Exposure and Fate of Neonicotinoid Insecticides in Cocoa plantations in Ghana

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Declaration

This thesis summarizes the results of research work undertaken by Enock Dankyi at the Department of Chemistry, University of Ghana, Legon and Aarhus University, Flakkeberg, Denmark, under the supervision of Prof. Derick Carboo (University of Ghana), Prof. Chris Gordon (University of Ghana) and Prof. Inge S. Fomsgaard (Aarhus University).

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Abstract

Neonicotinoids belong to the most important class of insecticides currently used in crop production and account for about a fourth of the insecticide market. They are considered highly effective against insect pests, safe to mammals, and possess multiple means of application for convenience and widespread usage in diverse crop protection. Neonicotinoids are systemic insecticides - they are absorbed into plants, travel through the vascular tissue, and help protect the plant from piercing and sucking insects. Their mode of action is through agonist activity at the postsynaptic nicotinic acetylcholine receptor (nAChRs) sites in the nervous system.

In recent years, reports have emerged on possible harmful effects of neonicotinoid insecticides on bee health. Across Europe, America and Australia, there has been calls for ban and re-evaluation of the use of neonicotinoid insecticides. In Europe, a ban has been in place for almost two years following a scientific report by the European Food Safety Authority (EFSA) which identified "high acute risks" for bees. However, the availability of conflicting reports on the effects of neonicotinoids on bees has prompted intense research on these class of insecticides. Quite clearly, understanding the effects of neonicotinoids on organisms in the ecosystem will rely on accurate knowledge of their exposure in the environment.

In Ghana, neonicotinoids are one of the most widely used class of insecticides particularly in cocoa production, where they are important for the control of mirids. Insect pests are a major concern in crop production in tropical conditions, due to the prevailing conducive environment for their growth. In cocoa production, insect pests such as mirids contribute to significant loses in yield. To address this concern, the government of Ghana introduced a free mass application policy on insecticides in cocoa farming, which has contributed to substantial improvements in yields of cocoa beans. Under the program, neonicotinoids are the major class of insecticides used.

In spite of the remarkable contribution of neonicotinoids to cocoa production, concerns about the environment and food safety have arisen due to widespread and intensive use of these chemicals in cocoa farms. Addressing these concerns requires accurate knowledge of the behavior and fate of these class of insecticides in the Ghanaian environment. The primary goal of this work was to assess the extent of exposure, behavior and fate of neonicotinoids in the Ghanaian environment, particularly in cocoa plantations where they are extensively used.

To achieve this, concentrations of neonicotinoid residues in soils across all the cocoa producing regions of the country were studied to examine their environmental exposure. Their fate in soils was studied by investigating their dissipation and sorption behavior using established kinetic models and isotherms. Finally, the exposure to cocoa beans (food) was studied to ascertain concerns for food safety. Analytical methods and instrumentation was an important aspect of this work to ensure accurate and reliable data. To this end, the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure was optimised and used for the diverse matrixes understudy. Quantification of analytes involved the use of liquid chromatography- tandem mass spectrometry (LC-MS/MS) for high sensitivity and low detection limits in the complex soil and cocoa matrixes used.

The findings from the study suggest that, neonicotinoids are persistent in the Ghanaian soils studied and may be found in soils several months to years after application. Sorption studies revealed that, sorption coefficients of neonicotinoids are generally low, with a high potential for leaching into surface and underground water systems. Due perhaps to their systemic nature and high application rates, neonicotinoids may accumulate to high levels in cocoa beans, particularly in cocoa shells.

The findings from this study has implications for pesticide application and current policy on pesticide usage, and reveal that, efficient application regimes are needed in cocoa production to ensure food and environmental safety.

Table of content

List of Figures

List of Tables

List of Abbreviations

QuEChERS	quick, easy, cheap, effective, rugged and safe
nAChR	nicotinic acetylcholine receptor
Log P	logarithm of partitioning co-efficient (in an octanol-water system)
CCD	colony collapse disorder
d-SPE	dispersive solid-phase extraction
PSA	primary secondary amines
GCB	graphitized carbon black
LC-MS/MS	liquid chromatography tandem mass spectroscopy
ESI	electrospray ionisation
MRM	multiple reaction monitoring
XIC	extracted ion chromatogram
DP	declustering potential
EP	exit potential
CE	collision energy
CEP	collision cell entrance potential
SFO	simple first order
FOMC	first order multi component
DFOP	double first order in parallel
TXM	thiamethoxam
IMI	imidacloprid
CLO	clothianidin
ACE	acetamiprid
TIA	thiacloprid

Structure of thesis

The results from the thesis have been presented in chapters based on various experiments conducted. These chapters are intended as manuscripts for submission to international peer-reviewed journals.

Chapter 1 gives a general background to the thesis and presents pertinent literature on the topics understudy. While literature may be replete on neonicotinoids and other topics discussed in this thesis, these topics cannot be exhaustively discussed. As such, the literature most relevant to the studies conducted have been presented here. Further background and literature are presented in chapters 2 to 5 based on the study being conducted.

Chapter 2 examines the occurrence of neonicotinoid insecticide residues in diverse soil types found in cocoa farms across the entire cocoa-growing belt of Ghana. Optimization of the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure for analysis of neonicotinoids in the soils being studied is discussed.

Chapter 3 presents result of analysis of applied neonicotinoid insecticide residues in cocoa beans obtained from cocoa farms across Ghana. The methodology employed in the analysis of residues in the complex matrix of cocoa beans with high fat and high pigments is described. The distribution of insecticides in cocoa shells and nibs (de-shelled beans) is also examined.

Chapter 4 describes a study of dissipation and persistence of the two most widely used neonicotinoids (imidacloprid and thiamethoxam) in representative soils from cocoa farms in Ghana. Confirmation of dissipation rates were performed using deuterated compounds of each of the insecticides studied concurrently. Estimated half-lives were obtained by use of established pesticide degradation kinetic models.

Chapter 5 presents an assessment of the sorption and desorption behavior of neonicotinoids in representative soil types. The influence of soil properties, potential mobility, and leaching of neonicotinoids in the environment is discussed.

Chapter 6 summarizes the main findings from the thesis and assesses its implications for cocoa production, pesticide usage and policy in Ghana. Perspectives and further research needs are presented.

1. General Introduction

1.1 Neonicotinoids – A new class of insecticides

1.1.1 Discovery

The insecticide industry essentially began in the 1940s with the discovery of DDT and other chlorinated hydrocarbons with remarkable insecticidal properties for the control of insect pests. They contributed significantly to increases in crop production and improved health care. However these gains were accompanied by unintended environmental and toxicological effects leading to restrictions in usage and subsequent bans in many countries worldwide (Adami et al., 1995; Frigo et al., 2002; Longnecker et al., 1997). The need for the creation of new chemicals with high selectivity and less environmental persistence led to the discovery of organophosphates, synthetic pyrethroids, and later methyl carbamates (Casida and Quistad, 1998). These new classes of chemicals greatly enhanced the diversity and arsenal of insecticides available for the control of insect pests. However, over time, challenges of resistance due to intensive usage and low sensitivity of molecular targets created the need for insecticides with different and more sensitive molecular targets (Casida and Quistad, 1998; Tomizawa and Casida, 2009).

In this regard, the discovery of neonicotinoid insecticides with a new mode of action, very low toxicity to mammals, high toxicity to insect pests and remarkable systemic characteristics has been considered a milestone in insecticide research (Jeschke and Nauen, 2008; Jeschke et al., 2013). The successful introduction of imidacloprid, the first neonicotinoid by Bayer Crop Science triggered a new era in the insecticide industry and intense research into modern agrochemicals (Jeschke et al., 2011; Kagabu, 2011). Imidacloprid was followed by thiamethoxam (Maienfisch et al., 2001) and then clothianidin (Meredith et al., 2002). Subsequently, four other neonicotinoids have been introduced to the market worldwide with

newer generations of neonicotinoids in development (Cutler et al., 2013; Jeschke et al., 2013; Tomizawa and Casida, 2011a; Wakita et al., 2003). Since their introduction in the 1990s, neonicotinoids have been considered the fastest-growing class of insecticides in modern crop protection and are currently the most widely used class (Jeschke et al., 2011). As at 2011, neonicotinoids were estimated to have a share of 28.5% of the insecticide market worth $12.75 billion and were registered in more than 120 countries worldwide (Jeschke et al., 2013). Imidacloprid is currently the best-selling insecticide worldwide and estimated to account for over 41.5% of the neonicotinoid market.

Table 1. Common, product and systematic names of commercially available neonicotinoids

Common name	Product name (s)	Systematic name
Acetamiprid	Prize Assail	N-[(6-chloropyridin-3-yl)methyl]-N'-cyano-N-methylethanimidamide
Clothianidin	Poncho Prosper	1-[(2-chloro-1,3-thiazol-5-yl)methyl]-2-methyl-3-nitroguanidine
Imidacloprid	Confidor Admire	N-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide
Thiacloprid	Calypso Biscaya	[3-[(6-chloropyridin-3-yl)methyl]-1,3-thiazolidin-2-ylidene]cyanamide
Thiamethoxam	Actara Cruiser	N-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene]nitramide
Dinotefuran	Scorpion Vectra	2-methyl-1-nitro-3-(oxolan-3-ylmethyl)guanidine
Nitenpyram	Capstar Bestguard	(E)-1-N'-[(6-chloropyridin-3-yl)methyl]-1-N'-ethyl-1-N-methyl-2-nitroethene-1,1-diamine

Neonicotinoids act as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) in the nervous system in both insects and mammals (Bai et al., 1991; Zhang et al., 2000). However, there is distinctive selectivity of neonicotinoids for nAChRs in insects as compared

to mammals due to their higher affinity for receptors in insects. This results in outstanding toxicity and selectivity to insect pests while being essentially safe to mammals, hence enabling their use not only in crop production but also in the control of pest on household pets (Kagabu, 2008; Tomizawa and Casida, 2011b, 2009, 2005; Tomizawa et al., 2000). This makes neonicotinoids safer to use compared to other classes of insecticides. Their unique mode of action also ensures there is no cross-resistance to other insecticide classes including chlorinated hydrocarbons (Na^+,Cl^- channel modulators); organophosphates and carbamates (acetylcholine esterase inhibitors); and pyrethroids (Na^+ channel modulators) (Table 2). These remarkable features has ensured the preferential use of neonicotinoids over other class of insecticides, gradually replacing them in the control of major insect pests in agriculture (Jeschke et al., 2011).

Table 2. Comparison of neonicotinoids with other classes of insecticides (Tomizawa and Casida, 2005)

Class	Log P	Systemic action	Nerve target	Potency (LD_{50}, mg/kg)		Selectivity factor*
				Insects	Rats	
Neonicotinoids	-0.7 to 1.3	+	nAChR	2.0	912	456
Organophosphates	1 to 5.5	±	AChE	2.0	67	33
Methyl carbamates	-1 to 3	±	AChE	2.8	045	16
Organochlorines	5.5 to 7.5	-	Na^+ or Cl^- channels	2.6	230	91
Pyrethroids	4 to 9	-	Na^+ channel	0.45	2000	4500

* LD_{50} in rats/LD_{50} in insects

1.1.2 Structure and activity of neonicotinoids

Neonicotinoids may be classified into two general groups based on their structure: (a) containing ring systems, and (b) having noncyclic structures (Nauen et al, 2001) (Figure 1).

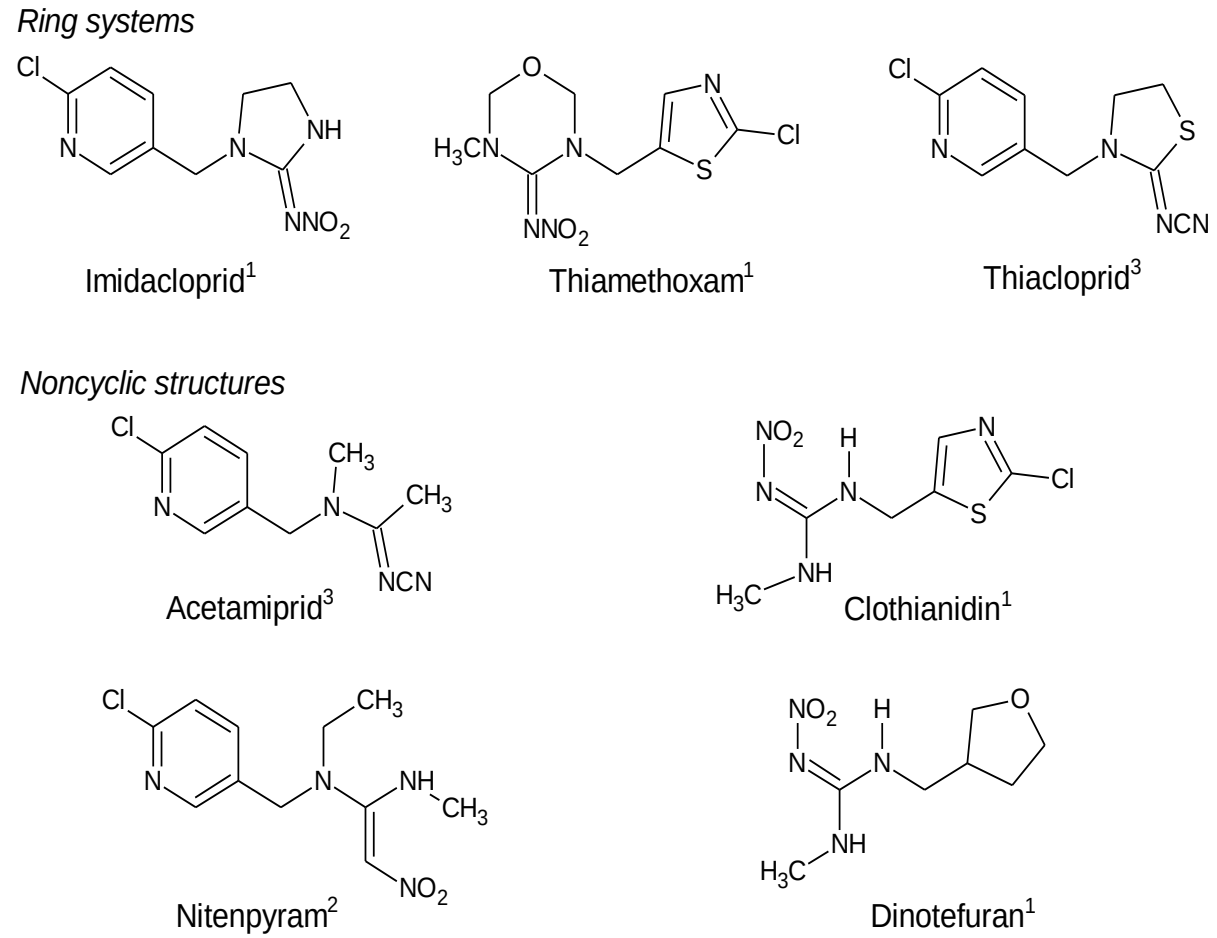

Figure 1. Structures of commercially available neonicotinoids. [1]Nitroguanidines, [2]Nitroenamines (Nitromethylenes), [3]Cyanoamidines

Neonicotinoids poses a common pharmacophore that can be represented as:

$$[- N - C (E) = X - Y]$$

Where X – Y is an electron withdrawing group (CH – NO_2, N – NO_2, N – CN) and

E is an NH, NR', CH_2, O, or S moiety.

