it appeared only 20 days after the first treatment and at low concentration. The main difference was the application of the insecticide. In fact, with seed coating before the seedling emergence, thiamethoxam could be degraded by soil bacteria causing clothianidin formation. Instead, with ornamental plant thiamethoxam was immediately up-take at high concentration and there was no time for clothianidin formation. Similar results were obtained for the other thiamethoxam metabolites, that were not identified in leaf samples, but only in ornamental guttation drops. These results confirmed that guttation analysis is a sensitive and fast method to identify insecticides metabolites potentially present in plants. Furthermore, it is a powerful technique to assess plants contamination due to pesticides presence in soil. However, insecticides concentration in guttation drops could be higher compared to leaf and so concentration in guttation drops is not representative of insecticides concentration in plant tissues.

Chapter 6

Conclusion

One goal of this study was to use guttation analysis in order to identify insecticide metabolite with systemic proprieties. These compounds may still be toxic and their quantification is an essential step in order to assess the effects of an insecticide for non-target animals. The developed method for guttation drops analysis allowed to identify insecticides used for seeds coating and their degradation products. The accurate masses, collected with HRMS, were used in order to identify the suspect compounds and the fragmentation spectra collected in the data dependent acquisition allowed to confirmation of their structures. In addition, analysis of guttation samples resulted to be an easy and powerful technique to provide information about insecticides degradation in plants and our study prove that guttation drops analysis is very useful to study which degradation products are more common in plant metabolism. In particular, this analysis can easily give important information about systemic proprieties of insecticides, because only compounds able to reach the apical part of the plant were detected. The main advantage of guttation is that they are a simple matrix (water solution) and the concentration of systemic compounds is very high. Therefore, they are a useful tool to screen the presence of systemic AI or metabolites in plant tissues.
The insecticides and metabolites identify in the guttation drops were also quantified and interesting information about their systemic proprieties were obtained. The results of corn analysis are summarized in Figure 6.1. In particular, the systemic insecticides thiamethoxam and thiacloprid were detected in corn guttation drops. Methiocarb has different chemical proprieties with respect systemic pesticides and it is not present in guttation drops at relevant concentration. Nevertheless, its degradation product methiocarb sulfoxide behave like a systemic pesticide. Fipronil is often grouped with neonicotinoids as a systemic insecticides, but according to the fipronil concentration measured in corn guttation drops is possible to say that the systemic proprieties of the insecticide fipronil are completely different from the neonicotinoids.
In addition, an extraction procedure for pesticides quantification in leaf was fully validated and coupled with the UHPLC-MS^2 method optimised it allowed the accurate quantification of insecticides and their degradation products in corn and *Aglaonema commutatum* leaf. Several insecticide metabolite identified in the guttation drop were also observed and quantified in corn leaves. High concentration of thiamethoxam and thiacloprid were observed. In addition, the metabolite

clothianidin, methiocarb sulfoxide and methiocarb sulfoxide phenol were also observed at high concentration. Therefore, the presence of these compounds in corn seedling confirmed that degradation products must be considered in the risk assessment related to systemic agrochemical used for seeds coating.