Based on currently available neonicotinoids, the pharmacophore may be grouped as:

(a) nitroenamines, [– N – C (E) = CH – NO_2; E = S, N]

(b) nitroguanidines [– N – C (N) = N – NO_2; E = N]

(c) cyanoamidines, [– N – C (E) = N – CN; E = S, Me]

The high affinity and selectivity of neonicotinoids towards insect nAChRs is attributable in part to differences in interactions and binding conformations at the receptor sites in insects and mammals particularly for nitroguanidine and cyanoamidine pharmacophores (Tomizawa and Casida, 2011b, 2009). It has been suggested that, a single dominant binding orientation is responsible for the high binding potency in insects, while in mammals, binding is associated with poor multiple binding conformations (Tomizawa and Casida, 2009, 2003). Studies show that, the binding affinity is strongly correlated with agonistic and insecticidal properties of molecules. However, binding interactions may differ based on type of neonicotinoid, species or organism and nAChR binding sub-sites (Zhang et al., 2000). Agonist behavior induces the continuous opening of nAChR channels leading to sustained excitation of neuronal membranes resulting in paralysis, cell energy exhaustion and death.

Probably more important to the action and success of neonicotinoids is the understanding of the nAChRs binding sites, subunits and recognition that confers selectivity. Since the discovery of the efficacy of imidacloprid, nAChRs have been extensively researched as perhaps the most attractive biochemical target site for exploration and discovery of modern agrochemicals (Jeschke et al., 2013). Currently other classes of insecticides including spinosyns which are natural compounds obtained from microorganisms, have been developed based on their action at nAChRs target sites (Kirst, 2010). Similarly, several new insecticides are in various stages of development based on nAChRs as a molecular target (Jeschke et al., 2013).

1.1.3 Physicochemical properties

The physicochemical properties of neonicotinoids conferred by their structures and functional groups also play a significant role in their field application and activity. For instance, the high excitation energy gap from ground state to a single excited state for the different functional groups ensures good photostability in field application (Kagabu and Akagi, 1997). Photostability increases in the order: [=CH-NO_2] < [=N-NO_2] < [=N-CN]. Thus the cyanoamidines including acetamiprid and thiacloprid show the best photostability in field applications.

Compared to other classes of insecticides, neonicotinoids exhibit good water solubility due to the presence of polar functional groups. Polarity and water solubility appears to increase in the order: [=N-NO_2] < [=N-CN] < [=CH-NO_2] as shown in Table 3. As a result of the high polarity, neonicotinoids have low lipophilicity. Lipophilicity is estimated by their log P_{ow} values, a logarithm of their partition coefficient in 1-octanol/water systems (Table 3) and is generally low compared to other classes of insecticides. The 'E' moiety present in the chromophore [–N–C (E) = X – Y] appears to be important in lipophilicity and increases in the order: NH < O < C < S].

Table 3. Physical properties of neonicotinoids (Tomizawa and Casida, 2005)

Neonicotinoid	Molecular weight	Water solubility (g/l)	Log P_{OW}*
Acetamiprid	222.7	4.25	0.8
Clothianidin	249.7	0.30-0.34	0.7
Dinotefuran	202.2	54.3	-0.64
Imidacloprid	255.7	0.61	0.57
Nitenpyram	270.7	>590	-0.66
Thiacloprid	252.7	0.185	1.26
Thiamethoxam	291.7	4.1	-0.13

*P_{OW} = 1- octanol/water partition

These properties of high water solubility and low lipophilicity of neonicotinoids have been found to be essential for membrane permeability, transport and translocation in living systems. Consequently, neonicotinoids possess excellent systemic properties, facilitating their effective usage in the control of piercing and sucking insects such as aphids, white flies and plant hoppers. They have been applied extensively in the control of these insects in a wide range of crops including rice, corn, cotton, soybean, oilseed rape, fruits and vegetables (Akamatsu, 2011). Once applied, neonicotinoids are taken up, move within the xylem and are translocated to growing parts of the plants where they provide long-term protection against insects. Besides their outstanding systemic activity, their physical and chemical properties also ensure broad-spectrum activity including contact and stomach activity on target pest.

It is worth noting that, while properties such as low P_{OW} values and good solubility in water may reduce their potential of accumulation in biological systems, their mobility in plants and the environment is enhanced, thereby presenting a greater environmental threat than less mobile insecticides (Banerjee et al., 2008; Fossen, 2006).

Perhaps, more important to the success and widespread use of neonicotinoids is the high versatility of application methods and techniques available, including foliar spraying, seed treatment, soil drench, stem application and irrigation water in a wide range of crops (Table 4). According to Jeschke et al, (2011) approximately 60% of all neonicotinoid insecticide applications are based on soil and seed treatment (Jeschke et al., 2011). Spray applications are targeted against pests in cereals, corn, rice, vegetables, sugar beet, potatoes, cocoa and others (Elbert and Nauen, 2004; Elbert et al., 2008).

Table 4. Versatility of application of neonicotinoid insecticides in crop production (Elbert et al., 2008)

Neonicotinoids	# of crop uses	Target pests	Foliar uses	Soil uses	Seed treatment
Imidacloprid	140	Thrips, mealybugs, leafminers, termites	++(+)	+++	++(+)
Nitenpyram	12	-	++	+	-
Acetamiprid	60	Codling moth, diamondback moth	+++	+	-
Thiamethoxam	115	Mealybugs, plant bugs, leafminers, termites	+++	+++	++
Thiacloprid	50	Codling moth, pollen beetle	+++	-	-
Clothianidin	40	Wooly aphid, oriental fruit moth, corn rootworm	++(+)	++	+++
Dinotefuran	35	Soft scales, thrips, mealybugs	+++	++	-

+++ broad use ++ good use + limited use - not relevant

1.2 Neonicotinoid insecticides in the environment

The benefits of the insecticide industry in general and neonicotinoids in particular to crop production cannot be overemphasized. This is evident in the extensive use of this class of insecticides as perhaps the most important agrochemical in crop production in just over two decades. However, negative environmental concerns have arisen despite their good attributes. This is because substantial amounts of applied neonicotinoid insecticides end up in the environmental media including soil, vegetation, water and air, which may have negative effects on the ecosystem. In general research has shown that, very little of applied pesticides actually reach the target pest with substantial portions ending up in the environment (Pimentel, 2005).

In the case of neonicotinoids, their mobility may have a major influence in their environmental behaviour (US EPA, 2003, 2002). Residues of neonicotinoid insecticides have been reported in soil (Dankyi et al., 2014; Jones et al., 2014), water (Schaafsma et al., 2015; Starner and Goh, 2012), bees and bee products (Laycock et al., 2012; Tanner and Czerwenka, 2011a; Yáñez et al., 2013), animals (Frew and Grue, 2012; Xiao et al., 2013, 2011) as well as in food crops

including fruits and vegetables (Chen et al., 2014; Obana et al., 2002; Wang et al., 2012a). A recent study by Chen et al (2014) has demonstrated the widespread exposure of neonicotinoid insecticide residues in several fruits and vegetables with possible implications for human exposure (Chen et al., 2014). Notwithstanding the relatively low toxicity of neonicotinoids to humans, continuous assessments of residue levels in food and their possible health implications need to be pursued to ensure food safety and health.

Irrespective of the method of application, neonicotinoid insecticides once applied, may enter soil, vegetation and water. Soils represent the most important matrix for neonicotinoids in field application. Sur & Stork (2003), have observed that, substantial amounts (80-98.4%) of seed applied neonicotinoid insecticides may end up in soil (Sur and Stork, 2003). In general, the soil compartment plays a key role in determining the fate and behaviour of applied insecticides. Knowledge of the presence, concentrations and behaviour of insecticides in soils is therefore paramount. For neonicotinoids, their high water solubility significantly influences their behaviour in soils with a high potential for leaching and contamination of surface and groundwater being reported (Gupta et al., 2008; Selim et al., 2010). Both the United States Environmental Protection Agency (US EPA) and Canada's Pesticide Management Regulatory Agency (PMRA) have classified one or more neonicotinoid as having "high" leaching potential or "mobile to highly mobile" (Anderson et al., 2015).

Based perhaps on the properties of neonicotinoids, soils and environmental conditions, a wide variability in dissipation rates have been reported in literature with no clear understanding of the factors accounting for the wide variation in published values (Goulson, 2013). In a review by Goulsen (2013), imidacloprid, thiamethoxam and clothianidin for instance have their reported dissipation half-lives in soils ranging from 28 – 1250, 7 – 3001 and 148 – 6931 days respectively (Goulson, 2013). As a result, neonicotinoids have been labelled as persistent with

high potential for accumulation in soils particularly after repeated applications (Dankyi et al., 2014; PMRA, 2001).

In general, dissipation of neonicotinoids in the environment is influenced by transformation and mobility. Neonicotinoids may undergo chemical or microbial decomposition into a great number of complex products based on interactions influenced by properties of chemical and soil, microorganisms and environmental conditions (Casida, 2011; Dai et al., 2010; G. Wang et al., 2013). Sorption has been identified as one of the major factors controlling the mobility and behaviour of neonicotinoids in soils and other media. In most studies neonicotinoids have been reported to exhibit low sorption coefficients and medium to high potential for leaching (Banerjee et al., 2008; Carbo et al., 2007; Kurwadkar et al., 2013a). Understanding the fate and behaviour of neonicotinoid insecticides in soils is not only central to the useful assessment of their concentrations but also significant in their management to ensure their safe use.

1.2.1 Effect of neonicotinoids on non-target organisms

An important aspect of concern for neonicotinoid insecticides in the environment and urgent calls for their re-evaluation is their potential effects on non-target organisms (Anderson et al., 2015; Pisa et al., 2014). An increasing number of reports have emerged on the possible toxicity of neonicotinoids to various non-target and beneficial organisms in the ecosystem including butterflies, natural predators and aquatic organisms (He et al., 2012; Lanteigne et al., 2014; Li et al., 2014; Roessink et al., 2013; Uğurlu et al., 2015; Uhl et al., 2015; Wang et al., 2015; Yao et al., 2015; Zhang et al., 2014).

In recent years, concerns have been largely based on reported harmful effects of neonicotinoids on bee health. The seriousness of this concern does not only stem from an environmental perspective but also an economic one. Bees are considered the most important group of pollinators in world food crop production and play a key role in the maintenance of biodiversity

(van der Sluijs et al., 2013). It has been estimated that, about 35% of world food crops comprising 87 leading food crops rely on animal pollinators particularly bees (Klein et al., 2007). Long-term decline in bee populations have been linked with adverse effects of neonicotinoid insecticides with reported cases of acute and chronic lethal toxicity as well as sub-lethal effects on reproduction and behavior of bees (Blacquière et al., 2012b; Goulson, 2013; Kasiotis et al., 2014; Soares et al., 2015; Tapparo et al., 2012; van der Sluijs et al., 2013). Calls for suspension in usage and re-evaluation of neonicotinoids have been rife in many developed economies worldwide. This has led the European Commission to adopt a proposal (Regulation (EU) No. 485/2013) to restrict the use of 3 neonicotinoid insecticides; clothianidin, imidacloprid and thiamethoxam for a period of two years following a European Food Safety Authority's (EFSA) scientific report which identified "high acute risks" for bees (European Food Safety Authority, 2012). Prior to this, restrictions were already in place in some European countries including Germany, France, and Switzerland. In the United States, a "save America's pollinators act" has been sent to congress seeking a ban on neonicotinoids until thorough independent scientific studies are conducted. Conclusions from research on the possible association between neonicotinoids and bee health are however far from certain. While the phenomenon of bee colony collapse disorder (CCD) is generally accepted, arguments of subjection of bees to high field-unrealistic levels of neonicotinoids during experiments, as well as other causal factors for CCD have been advanced (Dively et al., 2015; Hawthorne and Dively, 2011; Staveley et al., 2014; USDA, 2012). Quite clearly, the outcome of further investigations into possible effects of neonicotinoids on bee health and on other non-target organisms will rely on a deeper knowledge of their concentrations in the environment and an understanding of their environmental behavior. This is an area of intense current research and debate (Anderson et al., 2015; Hopwood et al., 2012; Morrissey et al., 2015; Simon-Delso et al., 2015).

1.2.2 Neonicotinoid insecticide usage in Ghana's cocoa production

In spite of concerns with chemical usage in general, the pesticide industry has become so integrated with crop production such that, many farmers believe that productivity cannot be sustained without their use. In Ghana, pesticide usage is considered to be generally low albeit intensive in areas where usage occurs (Gerken et al., 2001). Application is concentrated in cocoa, vegetable and fruit production with reported cases of overuse and misuse instigated by ignorance or a lack of safety concerns, as well as a general lack of effective regulations on chemical usage (Fianko et al., 2011; Ntow et al., 2006; Ondieki, 1996).

In general, insecticides play a critical role in food production and neonicotinoid insecticides comprise a significant and increasing proportion of insecticides applied. Based on data from Ghana's EPA, insecticides constitute more than 50% of active ingredients of all pesticides registered in the country; with neonicotinoids constituting the highest percentage of all insecticide formulations (EPA Ghana, 2012). Currently imidacloprid, thiamethoxam, acetamiprid and thiacloprid are approved for use in cocoa, cotton, cereals, vegetables and fruits. They are applied mainly as foliar sprays with limited seed application. In cocoa production, neonicotinoids and to a lesser extent pyrethroids have effectively replaced the relatively more harmful organochlorines and organophosphates that have been used for several years (EPA Ghana, 2007).

Cultivation of cocoa in Ghana predominantly occurs in the forested agrochemical zones of the country, located in the south-western portion, and comprises six out of the ten political regions of the country. Cocoa production is said to account for over 36% of cropped land and about 75% of agricultural exports (EPA Ghana, 2007; MOFA, 2013). As the second largest production of cocoa beans worldwide, cocoa production is integral to the economy of the country and contributes significantly to gross domestic product (GDP) and jobs for farmers.

Pest and diseases have presented the most significant challenge to the cocoa industry and have led to substantial yield loses (Aneani and Ofori-Frimpong, 2013).

Table 5. Product name, active ingredient and uses of fully registered neonicotinoid insecticides in Ghana*

Product name	Concentration of active ingredient	Crops/uses
Actara 240 WG	Thiamethoxam (250 g/kg)	Insecticide for the control of stem borer weevils in banana
Actara 240 SC	Thiamethoxam (240 g/L)	Insecticide for the control of capsids and insect pests in cocoa
Chemaprid 880 EC	Acetamiprid (6 g/L) + cypermethrin (72 g/L)	Insecticide for the control of insect pests in cotton
Confidor 200 SL	Imidacloprid (200 g/L)	Insecticide for the control of capsid bugs and insect pests in cocoa
Confidor 200 OD	Imidacloprid (200 g/L)	Insecticide for the control of capsid bugs and insect pests in cocoa
Dimiprid 200 SL	Imidacloprid (200 g/L)	Insecticide for the control of insect pests on vegetables
Proteus 170 OTEQ	Thiacloprid (150 g/L) + Deltamethrin (20 g/L)	Insecticide for the control of mirid and other pest in cocoa
Titan	Acetamiprid (25 g/L)	Insecticide for the control of aphids, thrips, leaf miners and white flies in tomatoes
K-Optimal EC	Lambda-cyhalothrin (16 g/L) + Acetamiprid (20 g/L)	Insecticide for control of insect pest on vegetables
Seedstar 440 DS	Anthraquinone + Imidacloprid + Metalaxyl	Insecticide for seed treatment
Rainimidac	Imidacloprid (350 g/L)	Insecticide for the control of insect pests on vegetables
Cardinal WS	Imidacloprid (5 g/kg) + Terbuconazole (4 g/kg)	Insecticide/fungicide for the control of insect pest on vegetables and cereals

*Data obtained from EPA, Ghana

As occurs in many tropical regions, climatic conditions in Ghana, and particularly in cocoa producing areas are characterised by high temperatures and heavy precipitation for prolonged periods of the year, with little or no seasonal variation (Lacher and Goldstein, 1997). While these conditions may be essential for cocoa production, they also create a conducive environment for insect pests including mirids, thereby posing significant challenges to

agriculture. In Ghana, *Sahlbergella singularis* Hagl and *Distantiella theobroma* (Dist.) have been identified as the two most important mirid species contributing to significant loses in yields of cocoa beans (Owusu-Manu, 2002). Various measures have been considered for their control including the use of pheromone traps which present a good environmentally safe option for pest control, albeit found to be ineffective (Sarfo, 2013). To date, insecticide application remains the single most important option (Adu-Acheampong et al., 2015).

To address the decline in yields, the government of Ghana set up the cocoa disease and pest control program (CODAPEC) in 2001. Under the program, insecticides are applied on cocoa farms or given to farmers for self-application at no financial cost. The program does not only ensure the control of pests in cocoa farms, but also ensures the use of approved insecticides on farms. The recommended rate of insecticide application is usually four times in a year in the months of August, September, October and December, based on high mirid populations during these months. These recommendations however, are said to be based on research conducted in the 1950s and have been found to be outdated. In a recent study on mirid population dynamics, Adu-Acheampong et al, (2014) have observed that, the current spraying regime does not adequately reflect the peak populations of mirids in cocoa farms (Adu-Acheampong et al., 2014).

During foliar application on cocoa farms, the insecticides are applied to the leaves and branches of trees by use of back-pack manual sprayers or motorised mist blowers. The tendency for farmers to misapply the insecticide is not only enhanced by the minimal financial burden but also the tacit approval by the Cocoa Research Institute (Tafo) in Ghana.

Under the CODAPEC program, neonicotinoids are the most predominantly used class of insecticides. In tree crops such as cocoa, where crop rotation is either restricted or impossible, the use of alternative insecticides with differing molecular target sites is essential to ensure the

avoidance or reduction in cross resistance. Neonicotinoids play a central role in this pest management approach, and is often used with the pyrethroid bifenthrin in alternating years. Among majority of cocoa farmers in Ghana, imidacloprid is the preferred choice of insecticide based on its perceived efficacy. It has been suggested that, low mirid populations during most parts of the year coupled with their less conspicuous occurrence, makes the use of systemic insects such as neonicotinoids even more important for the effective control of piercing and sucking mirids (Adu-Acheampong et al., 2015).

The "free" insecticide application program among other interventions have contributed to significant improvements in cocoa yields in Ghana, further entrenching her position as the second largest producer of cocoa beans worldwide. From a production of less than 400,000 metric tons per annum at the onset of the program in 2001, production steadily increased to a high of 1 million metric tons during the 2010/11 crop season (Figure 2). The current production of cocoa beans per annum is in excess of 800,000 metric tons. The high yields have contributed to huge economic gains for the country and improved livelihood among farmers.

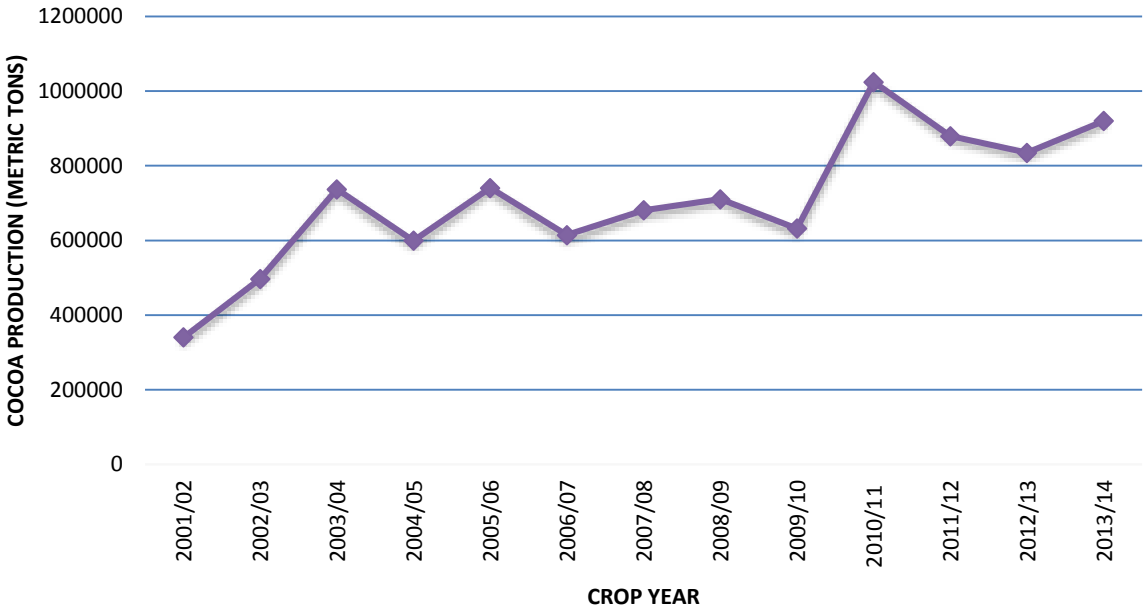

Figure 2. Ghana's cocoa production statistics since the inception of the free mass spraying exercise. Data was obtained from the Ghana cocoa board.

Despite the huge successes from this free mass application of insecticides program, the increased possibility of leaving substantial volumes of chemicals in the environment due to the widespread and intensive application rates presents a challenge for the environment, non-target organisms and food safety. Although neonicotinoid insecticides have been used for several years in the country, knowledge of their levels and behaviour in the Ghanaian and most African environment is unknown. Quite clearly, the use of insecticides may continue to be a part of cocoa and other crop production in Ghana and in other tropical climates. The challenge presented based on possible effects on the environment and non-target organisms will require uninterrupted efforts at assessing levels, behaviour and fate of these insecticides to aid in proper management and safety.

1.3 Analytical chemistry

1.3.1 Sample pre-treatment procedure

Accurate assessment of insecticide residue levels in food and the environment requires high quality methodologies and instrumentation. The complexity of these matrixes coupled with the need to quantify very low levels of residue in order to arrive at useful deductions further make methodologies critical. Added to this complexity is matrix variability and differences in chemical and physical properties of analytes which often warrant the use of different and/or more sophisticated extraction methodologies.

Prior to the separation and detection of analytes in matrixes, sample pre-treatment procedures are indispensable, and have a significant impact on the dependability of data produced. This usually involves the extraction of target analyte(s) from the sample matrix and the separation of target analyte(s) from co-extracts. In pesticide residue analysis including that of neonicotinoids, conventional liquid-liquid (shaking) extraction have been used for many years. Despite its simplicity and good efficiency, it remains laborious and solvent consuming (Zhang et al., 2012). The drawbacks have been largely overcome by newer and more sophisticated

extraction techniques such as ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), accelerated solvent extraction (ASE) and supercritical fluid extraction (SFE). Advantages over conventional methods may include high sample throughput, low solvent consumption, high recoveries, cleaner separation and the possibility of automation. While these new sample treatment procedures offer improved efficiency in analyte recovery and lower solvent usage, they invariably come at a cost due to the requirement of sophisticated instrumentation.

Solid-phase extraction (SPE) has always offered an alternative to liquid-liquid extraction in pesticide residue analysis (Boyacı et al., 2014; Wen et al., 2014). SPE usually involves the selective adsorption of analytes on solid phase materials (adsorbents) followed by elution with selected solvent(s). The procedure is rapid, simple and offers low consumption of solvents and have been especially applied in the clean-up of analytes from different matrixes as well as pre-concentration in water matrixes (Watanabe, 2012). Due to the selective retention of analytes or matrix components, the choice of sorbent is crucial in SPE. Several cartridges made up of pre-conditioned adsorbents including primary and secondary amines (PSA), graphitized carbon black (GCB), and C-18 are available and have been widely applied in pesticide residue analysis (Fritz and Macka, 2000; Hennion, 1999). While SPE may be used for the entire pre-treatment of analytes, the procedure has found more efficient usage as a clean-up step.

The need for a simple, efficient and less expensive extraction technique for sample pre-treatment for multi-class pesticide analysis led to the development of the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure by Annastassiades et al (2003) (Anastassiades et al., 2003) . The technique is based on an initial single-phase extraction in acetonitrile, followed by a liquid-liquid partitioning by addition of salts. In most cases, a clean-up of analytes is performed using a dispersive solid phase extraction (d-SPE) procedure involving the use of PSA, and other sorbents such as C18 and GCB. Magnesium sulphate

($MgSO_4$) is used in the removal of water during the clean-up step. The QuEChERS procedure combines the principles of liquid-liquid and solid-phase extraction into a single step that does not require solvent exchange or solvent evaporation (i.e. pre-concentration). Advantages over other conventional methods include simplicity, high recovery and accuracy, low solvent usage, high sample throughput and low costs. The original QuEChERS method has been widely verified in several laboratories and countries (Sack et al., 2011; Wong et al., 2010) and modified into two main methods: AOAC official 2007.01 method and European EN 15662 method. The QuEChERS method has been found to compare favourably with other conventional and more sophisticated methods in the extraction and clean-up of analytes from different matrices (Lehotay et al., 2005c; Lesueur et al., 2008; Park et al., 2011; Prestes et al., 2012). The different salting out procedures and the presence of different SPE sorbents have provided versatility (Bragança et al., 2012a; Dankyi et al., 2014; Lehotay et al., 2010) that ensures the application of the method to a wide range of analytes (Lian et al., 2010; Romero-González et al., 2011) in a wide range of matrixes including food (Hong-yan et al., 2007; Lee et al., 2011; Romero-González et al., 2011; Zhang et al., 2011), soil (Bragança et al., 2012a; Correia-Sá et al., 2012; Dankyi et al., 2014; Prestes et al., 2012) water (Brondi et al., 2011; Mantzos et al., 2013; Yang et al., 2010a) and animal tissues (Kamel, 2010; Lichtmannegger et al., 2015; Sapozhnikova and Lehotay, 2013). This flexibility was utilised in the current study as the procedure was employed in the extraction and clean-up of analytes in soil, food and water matrixes.

1.3.2 Liquid Chromatography- Tandem mass spectrometry (LC-MS/MS)

The high effectiveness of the QuEChERS procedure notwithstanding, good chromatographic separation and detection methods are required to satisfactorily determine the presence and quantities of analytes usually present in very low concentrations in complex food and environmental matrixes. In pesticide residue analysis, liquid chromatography-mass

spectrometry (LC-MS) quadrupole instruments have become the most widely used (Pico et al., 2004). This is due to the wide array of polar, thermolabile, non-volatile and non-GC amenable compounds available (Raina, 2011). Of the over 500 registered pesticides available worldwide for agriculture, majority are LC amenable. The increasing importance of LC-MS/MS is also reflected in the vast majority of recent publications on pesticide residue analysis (Gómez-Ramos et al., 2013; Pico et al., 2004).

In general GC and HPLC represent the two main chromatographic methods for separation of analytes. Neonicotinoids are better suited for HPLC as they are usually degraded by heat and hence require derivatization to be GC amenable. The use of HPLC does not require the additional step and simplifies the pre-treatment procedure. Prior to MS detection, ultraviolet (UV) detector, diode array detector (DAD), electron capture detector (ECD) and fluorescent detectors (FLD) have been widely used in pesticide residue analysis including neonicotinoids albeit with lower sensitivity and selectivity. The coupling of HPLC (or GC) with MS techniques such as single quadrupole, time-of-flight (TOF), ion-trap and triple quadrupoles in the past decade has revolutionised pesticide residue analysis and led to dramatic increases not only in the number of reported cases of analysis but also in the number of pesticides analysed per report (Lehotay et al., 2005a; Lian et al., 2010).

Atmospheric pressure ionisation sources (API) particularly electrospray ionisation (ESI) are the most common in LC and offers a softer ionisation compared to electron ionisation (EI) and chemical ionisation (CI) in GC. Tandem MS is performed in selected reaction monitoring (SRM) mode which offers significantly reduced background signals compared with single ion monitoring (SIM). Here, isobaric co-extracts even if present can be separated due to their different fragmentation in the collision cell often resulting in different product ions (Alder et al., 2006). Tandem MS thus offers excellent sensitivity and selectivity in residue analysis, providing for low limits of detection (LOD) and quantitation (LOQ) of analytes.

As occurs in other techniques, LC-MS and GC-MS are susceptible to matrix effects involving ion suppression or enhancement based on matrix, analyte, and sample pre-treatment procedure used and may adversely affect the accuracy of quantification (Kwon et al., 2012). A number of approaches such as use of cleaner matrixes, isotopically labelled internal standards, and matrix-matched standards are often used to reduce or eliminate the effect of matrix.

In the current study, LC-ESI-MS/MS was used for the analysis of all neonicotinoids due to reasons indicated above.

1.4 Aim

While neonicotinoid insecticides have contributed to substantial increases in the yield of cocoa beans in Ghana, the increased likelihood for exposure to crops and the environment as a result of the widespread and intensive usage presents a challenge both from the toxicological and regulatory points of view. This is even more imperative based on reports of possible detrimental effects of neonicotinoids on bees and other non-target organisms as well as reported widespread presence in food in other parts of the world. As a result of the extensive usage in Ghana, it becomes even more crucial to understand the effects on non-target organisms and health. A key part in the understanding of the effects of neonicotinoid insecticides in the environment, crops and non-target organisms is knowing their levels and fate in the environment. This study seeks to do that by:

a) Assessing the presence and residue levels of neonicotinoids in soils from cocoa farmlands across the country

b) Examining the concentrations of neonicotinoid insecticide residues in cocoa beans and their possible distribution in cocoa shells and cocoa nibs (deshelled beans)

c) Studying the fate of the two most widely used neonicotinoid insecticides (imidacloprid and thiamethoxam) in soils by measuring the dissipation rates and identification of possible metabolites

d) Estimating the sorption and desorption (leaching) potential of neonicotinoid insecticides as a means of predicting their mobility in soils

The study is focused on all four neonicotinoids currently registered and used in crop production in Ghana viz: Acetamiprid; thiacloprid; imidacloprid and thiamethoxam; as well as clothianidin. Although yet to be registered in Ghana, clothianidin is a known metabolite of thiamethoxam and hence was considered in the study based on interest in fate of neonicotinoids.