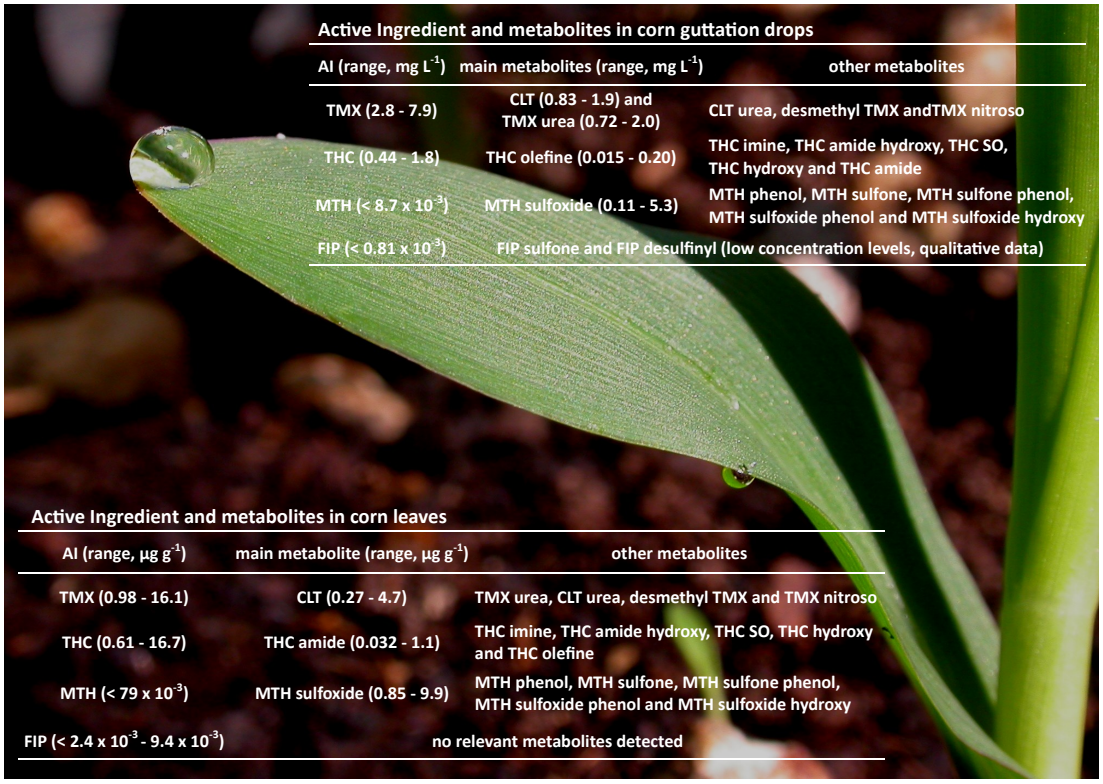


Figure 6.1: infographic summary of the results obtained from the analysis of corn guttations and leaves (TMX: thiamethoxam, THC: thiacloprid, MTH: methiocarb and FIP: fipronil).

Annex I. List of Abbreviations

a.u. Arbitrary Unit

ACN acetonitrile

AI Active Ingredient

CAS Chemical Abstracts Service

CCD Colony Collapse Disorder

d-SPE dispersive Solid Phase Extraction

d.p.r. Decreto del presidente della Repubblica

d.w. Dry Weight

DMSO Dimethyl Sulfoxide

ESI Electrospray Ionization

EU European Union

FIP fipronil

full-scan dd-MS^2 full scan data dependent MS^2

GABA gamma-aminobutyric acid

GAP Good agricultural practice

GCB Graphitized Carbon Black

GluCls glutamate-activated chloride channels

HCD Higher-energy Collisional Dissociation

HRMS High Resolution Mass Spectrometry

K_{ow} partition coeficient octanol water

KSCN Potassium thiocyanate

LC Liquid Chromatography

LD_{50} Lethal Dose for the 50 % of a population

LLE Liquid Liquid Extraction

MDL Method Detection Limit

MeOH Methanol

MQL Method Quantification Limit

MSX multiplexed MS

MTH methiocarb

MW Molecular Weight

nAChR nicotinic acetylcholine receptor

NCE Normalized Collision Energy

NMR Nuclear Magnetic Resonance

NOAEL No Observed Adverse Effect Level

OP organophosphorus

PRM Parallel Reaction Monitoring

PSA Primary Secondary Ammine

QuEChERS Quick, Easy, Cheap, Effective, Rugged, and Safe

SPE Solid Phase Extraction

THC thiacloprid

TMX thiamethoxam

UHPLC Ultra High Performance Liquid Chromatography

Annex II.
The Orbitrap mass analyser

The Orbitrap is a high resolution mass analyser and the instrument used for this thesis has a maximum resolution of 140 000. The High Resolution Mass Spectrometry (HRMS) allow to use the accurate mass in order to identify the raw structure of an analyte. However, the accurate mass alone is not sufficient to identify a compounds and also the fragmentation pattern obtained with MS^2 experiment is necessary. The instrument used for this thesis was a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap. The presence of a quadrupole installed before the Orbitrap mass analyser allow to isolate a precise scan range or a single precursor ion with a mass window of 1 Da to send it in the fragmentation cell as in common MS^2 experiment with triple quadrupoles. Using the full scan data dependent MS^2 (full-scan dd-MS^2) acquisition mode is possible to collected the full scan spectrum and the MS^2 spectrum of the most intense ions is preformed automatically. Coupling information of accurate mass and fragmentation spectra is possible to identify compounds also without the confirmation of an analytical standard.