2. Neonicotinoid insecticide residues in soils from cocoa farms

2.1 Introduction to chapter 2

This chapter discusses the analysis of the presence and concentrations of neonicotinoid insecticide residues in soils from cocoa farms. The QuEChERS procedure was optimized for the samples used in the analysis. This optimization based on salts and sorbents employed, as well as the choice of an optimum procedure has been argued in the narrative. Finally the outcomes from the application of the procedure in assessing concentrations of neonicotinoid insecticide residue in soils have been discussed. A summary of the work described has been published in Science of the Total Environment (2014, vol. 499, page 276-283) (Appendix A1).

2.1.1 Neonicotinoids in soil

The soil compartment does not only play a major role in the provision of nutrients to plants but also serves as an important sink for applied pesticides. Once a pesticide is applied to crops, its behaviour in the soil environment will depend on a number of complex interactions based on several factors including the properties of the soil and pesticide, the amount of pesticide applied, climatic factors and the presence of microorganisms. In general, the fate and behavior of pesticides in soils is determined by decomposition (chemical, photochemical or microbial), volatilization, adsorption and uptake by plants and organisms (Kah et al., 2007a). To a large extent, the concentration of a particular pesticide in soil at a given time following application is highly influenced by these factors.

The soil compartment is perhaps the most important sink for applied pesticides. For neonicotinoids, research shows that more than 60% of application is based on seed/soil treatment (Jeschke et al., 2011) with large amounts ending up in the soil (Sur and Stork, 2003). Despite these facts, literature on the occurrence of neonicotinoids in soil is low. Research in soils have been largely based on dissipation and leaching behavior without knowledge of the

influence of these factors on the occurrence and concentrations in fields. So far, Jones et al (2015) have reported clothianidin, thiamethoxam and imidacloprid levels of 0.02-13.6 µg/kg; < 0.02-1.5 µg/kg; and < 0.09 to 10.7 µg/kg respectively in arable soils following seed treatment (Jones et al., 2014).

In general the fate of neonicotinoid insecticides in soils is uncertain, with a wide range of dissipation rates reported in literature (El-Hamady et al., 2008; Juraske et al., 2009; Pitam et al., 2013; Ramasubramanian, 2013). In view of the complexity of pesticide-soil interactions, an understanding of residue levels in soils is central to a deeper understanding of their fate and behavior. This is particularly true in the study of pesticides in new environments often characterized by different soil types, properties and climatic conditions. Moreover, knowledge of occurrence and concentrations in soils is important due to the role of soils as a sink and the reported persistence of neonicotinoids in soils (Goulson, 2013).

2.1.2 Application of QuEChERS procedure to soils

The QuEChERS procedure has been originally developed and optimised for high moisture fruit and vegetable matrixes. In recent years, the technique has been applied to other food and environmental matrixes including animal products, water, sediments and soil (Angioni et al., 2011; Martins et al., 2013; Peña et al., 2011; Xia et al., 2010). The QuEChERS procedure has been shown to exhibit greater extraction efficiency of pesticides from soil matrixes compared to more sophisticated methods such as pressurized fluid extraction (PLE), also referred to as accelerated solvent extraction (ASE) and gel permeation chromatography (GPC) (Lesueur et al., 2008).

Several QuEChERS procedures exist including three extensively used salting out and sorbent clean-up procedures each. The existence of these diverse sorbents and salting out conditions has given much room for flexibility in utilising any combination of salts-sorbents for multi-

residue analysis often with good recoveries for most analytes. This flexibility is evident in the diversity of QuEChERS extraction and clean-up procedures that have been applied to one particular matrix such as soil (Asensio-Ramos et al., 2010; Caldas et al., 2011; Chen et al., 2010; Dong et al., 2009; Drozdzyński and Kowalska, 2009; Lesueur et al., 2008; Rashid et al., 2010; Shi et al., 2010; Yang et al., 2010a).

In nearly all cases of the application of these methods originally designed for food matrixes to other matrixes, no optimization has been done. This may have been due to the good recoveries associated with the QuEChERS procedures as well as the application of the method mainly to multi-class pesticide analysis and hence the lack of need to optimize for a particular class of analytes. Lehotay et al. (2010) have observed that, although some differences may exist in recoveries of analytes from different matrixes and under salting-out and clean-up conditions, these differences are often subtle and insignificant (Lehotay et al., 2010).

However, the absence of extensively researched data in the soil matrix particularly those being investigated, coupled with the availability of several QuEChERS procedures and no clearly defined procedures for soils make the need for optimization useful for the class of insecticides being investigated in this study. The purpose of the study therefore, was to determine the concentrations of five neonicotinoid insecticides (imidacloprid, thiamethoxam, clothianidin, acetamiprid and thiacloprid) in tropical soils from cocoa farmlands in Ghana, after extraction with an optimised QuEChERS procedure.

2.2 Materials and Methods

2.2.1 Soil sampling

Cocoa cultivation is concentrated in the south-western parts of Ghana, bordering with La Côte d'Ivoire, the largest producer of cocoa beans worldwide. Evidence of the high concentration of

cocoa production in this part of the country is seen by the fact that, the Western region alone accounts for about 60% of the country's total cocoa beans production.

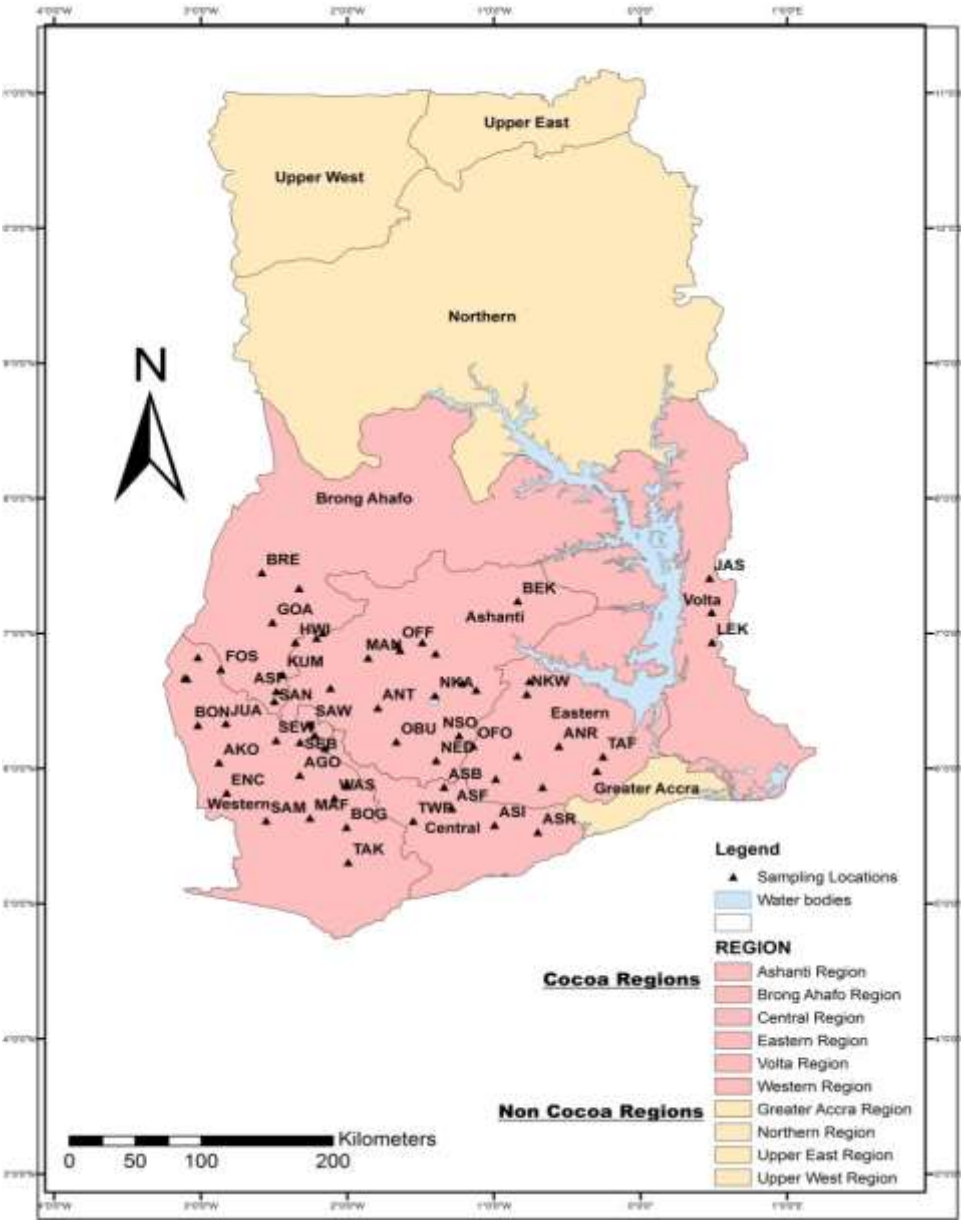

Figure 3. A map of locations of sampling in the major cocoa-growing regions of Ghana

Soils were sampled from all the six main cocoa-cultivation regions. The number of samples reflected the size, number of farmers, and amount of cocoa beans produced per region. Soil samples were obtained from a depth of 0-20 cm in cocoa farms. In each farm visited, samples were taken from between five to twelve different locations, 50 to 100 meters apart, depending

on the size of the farm. Samples obtained from a minimum of three to six different farms were aggregated to form a representative sample for a particular village/town. In all, 52 aggregate samples from more than 220 farms representing the six cocoa regions were employed in the study. Sample preparation involved the careful removal of litter, roots, stones and other exogenous objects. Soil samples were air-dried at room temperature and sieved using a 2 mm mesh, and stored at ambient temperature prior to analysis. In each region, where available, soil samples were collected from forest lands and abandoned cocoa farms. These samples were employed in method development and validation.

2.2.2 Reagents, Chemicals & Solutions

Acetonitrile and methanol obtained from Rathburn (Walkerburn, Scotland) and optima grade acetic acid (Fisher, Canada) were purchased for the study. Ammonium acetate and sodium chloride were purchased from Merck (Darmstadt, Germany). Sodium acetate, sodium citrate dihydrate (SCTD) and sodium citrate dibasic sesquihydrate (SCDS) were obtained from Sigma-Aldrich (Germany). Magnesium sulphate was purchased from BDH (Leuven, Belgium). 2ml sorbent tubes containing magnesium sulphate with combinations of PSA, C-18 and GCB were obtained from Phenomenex (CA, USA).

Insecticide standards (purity > 99%) of acetamiprid, imidacloprid, thiacloprid, clothianidin and thiamethoxam were obtained from Fluka (Germany). Deionized water was prepared using an EMD Millipore Milli-Q purification system (MA, USA).

A stock solution of each insecticide was prepared by dissolving a measured amount of solid compound in acetonitrile. In addition, a stock mixture of all insecticides was prepared from individual stock solutions by measuring and mixing appropriate volumes. All prepared standard solutions were stored at < 4°C prior to use.

2.2.3 LC-MS instrumentation

2.2.3.1 HPLC system

The chromatographic separation of analytes was performed on an Agilent 1200 HPLC system (Santa Clara, CA, USA) equipped with a BDS Hypersil reversed-phase C18 column with dimensions; 250 mm x 2.1 mm; 5 µm (Thermo Electron Co., UK). Analytes were run at a column temperature of 30°C. Gradient elution of analytes involved the use of two mobile phases 'A' and 'B'. Mobile phase 'A' consisted of 99% of a 10 nM ammonium acetate, with 1% methanol and mobile phase 'B' consisted of 90% of methanol, with 10% of 10 nM ammonium acetate. The chromatographic method began with an initial mobile phase composition of 10% for solvent B, increased to 100% over 10 minutes, held constant for a further 10 minutes and decreased to 10% for 1 min. 10µL of sample extract or standard was injected onto the column at a flow rate of 200 µL min^{-1} for a total run of 21 minutes. Optimisation of chromatographic conditions involved the use of different eluents, varying gradients and injection volumes in order to obtain good chromatograms in the shortest time possible.

2.2.3.2 Mass spectrometry

The HPLC was coupled with an AB Sciex (Forest City, CA) 3200 QTRAP mass spectrometer (MS), equipped with electrospray ionization (ESI). Multiple reaction monitoring (MRM) data was acquired and processed for all analytes in a positive ion mode. Optimized values of declustering potential (DP), exit potential (EP), collision energy (CE) and collision cell entrance potential (CEP) are listed in Table 6. The selection and optimisation of precursor ion and product ions for each analyte was carried out by direct injection of standards prepared in methanol: water (50:50 v/v) at a flow rate of 200 µL min^{-1}.

For all analytes, the efficiency of ionisation was best in positive mode. Optimal values of instrumental parameters were selected and applied to obtain the best MRM transition with the

highest intensities possible. The two most intense precursor-to-product ion transitions were chosen for each compound: the most intense being used for quantification and the other used for confirmation. This was particularly useful in the cases of imidacloprid and clothianidin, both of which produced two transitions of similarly high intensities.

Table 6. Instrument conditions and MRM transitions of precursor/product ions of analytes

Analyte	Ion transition (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)
Acetamiprid	223.14→126.00*	41.0	2.0	16.4	29.0
	223.14→99.10	41.0	2.0	16.4	53.0
Clothianidin	250.00 →169.01*	26.0	8.0	17.1	17.0
	250.00→132.00	26.0	8.0	17.1	19.0
Imidacloprid	256.13→209.10*	31.0	4.5	17.3	27.0
	256.13→175.00	31.0	4.5	17.3	23.0
Thiacloprid	253.06→126.00*	51.0	2.5	17.2	29.0
	253.06→99.00	51.0	2.5	17.2	57.0
Thiamethoxam	292.08→211.20*	26.0	9.5	18.3	17.0
	292.08→181.00	26.0	9.5	18.3	31.0

* Transitions used in quantitation

The data obtained was processed using the Analyst software (version 1.5.2). A chromatogram of all compounds monitored showed good resolution of peaks except in the case of imidacloprid and Clothianidin (Figure 4). Nonetheless they were easily identified and quantified in MRM mode due to the differences in m/z values of precursor and fragment ions.