Even if the HRMS technique is ideal for qualitative purpose, it has become very useful also for quantitative analysis. From this point of view, the main advantage of the high resolution is the high sensitivity in the full-scan mode if compared to other classic detector. The HRMS allow to filter more chemical noise during the analysis, with an enhancement of the signal to noise ratio. However, in presence of a complex matrices and many analytes coming to the detector in a short time range, Orbitrap mass spectrometer could miss analytes fragmentation in the full-scan dd-MS^2 acquisition mode, in particular if the analytes are present at low concentration. The best quantitative performance in terms of sensitivity could

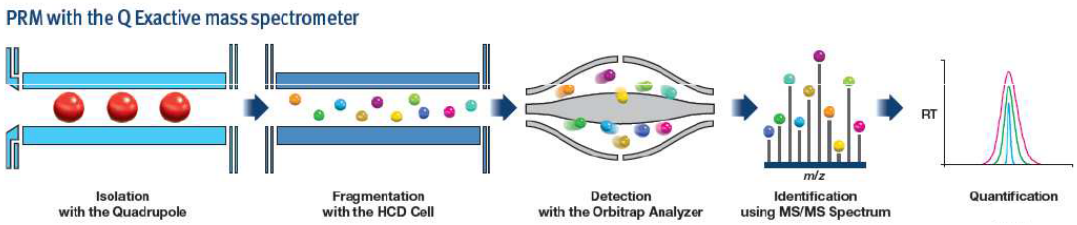


Figure 6.2: illustrative scheme of the PRM acquisition mode with an Orbitrap instrument. Adapted from thermofisher.com.

be achieved using Parallel Reaction Monitoring (PRM) acquisition mode. In this case, the Orbitrap works in a complete target mode and a list of target analytes is necessary (Li et al. 2015; Rajski et al. 2017). The precursor ion of the analyte is isolated by the quadrupole, fragmented in the HCD cell and then injected into the Orbitrap for acquisition (Figure 6.2). Working with UHPLC the peaks used to be narrow ($10 - 15$ seconds) and a fast acquisition rate is required. In order to improve the scan rate, MSX feature could be used (Kaufmann and Walker 2016). Therefore, more precursor ions were sequentially isolated by the quadrupole and stored inside the C-trap. Then they are fragmented together in the HCD cell and the fragments obtained were analysed simultaneously in the Orbitrap. In this way, the fastest possible scan rate is achieved and the high resolution allow to distinguish fragment ions generated by different precursors.

Annex III.
Multi-class pesticides screening in ornamental plants

Introduction

The ornamental plants are often highly attractive for pollinator insects and they could contribute to expose these insects to pesticides. Therefore, not only leaves, but also pollen and nectar samples were collected and analyzed. The idea was to quantify pesticides residues in ornamental plants purchased from garden center. The contamination of ornamental plants was studied in collaboration with the research group of Professor Dave Goulson and Professor Ellizabeth Hill (University of Sussex - Brighton, UK) and the results of these studies are already reported in Lentola et al. 2017.
Compared with guttation leaf and pollen are a more complex matrix and they required a procedure of matrix removal in order to obtain a selective analytical method. Therefore, an extraction procedure from plant tissues based on QuEChERS method was develop (Anastassiades et al. 2003). Later, this procedure was used to evaluate the presence of pesticides in ornamental plants suitable for gardens, in order to assess the exposure to pesticides of non-target animals (pollinators in particular).

Materials and methods

Popular bee-attractive ornamental plants were purchased from local garden centers located in the East Sussex area (United Kingdom). Samples of foliage, nectar and pollen were collected during flower blooming (May-July) from 29 plant species (Table 6.5).
Prior to extraction, 10 g of leaves were ground with liquid nitrogen followed by manual homogenization using a micro-spatula. Pollen samples were collected from flowers dried in an incubator at 37 °C for 24 hours to facilitate pollen release from the anthers. After drying, flowers were brushed over food strainers to separate pollen from anthers and sifted through multiple sieves of decreasing pore size (from 250 to 45 μm). Collection of nectar from flowers was performed through capillary action into glass micro-capillaries (5 mL). For each species three leaf replicate of 100 ± 5 mg were extracted. Instead, a variable amount within $10 - 50$

Table 6.1: properties of pesticides analysed in ornamental plants.

Pesticide	class of pesticide	Systemic or contact action	CAS number
Acetamiprid	neonicotinoid insecticide	systemic	135410-20-7
Clothianidin	neonicotinoid insecticide	systemic	210880-92-5
Imidacloprid	neonicotinoid insecticide	systemic	138261-41-3
Thiacloprid	neonicotinoid insecticide	systemic	111988-49-9
Thiamethoxam	neonicotinoid insecticide	systemic	153719-23-4
Chlorpyrifos	organophosphate insecticide	contact	2921-88-2
λ-Cyhalothrin	pyrethroid insecticide	contact	91465-08-6
α-Cypermethrin	pyrethroid insecticide	contact	67375-30-8
Chlorothalonil	chloro-nitrile fungicide	contact	1897-45-6
Iprodione	dicarboximide fungicide	localized penetrant	36734-19-7
Epoxiconazole	DMI-fungicide (SBI, Class I)	acropetal penetrant	133855-98-8
Flusilazole	DMI-fungicide (SBI, Class I)	acropetal penetrant	85509-19-9
Metconazole	DMI-fungicide (SBI, Class I)	acropetal penetrant	125116-23-6
Prochloraz	DMI-fungicide (SBI, Class I)	localized penetrant	67747-09-5
Propiconazole	DMI-fungicide (SBI, Class I)	acropetal penetrant	60207-90-1
Tebuconazole	DMI-fungicide (SBI, Class I)	acropetal penetrant	107534-96-3
Carbendazim	MBC-fungicide	systemic	10605-21-7
Spiroxamine	morpholine fungicide (SBI, Class II)	systemic	118134-30-8
Fluoxastrobin	QoI-fungicide	acropetal penetrant	361377-29-9
Pyroclastrobin	QoI-fungicide	localized penetrant	175013-18-0
Trifloxystrobin	QoI-fungicide	localized penetrant	141517-21-7
Boscalid	SDHI fungicide	acropetal penetrant	188425-85-6
Carboxin	SDHI fungicide	systemic	5234-68-4
Siltiofam	thiophene carboxamide fungicide	systemic	175217-20-6

mg of pollen and nectar was used for the extraction.

In order to optimize the extraction method several recovery experiments were performed. Particular attention was posed to the extraction solvent, the d-SPE step and the solvent evaporation. Recovery and precision (relative standard deviation) were evaluated at two different concentration levels using spiked daffodil leaves. Method linearity was evaluated using the R^2 coefficient of the linear calibration curve both in matrix (leaves) and in solvent. Matrix-effect was defined as the difference between these two curves slope. The calibration range was 0.5-50 ng/g for LC and 10-1000 ng/g for GC. Method detection limit (MDL) and method quantification limit (MQL) were calculated as the concentrations where signal to noise ratio was 3 and 10 respectively.

The optimized extraction procedure consisted in extract on a multi axis rotator for 10 minutes 10-100 mg of ground leaves/pollen/nectar with 500 µL of acetonitrile with acetic acid 1% and 400 µL of water. Then, 250 mg of a salt mixture ($MgSO_4$ and sodium chloride; 4:1) was added to obtain a complete phase separation and the organic phase was removed into an Eppendorf containing 50 mg of a d-SPE phase (PSA/C18/ENVI-Carb). The extract was mixed on a multi axis rotator for 10 minutes and then the supernatant was removed and the d-SPE phase was extracted with 200 µL of a ACN/toluene (1/3) solution. After centrifugation, the supernatant was combined with the previous one and spin filtered. The extract was split for the LC (400 µL) and GC (200 µL) analysis and it was evaporated

to dryness using a vacuum concentrator for LC and a nitrogen stream for GC. Finally, the extract was reconstituted with 10 µL of toluene for GC and 50 µL of ACN/water (30:70) for LC. UHPLC-MS/MS analyses were carried out using a Waters Acquity UHPLC system equipped with a reverse phase Acquity UHPLC BEH C18 column (1.7 µm, 2.1 mm x 100 mm) and coupled to a Quattro Premier triple quadrupole mass spectrometer. GC-MS/MS analysis were carried out using a Trace GC Ultra (Thermo Scientific) equipped with an Agilent DB-5MS UI column (30 m $\times$ 0.25 mm, 0.25 μm film thickness) and linked to an ion trap mass spectrometer (ITQ1100, Thermo Scientific). Information about ions used for quantification are reported in Table 6.2.