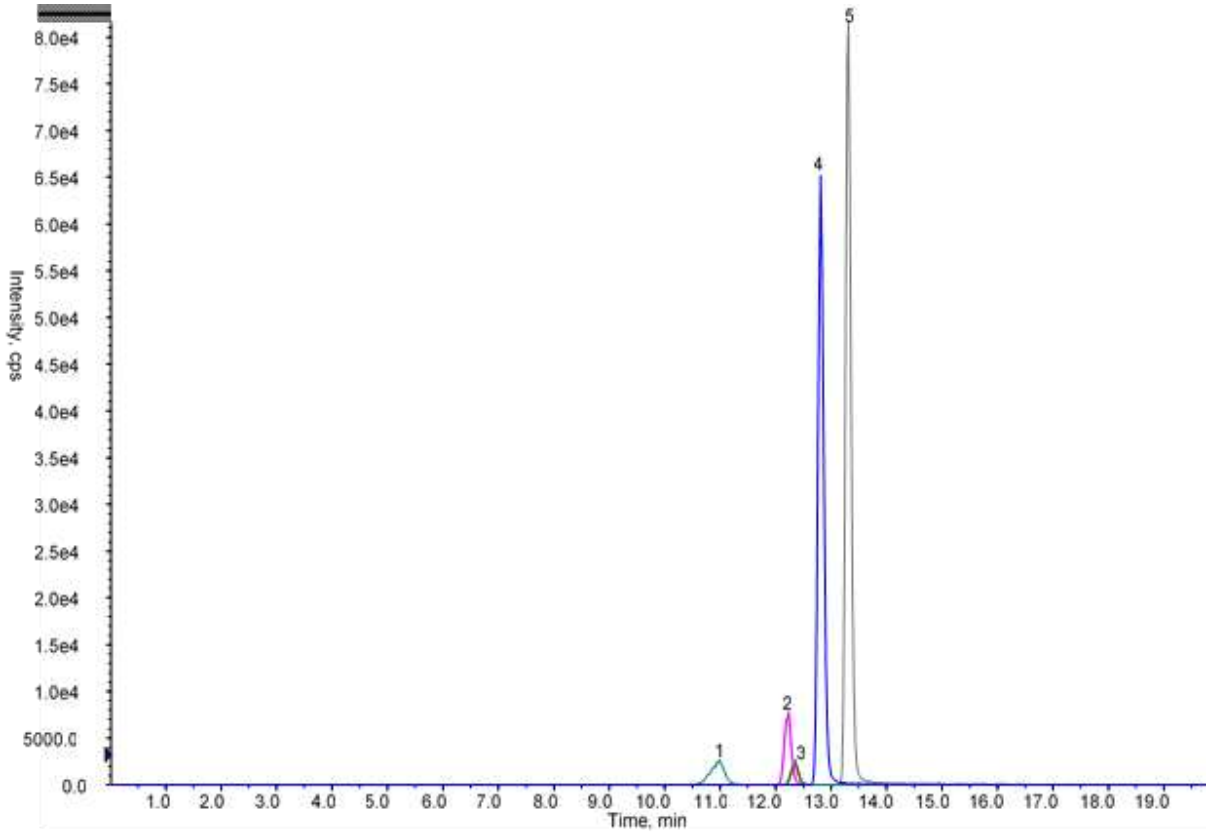

Figure 4. A chromatogram showing the five neonicotinoids of interest being analysed. Intensities represent a blank soil matrix spiked at 100 µg kg^{-1}. 1-Thiamethoxam; 2-Imidacloprid; 3-Clothianidin; 4-Acetamiprid; 5-Thiacloprid

2.2.4 Soil Physical and Chemical Characteristics

The soil characteristics measured include pH, soil organic carbon (SOC) and texture. Soil pH was measured using a pH meter in a 0.01 M calcium chloride solution, using a soil-solution ratio of 1: 2.5. The percentage soil organic matter content was estimated using the Walkley-Black method (Walkley and Black, 1934). The texture of soil samples was estimated using Calgon solution (Sodium hexametaphosphate) and 50g of sample, employing the hydrometer method (Bouyoucos, 1951).

2.2.5 QuEChERS procedure

2.2.5.1 Salting-out Extraction

To determine an optimum procedure for the extraction of analytes in the soil matrix being studied, experiments were conducted using the various salting-out procedures under the QuEChERS method. Five grams of blank soil samples were used. Samples were placed in 50 ml Falcon tubes and spiked with known concentrations of analytes (spike 1, Figure 5). After allowing them to stand for about 45 minutes, 5 ml of deionised water was added and mixed with the sample. 10ml of acetonitrile in 1% acetic acid was then added and the resulting mixture hand-shaken vigorously for one minute.

Three different mixtures of salts are commonly used in the QuEChERS procedure. These are: (i) 4.0 g $MgSO_4$ & 1.5 g NaOAc (ii) 4.0 g $MgSO_4$ & 1.0 g NaCl (iii) 4.0 g $MgSO_4$, 1.0 g NaCl, 1.0 g SCTD & 0.5 g SCDS. All three were assessed by adding each mixture to separate blank samples. The resulting mixtures were hand-shaken vigorously for one minute and then centrifuged at 4000 RPM for 5 minutes. The supernatant was separated and used in the clean-up step. Six replicates were prepared for each salting out procedure. A schematic diagram of the procedure is as shown in Figure 5.

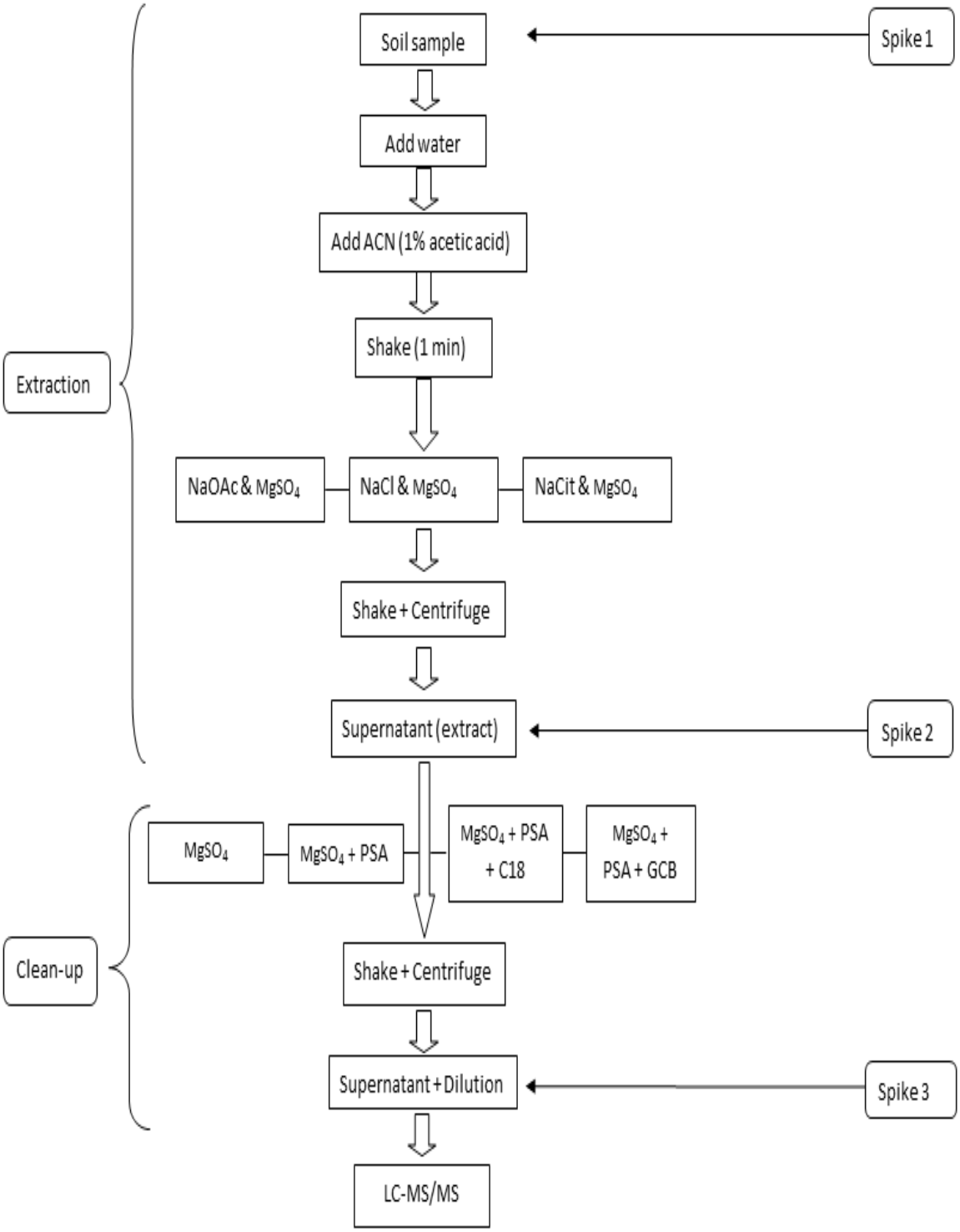

Figure 5. A schematic diagram of the QuEChERS procedure for sample extraction and clean-up. Spikes 1, 2 and 3 ensured the estimation of recoveries from the whole QuEChERS procedure, recovery from the clean-up procedure and matrix-matching respectively.

2.2.5.2 Sample clean-up

During the clean-up process, the three most widely used solid-phase sorbents viz; PSA, C-18 and GCB were examined for their efficiency of clean-up of the matrix from each of the three salting-out extraction procedures. The sorbents were added with the following compositions: (i) 25 mg PSA + 150 mg $MgSO_4$; (ii) 25 mg PSA + 25 mg C18 + 150 mg $MgSO_4$; (iii) 25 mg PSA + 7.5 mg GCB + 150 mg $MgSO_4$. Each sorbent/sorbent mixture was added to a 1ml aliquot of salted-out extract, then vortex-mixed for one minute and centrifuged at 4000 RPM for 3 minutes. To assess the efficiency of the clean-up process, spiking (spike 2) was performed prior to the addition of the sorbents. The supernatant produced was then separated and filtered through a 0.45 µm PTFE syringe filter. Aliquots of this solution were diluted with an equal volume of water prior to injection onto the LC column. To assess the need for a clean-up step or otherwise, aliquots of the solution obtained from the salting-out step were injected directly onto the column after addition of 150 mg of $MgSO_4$, followed by filtration and dilution without the use of the clean-up sorbents.

2.2.6 Method Validation

The performance of the various salting out and clean-up procedures was assessed by evaluating parameters including accuracy, precision, matrix effects, limit of detection (LOD) and limit of quantification (LOQ). For each procedure, the accuracy and precision were evaluated by estimating percentage recoveries and relative standard deviations (RSDs) at two fortification levels (8 and 80 µg kg^{-1}) and for six replicates of all neonicotinoids.

The linearity of the analytical procedures was studied using matrix-matched calibration solutions prepared in blank soil extracts, from the different extraction and clean-up procedures at a concentration range between 1.56 and 400 µgL^{-1}. The LOD and LOQ were estimated by injection of matrix-matched standard solutions at the lowest concentrations that yielded a signal

to background noise (S/N) ratio of three and ten respectively. An assessment of the influence of matrix was done by comparing the responses of matrix-matched and solvent standards (Pizzutti et al., 2007). The absolute matrix effect was estimated by comparing the slopes obtained from calibration solutions prepared in matrix and in solvents.

2.3 Results & Discussion

2.3.1 Soil Physico-chemical properties

Soils from tropical climates are often characterised by a high rate of decomposition and depletion of organic matter, due to the prevailing high temperatures. In this study however, soil samples employed showed moderate to high SOC content, most likely as a result of the high influx of litter. In most farms visited, litter was greater than 5 cm in thickness and completely covered soil surfaces. The litter content in farms is further enhanced during pruning of cocoa trees, an essential agronomic practice which does not only ensure larger cocoa pods, but also reduced humidity thereby reducing the likelihood of diseases and pest attack. Percentage SOC ranged from 1.28 to 7.43 (Average, 2.94) for all samples studied (Table 7, appendix B). The range of values may reflect several factors including age of farms, frequency of pruning, rate of litter fall and rate of decomposition of litter.

In general, measured pH ($CaCl_2$) of soils were slightly more acidic and had a range of 4.46-7.54 (Average, 6.20). The range of pH values may represent the varying soil types and their associated properties in the various regions of the country.

Table 7. Soil organic carbon, pH and clay content of soils.

Sample	Farm (n=52)	Blank**
SOC (%)	1.28 – 7.43 (2.94)	3.63
pH ($CaCl_2$)	4.46 – 7.54 (6.20)	6.52
Clay content (%)	12.4 – 49.5 (20.4)	27.3

** Properties of soil used in method development

Soil texture classification based on grain size suggests soils from the cocoa farms as predominantly sandy-loam, loam or sand-clay-loam (Figure 6) according to the United States Department of Agriculture (USDA) classification system (USDA, 1987). In general the content of sand was greater than 50% in the majority of soil samples analysed and varied widely whereas clay content was lower than 20% in about half of the samples analysed. The low variability of clay and high variability of sand across farms in the various geographical location of the country is seen in Figure 6. With the exception of one sample (DOR) with 49.5% clay content, all other soils were generally low in clay content, ranging between 12.4% and 37.4%. The physico-chemical properties of the soils may play an important role in determining the fate of neonicotinoids in the soils. These are discussed in detail in chapter 5. Nonetheless, the low variability of important physico-chemical properties such as clay content, SOC and pH across the cocoa-growing regions of the country may help in predicting insecticide fate and management across the country.

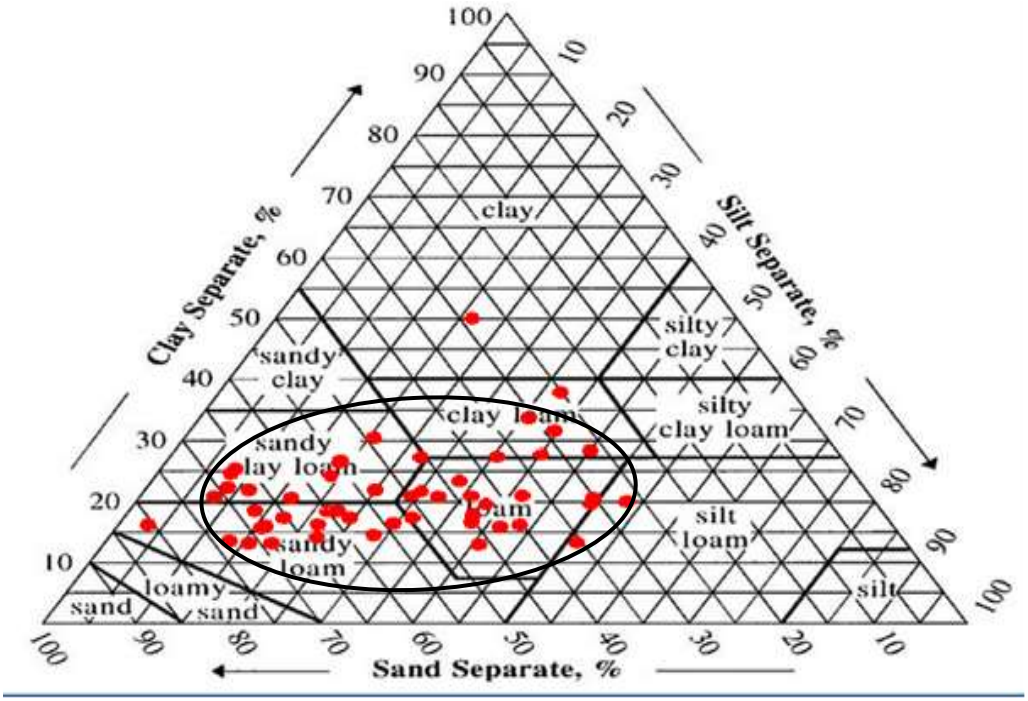

Figure 6. Soil texture classification based on the USDA textural triangle

2.3.2 Salting-out extraction procedure

Average recoveries obtained from extraction of neonicotinoids in soils with various salts and without a subsequent clean-up step are presented in Table 8. Good recoveries of analytes (77.5 - 96.8 %; RSD ≤ 9.0) were obtained for all salts used at the 80 µg kg^{-1} fortification level without the need for clean-up sorbents (SANCO/12571/2013, 2013). However analyte recoveries at the 8 µg kg^{-1} fortification level were relatively low with higher relative standard deviations (48.6 to 87.4 %; RSD ≤ 17.7) especially when citrate salts were used during the extraction procedure. Better yields were obtained from extracts employing both NaCl & $MgSO_4$ (76.5 – 87.4; RSD ≤ 14.2) and NaOAc & $MgSO_4$ (64.9 – 86.3; RSD ≤16.2) salts in all analytes. The lower recoveries and higher relative standard deviations for analytes at the lower fortification level may have been due to a higher influence of matrix at that low concentration of analytes. This may occur in different matrixes and analytes as the concentration of analytes approach the limits of quantitation.

Differences in recoveries for the three salts were minimal as shown in Figure 7 and 8 for the 80 and 8 µg kg^{-1} level of fortification respectively. Based on a one way analysis of variance (ANOVA) at a 95% confidence interval (Appendix x), the differences were found to be statistically significant in all analytes except thiamethoxam. Bonferroni post hoc comparisons suggest the use of NaCl & $MgSO_4$ for extraction resulted in significantly higher yields in almost all analytes as compared to the other two salt mixtures. The peculiarity in the results of thiamethoxam may have been due to the high variability among replicates which led to higher values of RSDs.

Table 8. Percentage recoveries of neonicotinoids with varying salting out extraction procedures at the two fortification levels (µg kg^{-1}) (n = 6 for each treatment).

Salts	Fortification	Imidacloprid	Thiacloprid	Thiamethoxam	Acetamiprid	Clothianidin
NaOAc &	8	71.0 ±9.6	64.9±16.2	86.3±10.9	72.0±10.7	76.5±11.4
$MgSO_4$	80	83.8 ±3.3	80.8±2.8	84.9±6.7	92.4±2.4	88.9±1.3
NaCl &	8	77.0±13.8	76.3±8.9	87.4±14.2	76.5±6.0	77.3±14.0
$MgSO_4$	80	91.2±5.0	89.3±3.5	86.1±6.9	93.9±4.2	96.8±1.3
NaCitrate	8	53.6±17.7	48.3±11.4	72.9±8.6	55.0±10.4	65.4±14.4
& $MgSO_4$	80	85.2±2.7	77.5±5.5	90.1±9.0	86.7±3.6	90.1±8.0

Besides inducing phase separation, the type of salts applied for extracting analytes under the QuEChERS procedure may impact pH, matrix polarity and matrix constitution thereby influencing recovery. Although addition of the various salts impacted on the pH of the matrix (citrate, chloride and acetate salts in increasing order of pH), its influence on analytes as well as matrix components is unclear in the current study. It was however observed that soil pH had less of an influence on the final pH of the matrix after extraction. Under the QuEChERS procedure, the relevance of pH influence has been found to be mostly dependent on the pH sensitivity of the analytes under studied. This is often the case for acid or base sensitive analytes. In generally, neonicotinoids have been found to be stable over a wide range of pH and hence the observed low influence of pH in the matrix under study (Campbell et al., 2005; Guzsvány et al., 2006).

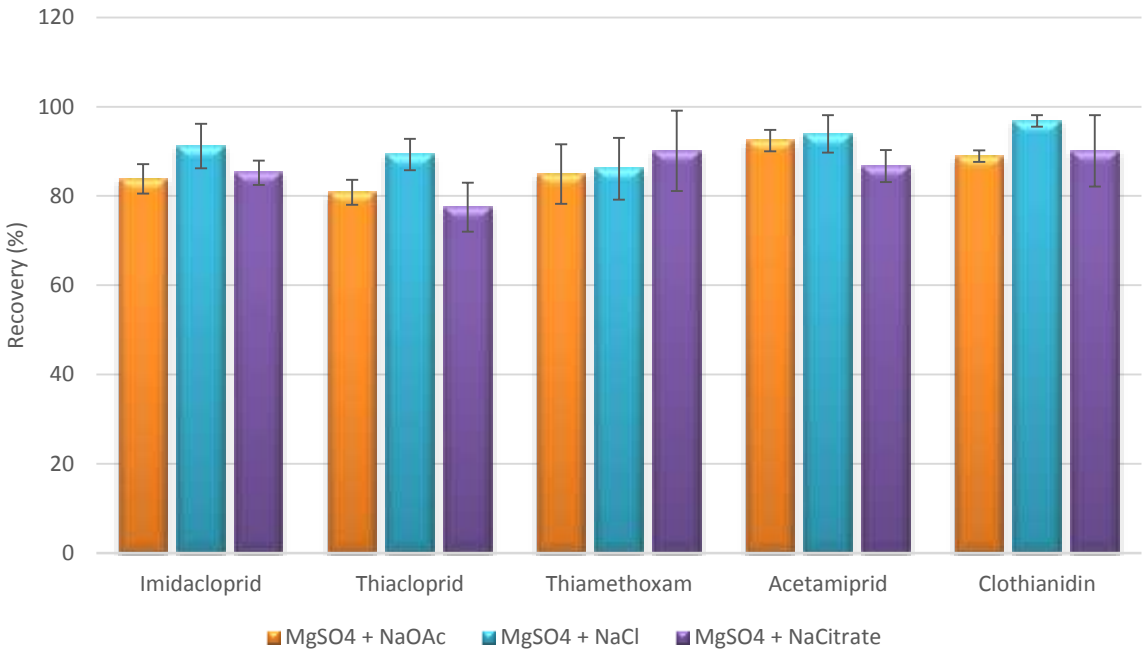

Figure 7. Percentage recoveries of neonicotinoids showing RSDs after extraction with different salts at 80 µg kg^{-1} level of fortification (n=6).

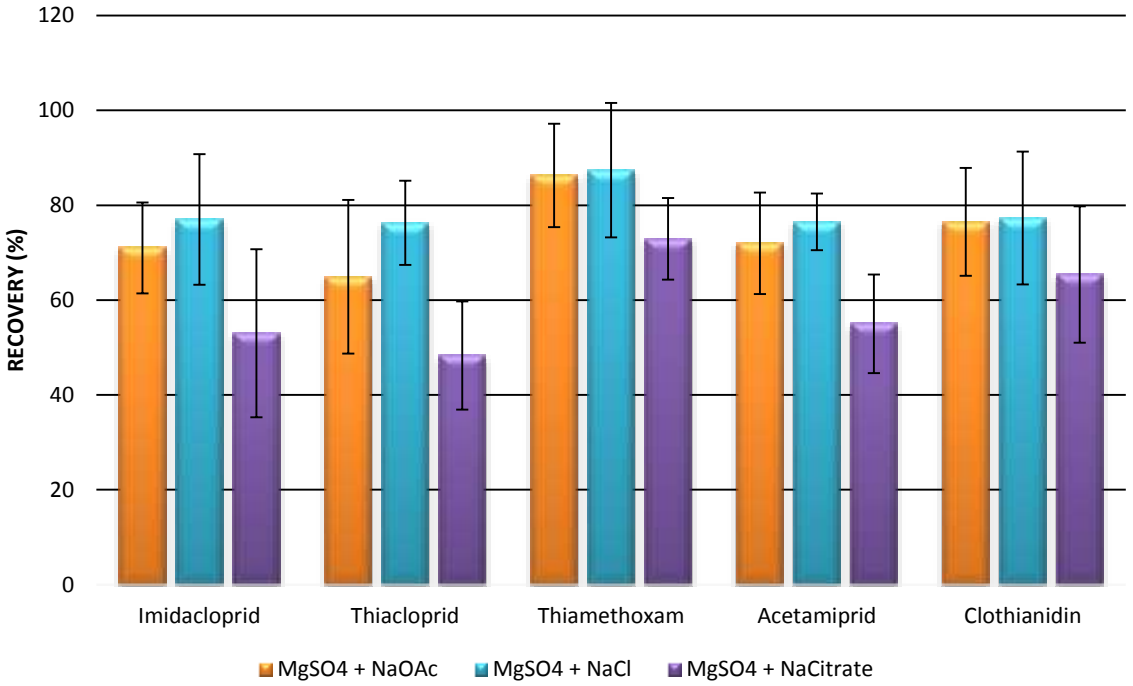

Figure 8. Percentage recoveries of neonicotinoids showing RSDs after extraction with different salts at 8 µg kg^{-1} level of fortification (n=6).

The important differences in the various salts used under the QuEChERS procedure are the resulting pH of the medium and the presence or absence of buffering. The original salting-out procedure employed the use of NaCl & $MgSO_4$ in acetonitrile (Anastassiades et al., 2003). However, low recoveries of some pH sensitive analytes prompted the need for a buffering medium. The result of the application of sodium acetate and sodium citrate in response to this need increased recoveries remarkably leading to the adoption of both as official methods: AOAC 2007.01 and EN 15662 respectively (Lehotay et al., 2005b).

The results obtained in this study however, do not suggest the need for a buffering medium for neonicotinoids in the matrix being studied. Despite the minimal differences, NaCl & $MgSO_4$ was chosen as optimum for extraction due to the higher yields and lower RSDs compared to the other salts. Shi et al (Shi et al., 2010) and Dong et al (Dong et al., 2009) have reported similar salt choices in acetonitrile extraction of oxadiargyl and metaflumizone respectively from soils without the need for buffering. While acetate salts have been used in some studies (Rashid et al., 2010), the use of citrate salts appear to be the most commonly reported in literature for soil matrix (Asensio-Ramos et al., 2010; Lesueur et al., 2008; Qiao et al., 2011; Yang et al., 2010b). It is quite obvious that the important considerations in the choice of a particular salting out procedure is largely based on the nature of analytes and matrix under study (Lehotay et al., 2005b).

2.3.3 Clean-up procedure

Recovery of analytes following d-SPE ranged from 78.5 to 104.8% and 48.6 to 87.2 % at 80 and 8 µg kg^{-1} fortification levels respectively (Table 9). Thus improved recoveries of analytes were obtained after the clean-up step. This was evident in almost all analytes and sorbent types particularly at the 80 µg kg^{-1} level of fortification. Recoveries were highest with PSA and appeared to decrease upon addition of other sorbents (C-18 and GCB). With the exception of

thiamethoxam, analysis of variance indicated higher yields of statistical significance for all analytes upon PSA clean-up at the 80 µg kg^{-1} level of fortification. The apparent effectiveness in the use of PSA as sorbent was evident in all salt matrixes. However analytes appeared less amenable to d-SPE clean-up at the relatively low concentration of 8 µg kg^{-1} (Table 9). This may probably be due to the relative increase in matrix influence at lower concentrations of analytes, closer to their limits of quantification as suggested in section 3.2.

Table 9. Percentage recoveries of neonicotinoids from varying d-SPE clean-up conditions (n = 6 for each treatment)

Level	Salts	Sorbent	Imidacloprid	Thiacloprid	Thiamethoxam	Acetamiprid	Clothianidin
80 µg kg^{-1}	NaOAc & $MgSO_4$	PSA	97.8±2.7	92.3±1.2	100.5±2.5	101.2±2.4	100.0±5.6
		PSA+C18	93.2±3.5	89.3±3.9	92.5±9.3	96.7±2.6	98.3±1.8
		PSA+GCB	92.8±2.7	84.5±2.8	87.9±12.9	94.3±1.6	94.0±1.9
	NaCl & $MgSO_4$	PSA	98.6±3.1	97.1±1.9	94.0±9.0	102.9±1.7	104.8±2.1
		PSA+C18	96.3±1.9	95.5±3.5	92.4±10.3	99.6±3.9	98.6±3.9
		PSA+GCB	94.5±1.6	91.7±3.3	85.3±6.9	96.5±1.1	97.3±4.3
	NaCitrate & $MgSO_4$	PSA	89.8±5.3	81.0±6.9	92.2±5.5	93.4±4.5	89.3±6.7
		PSA+C18	86.5±3.7	80.3±5.6	84.8±11.7	89.6±4.4	91.8±2.8
		PSA+GCB	84.7±3.7	78.5±5.5	87.4±9.6	88.9±2.9	88.4±9.5
8 µg kg^{-1}	NaOAc & $MgSO_4$	PSA	65.2±13.5	63.8±19.1	76.1±14.1	72.0±12.9	70.6±13.7
		PSA+C18	67.0±20.9	63.8±16.5	80.2±13.3	70.7±13.0	74.1±18.6
		PSA+GCB	60.2±19.4	60.9±17.2	82.3±10.3	69.1±12.4	68.0±18.8
	NaCl & $MgSO_4$	PSA	79.3±11.3	79.6±8.7	85.8±15.0	81.3±4.8	72.0±14.4
		PSA+C18	78.5±17.2	77.4±6.8	86.4±9.1	76.7±7.3	69.3±13.9
		PSA+GCB	87.2±7.5	74.2±6.5	85.2±11.0	75.3±5.0	74.5±13.2
	NaCitrate & $MgSO_4$	PSA	58.2±13.4	51.0±8.8	75.2±5.9	56.8±8.5	60.7±6.3
		PSA+C18	62.5±13.6	50.6±11.1	74.6±9.9	55.0±9.1	63.9±20.8
		PSA+GCB	48.6±17.7	48.8±8.6	75.4±8.4	54.3±6.1	49.8±21.9

The QuEChERS procedure as originally developed by Anastassiades (Anastassiades et al., 2003) comprises a salting out extraction followed by a d-SPE clean-up procedure. The addition of the clean-up step was important in removing most matrix co-extracts. Soil as a matrix is very complex and diverse. This complexity is further enhanced by high levels of organic matter which has been reported to decrease extraction efficiency (Bragança et al., 2012b; Correia-Sá

et al., 2012) often warranting the use of a clean-up procedure. PSA has been employed often in the removal of organic acids, fatty acids and sugars from diverse sample matrixes. The presence of primary and secondary amines in its structure helps in the retention of many polar compounds including sugars, organic acids and fatty acids which may be components in the soil matrix studied. Unlike PSA, the addition of C-18 commonly used in the removal of fats and other non-polar compounds as well as GCB often used in the removal of pigments tends to decrease the recovery of analytes suggesting perhaps the absence of these as matrix components and a possible adsorption of analytes by these sorbents.

In literature, PSA have been the most predominantly used sorbent material in clean-up extraction of various analytes from soil matrixes (Asensio-Ramos et al., 2010; Dong et al., 2009; Lesueur et al., 2008; Yang et al., 2010b). Reported recoveries however vary, perhaps based on analytes and matrix composition. The use of PSA & C-18 as sorbents has been reported by Drozdzynski and Kowalska with recoveries ranging from 83-104 % (Drozdzyński and Kowalska, 2009). However, not all studies have employed sorbents. Shi et al (Shi et al., 2010) have reported recoveries ranging from 82.9 to 112 % for oxadiargyl residues in soils after a salting out extraction with $MgSO_4$ and NaCl without the need for clean-up. Similarly Caldas et al (Caldas et al., 2011) have reported recoveries of 70.3 to 120 % in the QuEChERS extraction of multiple classes of pesticides from soils without clean-up sorbents. In this study, the importance of a clean-up procedure was evident at both levels of fortification, and especially so at higher concentrations (80 µg kg^{-1}) of analyte. Although PSA was found to be the best sorbent for the clean-up of analytes in the matrix studied, the choice of a clean-up step will be largely based on the degree of precision required. In the current study, a clean-up procedure using PSA as sorbent was not only important in providing higher recoveries and precision for analytes, but also provided cleaner matrixes and chromatograms.