Results

The developed analytical method for leaves extraction was used to quantify pesticides belonging to several different agro-chemical classes (Table 6.1). Some of them required particular attention in order to obtain high recovery factors. Chlorothalonil resulted to be sensitive to alkaline environment and only adding acetic acid in the extraction solvent good recoveries were obtained. Furthermore, several hydrophobic compounds were retained by the d-SPE used for matrix removal. Therefore, it must be washed with high amount of toluene to obtain high recoveries. Finally, chlorpyrifos was lost during the solvent evaporation in vacuum condition. Therefore, a nitrogen stream at atmospheric pressure was used to concentrate the portion of extract used for GC analysis. The method performance in terms of recoveries, bias, precision and linearity were satisfactory (Table 6.3) However, for three analytes (chlorothalonil, chlorpyrifos and iprodione) a significant matrix effect was observed and therefore a matrix-matched calibration curve was used for an accurate quantification of these compounds.
Pesticides residues were detected in 27 of the 29 analysed plants. Most of the plants contained more than one pesticide (Table 6.5). Neonicotinoids were the most common insecticides detected and boscalid, spiroxamine and DMI-fungicides were detected in more than 38% of plants. A wide concentration range was observed in leaves from different plant species (Table 6.6). This result shows that different plant species were treated with different amount of agrochemical products or in a different period. The same variability was observed also in pollen samples (Table 6.4) and in nectar samples only acetamiprid (in just one specie, below the method quantification limit (MQL= 0.14 ng/g)), imidacloprid (in four species at 1.4 ± 2.2 ng/g) and thiacloprid (in just one specie, below the MQL (0.15 ng/g)) were detected. Compared to contact and penetrant pesticides, systemic pesticides results to be more present in pollen and nectar samples, sometimes at relevant concentrations for eco-toxicological effects. In addition, even if these concentrations are not high enough to cause acute toxic effect, they could contribute to expose non-target animals to toxic chemicals and cause sub-lethal toxic effects (Desneux et al. 2007). Particularly interesting is the presence of some of these compounds (e.g. imidacloprid) in pollen collected from bumble-bees nests located in the Sussex area (David et al. 2016). Ornamental plants are usually rich of flowers and therefore bees and other pollinator insects are highly attracted by these plants.

Table 6.2: retention times and optimized parameters for pesticide quantification by either GC-MS/MS or UHPLC-MS/MS. Superscript letters denote which internal standard was used for analyte quantification.

Pesticide	instrument	tR (min)	Q1 precursor ion (m/z)	Q3 quantification ion (m/z)	Q3 confirmation ion (m/z)
Chlorothalonil(a)	GC	15.71	266	170	231
Chlorpyrifos(a)	GC	17.24	314	258	286
Iprodione(b)	GC	19.48	244	187	159
λ-Cyhalothrin(c)	GC	21.68	197	141	161
α-Cypermethrin(c)	GC	23.22	163	127	91
Pyrene (IS)(a)	GC	18.57	202	174	200
Chrysene (IS)(b)	GC	21.07	228	224	202
Trans-permethrin-d6(c)	GC	22.44	183	168	165
Carbendazim(d)	LC	3.96	192	160	131
Thiamethoxam(e)	LC	6.01	292	211	180
Clothianidin(f)	LC	7.23	250	169	132
Imidacloprid(g)	LC	7.81	256	209	175
Acetamiprid(g)	LC	8.77	223	126	56
Thiacloprid(h)	LC	10.98	253	126	186
Carboxin(h)	LC	13.26	236	143	86
Spiroxamine(h)	LC	14.31	298	144	100
Epoxiconazole(k)	LC	15.53	330	121	70
Boscalid(k)	LC	15.89	343	140	308
Tebuconazole(k)	LC	16.24	308	70	125
Flusilazole(j)	LC	16.45	316	165	246
Prochloraz(j)	LC	17.01	377	70	309
Metconazole(j)	LC	17.32	320	70	125
Fluoxastrobin(j)	LC	17.75	459	427	460
Propiconazole(j)	LC	17.88	342	159	69
Siltiofam(j)	LC	18.59	268	138	73
Pyroclastrobin(j)	LC	22.29	388	164	195
Trifloxystrobin(j)	LC	23.58	409	186	205
Carbendazim-d3(d)	LC	3.94	195	160	-
Thiamethoxam-d3(e)	LC	5.96	295	214	132
Clothianidin-d3(f)	LC	7.18	253	172	132
Imidacloprid-d4(g)	LC	7.76	260	213	179
Carbamazepine-d10(h)	LC	12.42	247	205	-
Tebuconazole-d6(k)	LC	16.16	314	72	-
Prochloraz-d7(j)	LC	16.91	384	316	70

In conclusion, pesticides concentration in ornamental plants varied widely due to different type (foliar or soil applied) and timing of treatment applied. However, these plants resulted to be contaminated with a complex mixture of insecticides and fungicides. In some cases their concentration in pollen are sufficient to have negative effects on pollinators insects.