2.3.4 Validation of QuEChERS procedure

The performance of the chosen procedure ($NaCl+MgSO_4$ salting out combined with PSA clean-up) was evaluated for linearity, precision, LOD and LOQ and possible matrix interference was taken into consideration by calculating concentrations of neonicotinoids from matrix-matched calibration standards using the peak area with a weighting of $^1/x$. Calibration curves were linear over a range of 1.56 to 400 μgL^{-1} for all neonicotinoids with correlation coefficients ≥ 0.9986 (Table 10). Percentage recoveries ranged from 72.0 to 104.8 with RSD $\leq$ 15.0 for all neonicotinoids at both 8 and 80 μgkg^{-1} fortification levels indicating good recovery and precision as recommended by the DG SANCO guidelines (SANCO/12571/2013, 2013). LOQ was estimated between 2.0 and 9.0 µg kg^{-1} based on analyte by injection of matrix-matched standard solutions at the lowest concentrations that yields a signal to background noise (S/N) ratio of three and ten respectively. Dong et al have reported LOQ of 4.0 µg kg^{-1} in the analysis of metaflumizone from soil using a similar procedure on an UPLC-MS/MS (Dong et al., 2009). Ramasubramanian reported LOQ of 10 µg kg^{-1} using HPLC with diode array detection in clothianidin analysis employing a similar salting out procedure without further clean-up (Ramasubramanian, 2013). Even lower LOQ of < 1 μgkg^{-1} has been reported for this procedure using UPCL-MSMS (Mei et al., 2011). In general the results obtained were comparable to results from similar procedures in literature despite the difference in analytes and some parameters.

Matrix influence were low particularly when sodium chloride and magnesium sulphate were used in extraction in combination with PSA clean-up (Table 10). The effects were more pronounced at lower concentrations of analytes especially when citrate salts were used during the extraction procedure. The low matrix influence may have been due to the use of clean-up

sorbents as well as the high selectivity of the LC-MSMS process. Nonetheless, all calibrations of analytes were performed in matrix-matched standards.

Table 10. Matrix effects and LOQ in optimised procedure: Salting out extraction with NaCl & $MgSO_4$ and clean-up with PSA

Analyte	Solvent (%) [a]	Matrix (%) [b]	Matrix effect [c]	R^2	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)
Imidacloprid	88.3	97.8	0.91	0.9998	2.0	4.0
Thiacloprid	94.6	97.1	1.01	0.9986	1.0	2.0
Thiamethoxam	96.8	94.0	1.01	0.9995	2.0	5.0
Acetamiprid	97.7	102.9	0.95	0.9992	1.0	2.0
Clothianidin	103.0	104.8	0.97	0.9999	3.0	9.0

[a] Analytes recovery using solvent calibrated standards

[b] Analyte recovery using matrix-matched calibration standards

[c] Matrix effect expressed as the ratio of the slopes of the calibration curves obtained from the matrix-matched standards and solvent standards

2.3.5 Application to soil samples from cocoa farms

The developed procedure was applied in the extraction and analysis of neonicotinoid insecticides in fifty two soil samples obtained from cocoa farmlands in Ghana. In all the farms visited, the most current application of neonicotinoids had occurred at least four months prior to sampling with some cases beyond two years. Samples were analysed using LC-MS/MS after extraction with magnesium sulphate and sodium chloride in acetonitrile with 1% acetic acid. Extracts were further cleaned up using PSA as sorbent. Quantities of salts, sorbents and solvents used were as described in the procedure development (sections 2.2.5). Results obtained are shown in Table 11.

Table 11*. Concentration of neonicotinoids (µg kg-1) in soil samples. Only values above the LOQ have been presented.

Sample	Imidacloprid	clothianidin	Sample	Imidacloprid	clothianidin
OBU	251.4	23.1	NKR	4.4	-
ASK	110.0	-	ASA	4.3	-
SAN	82.2	-	ASM	-	-
BRE	64.0	-	DUN	-	-
JUA	58.4	-	SAW	-	-
OFO	49.6	-	HWI	-	-
WAS	48.0	-	ASI	-	10.1
ASB	32.1	12.2	GOA	-	-
TWP	34.5	-	KAS	-	-
KPA	29.6	-	TWH	-	-
SEB	23.5	-	ANT	-	9.8
BEK	21.5	-	TAF	-	-
ANY	18.9	-	ENC	-	-
SAE	18.4	-	ASR	-	-
LEK	13.4	-	JAS	-	-
MIM	13.3	-	BON	-	-
DOR	11.0	-	KAJ	-	-
NSO	8.9	15.9	KON	-	-
ASU	8.4	-	MAN	-	-
FOS	8.0	-	SEW	-	-
SUN	7.2	-	NKA	-	-
BOG	6.6	-	NKW	-	-
KUM	6.3	-	ODA	-	-
AKO	6.2	-	OFF	-	-
TEP	5.7	-	ASF	-	-
AGO	5.1	-	NED	-	-

*Thiaclorid, Thiamethoxam and Acetamiprid were below quantification limits in all samples

Acetamiprid, thiacloprid and thiamethoxam were not detected in any of the samples analysed. While acetamiprid is not yet recommended for use in cocoa production, thiacloprid marketed as Proteus® (150 g/L thiacloprid + 20 g/L deltamethrin) and thiamethoxam marketed as Actara® are approved for cocoa production. However their use is quite limited based on responses from farmers and cocoa technical officers perhaps reflecting their apparent absence in cocoa soils sampled.

Clothianidin levels were quantified in approximately 10% of samples analysed. Values ranged from 9.8 to 23 µg kg^{-1}. The observed results are in spite of the absence of registration of the insecticide in any formulation in the country. However, apart from being an insecticide itself, clothianidin is a known metabolite of thiamethoxam which has been applied in relatively limited quantities in some cocoa farms. Thus the likelihood of its transformation from thiamethoxam is high.

In contrast, imidacloprid was quantified in more than 50% of samples with values ranging from 4.3 to 251.4 µg kg^{-1}. Undoubtedly imidacloprid marketed as Confidor® (200 g/L) is the most popular insecticide used. Together with bifenthrin (27 g/L), they are the most widely used insecticides under a free national cocoa spraying program in the country. The popularity of imidacloprid was evident in some farmers' perception of higher quality of beans following its usage. Its recommended rate of application on cocoa farms is four times in a year, mostly in the months of August, September, October and December. The rate of application is even higher in farms with less optimum agronomic practices.

The results obtained suggest that considerable levels of neonicotinoids may be found in soils months after application. Thus the tendency for non-point contamination is high under this circumstance. Although the variation in the levels of neonicotinoids may be due to the amount and frequency of application, interactions in soil is often determined by the physical and chemical properties of both insecticide and soil and may play a significant role in determining

their fate. Processes such as sorption, leaching and degradation may be significant in this regard. While sorption of neonicotinoids has been found to increase with increasing soil organic carbon content (Cox et al., 1997), their high solubility in water coupled with the high composition of sand in the samples analysed may increase the tendency of leaching also.

In the current study, the relationship between soil properties and concentrations of neonicotinoids in soils could not be established. This is due to the requirement of some equally important information such as amount of applied neonicotinoids per area of land, number and frequency of application as well as exact periods of application. A complete set of information was impossible to obtain from a number of farms visited at the time of sampling. In practice, complete information could only be obtained with prior knowledge of farmers who were eager to give "suitable" information to researchers rather than actual information that may not reflect positively on the farmer. Most farmers were quick to point to the use of recommended dosage on chemical containers, often with their own interpretation of the required dosage for application. Besides the recommended spraying periods of August, September, October and December, a number of farmers employed a ''spray on sight'' approach whereby insecticides were applied or re-applied whenever insects, particularly aphids were sighted. The inclination to increase the concentration of chemicals in an effort to reduce the frequency of spraying was also found to occur among farmers.

Concerns about the possible effects of neonicotinoids on bee health have been heightened in recent years prompting increased research on this class of insecticides (Blacquière et al., 2012a; Goulson, 2013). However, current knowledge on possible effects on other non-target organisms remains low and largely deduced (Goulson, 2013; Miranda et al., 2011). Quite clearly, a knowledge of concentrations of neonicotinoid insecticides in various environmental media will be important in providing a better understanding of their possible fate and effects on non-target organisms.

2.4 Conclusions on chapter 2

The QuEChERS procedure has been adapted successfully in extracting neonicotinoids in tropical soils from cocoa plantations. Sample extraction with sodium chloride & magnesium sulphate in acidified acetonitrile and clean-up using PSA was found to be the optimum conditions for analysis of neonicotinoids in the soils studied based on good accuracy and reproducibility. Yields for all analytes were high with low LOQs. From the application of the procedure to soil samples from cocoa farms in Ghana, the results suggest neonicotinoids particularly imidacloprid may be found in soils several months to years after application. The presence and concentrations obtained may suggest intensive usage and/or persistence in soil. Nonetheless, this knowledge will be essential in decision making regarding usage and environmental management of neonicotinoids in tropical environments. Further studies on fate of neonicotinoids in soil and the cocoa plant, particularly in cocoa beans will be important in this regard and have been discussed in chapters 3, 4 and 5.

3. Neonicotinoid insecticide residues in cocoa beans

3.1 Introduction to chapter 3

This chapter discusses the application of the QuEChERS procedure in the extraction of neonicotinoid insecticide residues from the cocoa beans matrix. The importance of this step was not only because the procedure had not been applied to the cocoa matrix, but the complexity of the matrix characterized by high fat and high pigments. The developed procedure enabled the analysis of the concentration of neonicotinoids in cocoa beans and has been discussed. Finally the chapter discusses the distribution of residues in both cocoa nibs (deshelled beans) and cocoa shells and its implications for food safety and pesticide management. A summary of the work described has been published in the Journal of Food Composition and Analysis (2015, vol. 44, page 149-157) (Appendix A2).

3.1.1 Neonicotinoids in food

Among the important concerns for pesticide usage in general, are their exposure and impact on non-target organisms (Pisa et al., 2014). In humans, this often occurs through the consumption of food exposed to chemicals used in the control of pests on farms. Although neonicotinoid insecticides exhibit low toxicity to mammals (Tomizawa and Casida, 2005), acute and chronic toxicity may result depending on levels and length of exposure due to interactions of neonicotinoids and/metabolites with nAChRs in mammals (Duzguner and Erdogan, 2012). A reported linkage of neonicotinoid exposure and lipid accumulation in adipocytes and its possible link with obesity in humans have emerged (Park et al., 2013). Acute inhalation has also been associated with severe gastrointestinal symptoms along with respiratory distress and neuropsychiatric features (Kumar et al., 2013). While levels of exposure in food may be low, continuous monitoring of residue levels is essential to ensure safety to man.

Compared to other insecticide classes, knowledge of neonicotinoid residue levels in food is low (Chen et al., 2014). In literature, much of the current knowledge on residues of neonicotinoids has been restricted to honey and honey bees due to reported detrimental health effects of this class of insecticides on pollinators (Blacquière et al., 2012b; Laycock et al., 2012; Tanner and Czerwenka, 2011b). Although some research in fruits and vegetables exist, they are mainly restricted to periodic market surveys or the application of newly developed procedures often in multi-class pesticides analysis (Bakırcı et al., 2014; Garrido Frenich et al., 2008; Gilbert-López et al., 2010; Obana et al., 2002; Wang et al., 2012b; Xie et al., 2011; F. Zhang et al., 2012). Chen et al (2014) have reported the widespread presence of neonicotinoid insecticide residues in some common foods including fruits and vegetables (Chen et al., 2014). However knowledge in tropical foods such as cocoa beans and actual sources of contamination remain low. Although neonicotinoids have been used extensively in the production of cocoa and other crops in Ghana and other West African countries for several years, their levels in food is not yet known.

In Ghana, neonicotinoid insecticides are widely used in food production particularly cocoa. Cocoa beans are the main raw material for cocoa products including chocolate. The cocoa tree is mainly grown in the tropics, particularly in West Africa where more than 60% of worldwide cocoa beans is produced (ICCO, 2014). Ghana is the second largest producer of cocoa beans and the industry is highly controlled by government regulations. This is because the cocoa sector contributes significantly to the country's gross domestic product (GDP) through export and provision of employment for farmers (Ghana Statistical Service, 2014; Global Agricultural Information Network, 2012). However, cocoa yields per hectare in Ghana are considerably lower than other top cocoa producing countries. Pest and diseases are said to contribute significantly to the low yields (COCOBOD, 1995). According to the international cocoa organization (ICCO), up to 40% of global annual cocoa production is lost to insect pests and

diseases (ICCO, 2013). In response, the Government of Ghana through the Ghana Cocoa Board introduced the Cocoa Diseases and Pests Control (CODAPEC) program over a decade ago (COCOBOD, 2012). Under this initiative, mass application of insecticides is performed on cocoa farms across the country, up to four times in a year at no financial cost to farmers. Neonicotinoids particularly imidacloprid are extensively applied under the program and have contributed to significant increases in yield (COCOBOD, 2012).

However, a drawback to the mass application of insecticide program is the tendency for crop and environmental contamination due to the extensive use and multiple application rates. In previous studies, we have demonstrated that, applied neonicotinoids (in particular imidacloprid) in cocoa farms may enter and persist in soils for several months following application (Dankyi et al., 2014). Unfortunately the fate of these insecticides in cocoa beans, which is the main product consumed is not yet known.

Whereas some research exist of pesticide residues in cocoa beans, they have been restricted to other classes of pesticides including organochlorines, organophosphates and carbamates which are less readily used in cocoa production in recent years, particularly in Ghana (Frimpong et al., 2012a; 2012b; 2012c; Owusu-Ansah et al., 2010). With the coming into force of market regulations seeking to limit pesticide usage in food crops, such as the new European Union (EU) Regulation 396/2005/EC on "maximum residue levels of pesticides in or on food and feed of plant and animal origin" (European Commission, 2008), the need for knowledge on pesticide levels in produce such as cocoa beans mainly produced for export to European and other markets cannot be overemphasized.

3.1.2 Application of the QuEChERS procedure to high fat matrixes

It is quite evident that the effective management of these insecticides in food from developing countries will require reliable quantitative and qualitative assessment based on simple, efficient

and less expensive techniques to ensure food safety. The QuEChERS procedure does not only offer this simplicity, reliability and effectiveness but also a high flexibility for application to a wide range of analytes and matrixes (Lehotay et al., 2010).