Table 6.3: validation parameters for the optimized method 1. Method performance of UHPLC-MS/MS and GC-MS/MS analyses. The MDL and MQL values are given as ranges depending on the mass of sample available for analysis (5-90 mg for pollen and 10-50 µL for nectar).

Analyte	instrument	R^2	matrix effect	leaves		pollen		nectar		recovery (%)			
				MDL	MQL	MDL	MQL	MDL	MQL	LC spike: 1 ng/g GC spike: 100 ng/g		LC spike: 10 ng/g GC spike: 1 µg/g	
			(%)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	mean	RSD	mean	RSD
acetamiprid	LC	0.9877	17	0.04	0.14	0.05 – 0.84	0.16 – 2.8	0.08 – 0.42	0.28 – 1.4	114	17	110	19
α-cypermethrin	GC	0.9951	1	22	72	24 – 430	80 – 1435	43 – 215	143 – 717	104	10	91	20
boscalid	LC	0.9965	17	0.41	1.0	0.45 – 8.2	1.1 – 21	0.82 – 4.1	2.1 – 10	102	7	109	14
carbendazim	LC	0.9817	−18	1.2	5.9	1.4 – 24	6.5 – 118	2.4 – 12	12 – 59	93	13	78	9
carboxin	LC	0.9875	−6	0.05	0.17	0.06 – 1.0	0.19 – 3.3	0.10 – 0.50	0.33 – 1.7	124	1	122	18
chlorothalonil	GC	0.9958	62[a]	76	230	84 – 1521	256 – 4609	152 – 760	461 – 2304	116	7	98	3
chlorpyrifos	GC	0.9985	55[a]	19	50	21 – 377	56 – 1000	38 – 188	100 – 500	104	10	111	12
chlothianidin	LC	0.9980	−13	0.11	0.38	0.12 – 2.2	0.42 – 7.6	0.22 – 1.1	0.76 – 3.8	119	16	120	6
epoxiconazole	LC	0.9959	−11	1.1	4.6	1.2 – 22	5.1 – 91	2.2 – 11	9.1 – 46	140	11	99	15
fluoxastrobin	LC	0.9963	21	0.01	0.04	0.01 – 0.27	0.05 – 0.89	0.03 – 0.13	0.09 – 0.44	109	6	106	7
flusilazole	LC	0.9954	−19	0.19	0.62	0.21 – 3.7	0.69 – 12	0.37 – 1.9	1.2 – 6.2	71	6	89	13
imidacloprid	LC	0.9911	−6	0.36	1.2	0.40 – 7.2	1.3 – 24	0.72 – 3.6	2.4 – 12	122	8	112	18
iprodione	GC	0.9936	80[a]	3.7	44	4.1 – 75	49 – 882	7.5 – 37	88 – 441	99	16	109	8
λ-chyalothrin	GC	0.9850	−5	10	52	11 – 202	57 – 1033	20 – 101	103 – 517	119	14	106	15
metconazole	LC	0.9887	−2	0.38	1.3	0.42 – 7.6	1.4 – 25	0.76 – 3.8	2.5 – 13	73	23	77	21
prochloraz	LC	0.9992	−18	0.30	1.0	0.33 – 6.0	1.1 – 20	0.60 – 3.0	2.0 – 10	84	23	72	12
propiconazole	LC	0.9973	−19	0.39	1.3	0.43 – 7.7	1.4 – 26	0.77 – 3.9	2.6 – 13	72	10	101	17
pyroclastrobin	LC	0.9996	−20	0.60	2.0	0.67 – 12	2.2 – 40	1.2 – 6.0	4.0 – 20	119	7	96	9
silthiofam	LC	0.9968	−20	0.08	0.27	0.09 – 1.6	0.30 – 5.4	0.16 – 0.81	0.54 – 2.7	108	14	91	21
spiroxamine	LC	0.9979	21	0.03	0.11	0.04 – 0.65	0.12 – 2.2	0.06 – 0.32	0.22 – 1.1	73	20	60	2
tebuconazole	LC	0.9838	−10	0.17	0.58	0.19 – 3.5	0.64 – 12	0.35 – 1.7	1.2 – 5.8	110	12	99	4
thiacloprid	LC	0.9848	−10	0.05	0.15	0.05 – 0.91	0.17 – 3.0	0.09 – 0.46	0.30 – 1.5	117	1	116	9
thiamethoxam	LC	0.9937	−15	0.09	0.46	0.10 – 1.8	0.51 – 9.2	0.18 – 0.89	0.92 – 4.6	107	10	99	1
trifloxystrobin	LC	0.9976	6	0.24	0.30	0.27 – 4.8	0.33 – 5.9	0.48 – 2.4	0.59 – 3.0	91	20	87	10

[a]matrix-matched calibration was used for the quantification of these compounds.

Table 6.4: Comparison between the mean concentration of pesticides in leaves and pollen of different ornamental plant species or varieties.

Pesticides grouped by translocation properties in the plant	Leaves (ng/g) Mean ± SD[a]	Pollen (ng/g) Mean ± SD[a]
Systemic		
acetamiprid	8.6 ± 23	0.45 ± 0.23
imidacloprid	3.8 ± 9.1	6.9 ± 16
thiacloprid	1.2 ± 1.9	0.78 ± 1.1
thiamethoxam	17 ± 35	11.0 ± 16
clothianidin	9.3 ± 4.9	11.0 ± 9.3
carbendazim	54 ± 79	57 ± 98
spiroxamine	0.54 ± 0.82	<0.20[b]
Acropetal penetrant		
boscalid	30 ± 66	0.53 ± 1.1
fluoxastrobin	8.0 ± 17	<MDL[c]
propiconazole	0.65 ± 1.1	<MDL[c]
tebuconazole	0.16 ± 0.23	<MDL[c]
Localised penetrant		
iprodione	2743 ± 4459	252 ± 496
pyroclastrobin	38 ± 85	9.8 ± 14
trifloxystrobin	0.27 ± 0.04	<MDL[c]
prochloraz	55 ± 104	4.9 ± 12
Contact		
chlorothalonil	485 ± 416	<MDL[c]
chlorpyrifos	146 ± 142	81 ± 115
cyhalothrin	121 ± 33	<MDL[c]
cypermethrin	844 ± 251	<111[b]

[a]Mean concentrations of pesticides were calculated for samples from all plant species/varieties where there were matching leaf and pollen samples. The concentrations over the MDL but below the MQL were assigned the MDL value, whilst concentrations below the MDL were considered to be zero.

[b]Below the MQL in all the analysed samples.

[c]Below the MDL in all the analysed samples.

Table 6.5: number of pesticides detected in leaves of different ornamental plants.

Common name	species and variety	retailer	insecticides	fungicides
Achillea	*Achillea millefolium* 'Desert Eve Deep Rose'	B&Q	1	3
Ageratum	*Ageratum houstonianum*	Aldi	3	4
Allium	*Allium hollandicum*	Wyevale	2	1
Bellflower	*Campanula portenschlagiana*	Wyevale	0	2
Catmint	*Nepeta cataria* 'Six Hill Giant'	Wyevale	2	3
Catmint	*Nepeta cataria* 'Walkers low'	Wyevale	1	2
Coreopsis	*Coreopsis grandiflora* 'Early Sunrise'	B&Q	1	3
Cosmos	*Cosmos bipinnatus* 'Casanova Violet'	Homebase	4	1
Crocus	*Crocus vernus* 'Golden Yellow'	Wyevale	1	1
Daffodil	*Narcissus jonquilla* 'Tete-a-Tete'	Wyevale	0	0
Dahlia	*Dahlia x hybrida* 'Gallery Art Fair'	Staverton's	0	1
Dahlia	*Dahlia x hortensis* 'Bishop of Llandaff'	Wyevale	1	0
Dahlia	*Dahlia x hybrida* 'Mystic Dreamer'	B&Q	2	2
Dutch iris	*Iris tingitana* × *I. xiphium*	Wyevale	1	3
Foxgloves	*Digitalis purpurea* 'Dalmatian White'	Wyevale	1	1
Grape hyacinith	*Muscari armeniacum*	Wyevale	1	5
Heathers	*Erica carnea*	Wyevale	5	5
Lavender	*Lavandula stoechas* 'Victory'	Wyevale	0	3
Lavender	*Lavandula angustifolia*	Wyevale	0	1
Lavender	*Lavandula stoechas* 'Papillon'	Wyevale	0	3
Salvia	*Salvia longispicata x S. farinacea* 'Mystic Spires'	Staverton's	1	0
Salvia	*Salvia nemerosa* 'Sensation Deep Rose'	Homebase	0	0
Scabious	*Scabiosa columbaria* 'Pink Mist'	Wyevale	1	1
Scabious	*Scabiosa columbaria* 'Butterfly Blue'	Homebase	3	2
Strawberry	*Fragaria* × *ananassa* 'Toscana F1'	Homebase	2	2
Thistles	*Cirsium atropurumeum*	Wyevale	2	1
Verbena	*Verbena x hybrida*	Aldi	3	3
Veronica	*Veronica spicata*	Staverton's	2	4
Wallflower	*Erysimum linifolium* 'Bowles's Manve'	Wyevale	1	1

Table 6.6: concentration of pesticides detected in leaves of different ornamental plant species or varieties.

Pesticide[a]	Number of plant species/varieties where the pesticide was detected (% of total plants analysed)	Mean[b] ± SD (ng/g)	Median (ng/g)	Range (ng/g)
Thiacloprid	14 (48)	1.0 ± 1.8	0.28	0 - 6.4
Boscalid	14 (48)	37 ± 61	7.7	0 – 223
Spiroxamine	12 (41)	0.65 ± 0.85	0.34	0 - 3.5
Imidacloprid	11 (38)	3.9 ± 8.4	0.36	0 – 29
Prochloraz	9 (31)	59 ± 99	3.5	0 – 308
Pyroclastrobin	7 (24)	39 ± 66	3.1	0 – 257
Acetamiprid	6 (21)	7.5 ± 21	0.04	0.04 – 85
Iprodione	5 (17)	1966 ± 3549	327	3.7 – 10593
Thiamethoxam	4 (14)	16 ± 35	0.77	0.09 – 119
Carbendazim	3 (10)	54 ± 79	9.6	1.2 - 213
Chlorpyrifos	3 (10)	108 ± 127	19	19 - 328
Chlorothalonil	2 (7)	486 ± 416	364	0 - 1190
Fluoxastrobin	2 (7)	8.0 ± 17	0.19	0.09 - 41
Tebuconazole	2 (7)	0.16 ± 0.23	0.09	0 - 0.60
Clothianidin	1 (3)	9.3 ± 4.9	11	3.8 - 13
λ-Cyhalothrin	1 (3)	121 ± 33	105	99 - 158
Cypermethrin[c]	1 (3)	844 ± 251	805	616 - 1113
Propiconazole	1 (3)	0.65 ± 1.1	0	0 - 2.0
Trifloxystrobin	1 (3)	0.27 ± 0.04	0.24	0.24 - 0.32

[a]The concentrations of the fungicides carboxin, epoxyiconazole, flusilazole, metconazole and siltiofam were all below MDL.

[b]Mean, median and range value were calculated using the concentrations measured in all the plant species/varieties where a specific compound was detected. The concentrations over the MDL but below the MQL were assigned the MDL value, whilst concentrations below the MDL were considered to be zero.

[c]detected 3 isomers, quantified as sum of the three peaks on calibration curve obtained from α-cypermethrin.

Annex IV. List of publication

- A. Lentola, A. David, A. Abdul-Sada, A. Tapparo, D. Goulson and E. M. Hill. Ornamental plants on sale to the public are a significant source of pesticide residues with implications for the health of pollinating insects. Environmental Pollution (IF: 5.552), May 2017. https://doi.org/10.1016/j.envpol.2017.03.084.

- Chiara Giorio, Anton Safer, Francisco Sánchez-Bayo, Andrea Tapparo, Andrea Lentola, Vincenzo Girolami, Maarten Bijleveld van Lexmond, e Jean-Marc Bonmatin. «An Update of the Worldwide Integrated Assessment (WIA) on Systemic Insecticides. Part 1: New Molecules, Metabolism, Fate, and Transport». Environmental Science and Pollution Research (IF: 2.741), November 2017. https://doi.org/10.1007/s11356-017-0394-3.

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