However, the QuEChERS method has been originally designed for low-fat food matrixes (Anastassiades et al., 2003). Application to high fat matrixes often present a challenge due to high lipid co-extractives that may not only adversely affect extraction and chromatographic efficiency but also instrumentation (Chamkasem et al., 2013). This is particularly the case for lipophilic pesticides, which tend to exhibit greater partitioning in lipophilic portions of fatty matrixes resulting in reduced extraction efficiency. In literature, conventional methods that are widely used for the extraction of pesticides from fatty matrixes include solid-phase micro-extraction (SPME) (Tsoutsi et al., 2006), matrix solid-phase extraction (MSPD) (Ferrer et al., 2005), gel permeation chromatography (GPC) (Fernández Moreno et al., 2006) and supercritical fluid extraction (SFE) (Juhler, 1998; King and Zhang, 1998).

In recent years, the QuEChERS method has been applied to matrixes of medium (≈ 15%) to high fat (> 40%) content including nuts, avocado, fish and animal foods with varying degrees of success (Chamkasem et al., 2013; Choi et al., 2015; Koesukwiwat et al., 2010; Lozano et al., 2014; Luzardo et al., 2013; Rajski et al., 2013; Sobhanzadeh et al., 2012). Lehotay & Mastovska (2010) have observed that, the QuEChERS method may compare favourably with established methods such as MSPD in the analysis of polar and semi-polar pesticides from low fat food matrixes but performed poorly (25-50% recovery) with nonpolar pesticides (Lehotay et al., 2005c). However, various modifications of the method have been performed to enhance the efficiency of QuEChERS extraction in high fat matrixes including the use of a freeze-out step (Koesukwiwat et al., 2010), higher solvent-sample ratios (Chamkasem et al., 2013), and zirconium sorbents (Lozano et al., 2014; Rajski et al., 2013; Sapozhnikova and Lehotay, 2013).

The challenge in the application of the method to the cocoa matrix may not only be due to its high fat content (> 40%) but also its highly pigmented nature (Torres-Moreno et al., 2015) (Krysiak, 2006; Zyzelewicz et al., 2014). In the current study, the QuEChERS procedure was explored in the extraction and clean-up of five neonicotinoid insecticide residues in cocoa beans and shells obtained from Ghana. An optimized procedure was then used to assess the levels of residues of neonicotinoid insecticides resulting from their use in the country's cocoa production. Cocoa shells comprise the thin outer covering (husk) of the beans, which are usually removed during the processing of cocoa. Various studies have reported the significance of shells, husks, peels and skin of food crops in pesticide distribution and accumulation (Placido et al., 2013; Teixeira et al., 2004; Xu et al., 2012). In the current study, the shells were analyzed separately from the de-shelled beans (cocoa nibs) to examine insecticide distribution.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Insecticide standards of thiamethoxam (99.6%), clothianidin (99.9%) and imidacloprid (99.9%) as well as labelled internal standards (IS) of imidacloprid-d_4 (99.9%) and thiamethoxam-d_3 (98%) were all purchased from Sigma-Aldrich (Steinheim, Germany). Acetamiprid (98.1%) and thiacloprid (98.0%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

Prepackaged 12 mL tubes containing one of the following: (a) 4 g of $MgSO_4$ & 1 g of NaCl; (b) 6 g of $MgSO_4$ & 1.5 g of sodium acetate (NaOAc); (c) 4 g of $MgSO_4$, 1 g of NaCl, 0.5 g sodium citrate dibasic sesquihydrate (SCDS), 1 g of sodium citrate tribasic dehydrate (SCTD) were purchased from Supel QuE product lines, Sigma-Aldrich. Dispersive solid phase clean-up sorbent tubes (2 mL) containing 150 mg of $MgSO_4$ together with one of the following sorbents: (a) 50 mg PSA only (b) 50 mg PSA & 50 mg C-18; (c) 50 mg PSA, 50 mg C-18 & 50 mg GCB; (d) 50 mg Z-Sep+ were all purchased from Sigma-Aldrich. Sodium hydrogen

carbonate ($NaHCO_3$) and ammonium acetate (NH_4Ac) were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile and glacial acetic acid were obtained from Rathburn (Walkerburn, Scotland) and VWR (Fontenay-sous-Bois, France) respectively. Deionized water was prepared using a Merck Millipore Milli-Q advantage A10 ultrapure water purification system (Darmstadt, Germany).

3.2.2 LC-MS/MS instrumentation

Chromatographic separation of analytes was performed on an Agilent 1260 Infinity HPLC system (Santa Clara, CA, USA) equipped with a BDS Hypersil reversed-phase C-18 column (250 mm x 2.1 mm; 5 µm) (Thermo Electron Co., UK) at a temperature of 30°C. Mobile phase A and B consisted of 5 mM ammonium acetate and 95% acetonitrile in 5 mM ammonium acetate respectively. The gradient was run at 300 µL min^{-1} for 16 minutes as follows: 10% B increased linearly to 100% in 8 minutes; held constant for 2 minutes; decreased back to 10% B in 1 minute; and maintained for an equilibration time of 5 minutes. The injection volume was 5µL. The mass spectrometer used was a 3200 QTRAP (AB Sciex, Forest City, CA, USA) equipped with electrospray ionization (ESI). The MS determination of all analytes was performed in positive mode with multiple reaction monitoring (MRM) of the two most intense precursor-product ion transitions for each analyte, one used for quantification and the other for confirmation. The source parameters employed were: curtain gas (CUR) of 20 psi, collision gas (CAD) of medium pressure, ion spray voltage of 4500 V, source temperature of 475 °C and both nebulizer and heater gas (GS1 and GS2 respectively) of 60 psi each. Optimized values of compound dependent parameters: declustering potential (DP), exit potential (EP), collision energy (CE) and collision cell entrance potential (CEP) are listed in Table 12. Data obtained was processed using the Analyst software (version 1.6.2). Analytes were quantified from matrix-matched standard curves using peak area and a weighting of 1/x.

Table 12. Instrument conditions, MRM transitions of precursor/product ions of analytes.

Analyte	Ion transition (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)
Acetamiprid	223.10 → 126.20[q]	46.0	5.0	20.0	27.0
	223.10 → 99.00	46.0	5.0	20.0	49.0
Clothianidin	250.00 → 169.00[q]	41.0	4.5	26.0	19.0
	250.00 → 132.00	41.0	4.5	26.0	19.0
Imidacloprid	256.10 → 209.10[q]	41.0	5.0	18.0	17.0
	256.10 → 175.10	41.0	5.0	18.0	21.0
Imidacloprid-d_4	260.03 → 213.00[q]	51.0	6.5	24.0	23.0
	260.03 → 179.00	51.0	6.5	24.0	19.0
Thiacloprid	252.98 → 126.10[q]	56.0	7.5	18.0	27.0
	252.98 → 99.10	56.0	7.5	18.0	55.0
Thiamethoxam	291.95 → 211.00[q]	31.0	6.0	20.0	19.0
	291.95 → 181.00	31.0	6.0	20.0	27.0
Thiamethoxam-d_3	294.95 → 213.90[q]	31.0	6.5	16.0	19.0
	294.95 → 184.10	31.0	6.5	16.0	27.0

[q] Transitions used for quantification

3.2.3 Sampling

Fermented and dried cocoa beans were obtained from all the major cocoa growing regions of Ghana. Sampling locations are indicated in Figure 9. Samples were obtained from cocoa farmers, licensed buying centres and depots in the various regions, towns and villages. The number of samples obtained from each region was based on relative production of cocoa beans. In total, 86 samples from the 6 cocoa-growing regions of the country were obtained for the study. Organic cocoa bean samples were obtained from farmers in parts of the Volta region of the country where no agrochemical was said to have been used. In all locations, only fermented and dried beans were sampled.

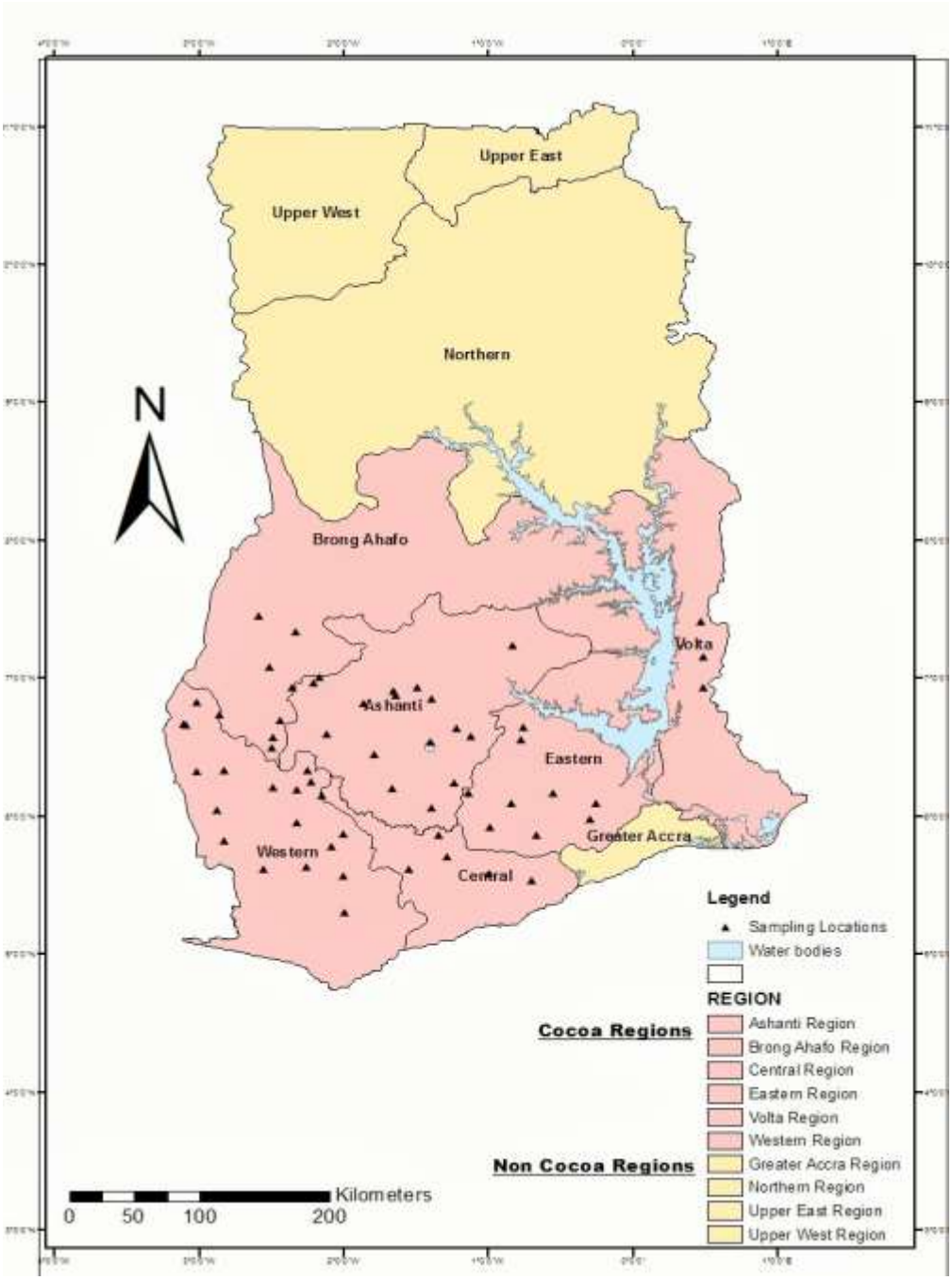

Figure 9. A map of Ghana showing cocoa-growing regions and locations of sampling.

3.2.4 Sample preparation

The fermented and dried beans were manually de-shelled and homogenized. Shells were homogenized separately. All samples were again air-dried at room temperature to a moisture content of < 5.0% to ensure uniformity of sample weight. When necessary, caked samples were disaggregated. The organic cocoa beans obtained were used as blank matrix samples for matrix

matching, method development and validation. Three grams of samples were used for method development and analysis. Recoveries at different levels of fortification: 10; 50; 100 and 200 µg/kg were prepared by spiking 3 g of sample with 30 µL of 1, 5 and 10 µg/mL and 60 µL of 10 µg/mL concentration of insecticide solution respectively. Samples were spiked with analytes and/or internal standards prior to acetonitrile extraction. For reagent blanks, 7 mL of deionized water was used without sample.

3.2.5 Sample extraction and Clean-up

Different QuEChERS extraction procedures were experimented with to determine the optimum procedure for the matrix under study. In each case, 3 g of sample was accurately weighed into 50 mL Falcon tubes and fortified with the appropriate standards for about 30 minutes. Approximately 7 mL of water was then added and allowed to soak up the matrix. Subsequently, 15 mL of acetonitrile was added and the mixture shaken for 2 minutes on a Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ, USA) at 1500 strokes/minute. After addition of salts (Table 13), the shaking process was repeated under the same conditions and the resulting mixture centrifuged at 4000 RPM for 5 minutes. Sample clean-up involved the addition of 1 mL of extract (from the acetonitrile layer) to various sorbent mixtures (Table 13) in pre-packed tubes to determine the most appropriate. The resulting mixture was shaken on a vortex mixer (Velp Scientifica, Usmate Velate, Italy) at 3000 RPM for 30 seconds and centrifuged at 4000 RPM for 5 minutes. Extracts were diluted 1:1 with water and filtered using a 0.22 µm PTFE syringe filter prior to injection onto the LC-MS.

Table 13. Salts and sorbents employed during the various experimentation under the QuEChERS procedure

Experiment 1	a (non-buffered)	b (acetate-buffered)	c (citrate-buffered)
Salts	4 g $MgSO_4$ & 1 g NaCl	6 g $MgSO_4$ & 1.5 g sodium acetate	4 g $MgSO_4$, 1 g NaCl, 0.5 g SCDS, 1 g SCTD

Experiment 2	a	b	c	d
Sorbents	50 mg PSA	50 mg PSA & 50 mg C-18	50 mg PSA, 50 mg C-18 & 50 mg GCB	50 g Z-Sep+

3.2.6 Validation of Procedure

Validation of an optimum QuEChERS procedure was performed by evaluating parameters including accuracy, precision, matrix effects, and limit of quantification (LOQ) based on the DG SANCO guidelines (SANCO/12571/2013, 2013). For each procedure, the accuracy and precision was evaluated by estimating percentage recoveries and relative standard deviations (RSDs) at four different fortification levels for all the analytes. The ruggedness of the procedures were assessed by examining the influence of various treatments such as manual shaking and use of Geno grinder, use of varying sample-solvent ratios and influence of pH by addition of base or acid. The linearity of the analytical procedures was studied using matrix-matched calibration solutions prepared in blank cocoa extracts. Calibration curves were plotted at 10 levels of concentration ranging between 1.56 and 800 μgL^{-1}. LOQ was estimated using matrix-matched standard solutions at the lowest concentration of analytes that yielded a signal to background noise (S/N) ratio of ten. Matrix influence was assessed by comparing responses of matrix-matched and solvent standards (Pizzutti et al., 2007). Absolute matrix effects was estimated by comparing the slopes in calibration solutions prepared in matrix and in solvents.

3.3 Results and discussion

3.3.1 Cocoa bean matrix

Cocoa beans are rich in polyphenols which are associated with color and flavor of the beans and their products (Niemenak et al., 2006). During fermentation, polyphenols in cocoa undergo oxidation and polymerization to form condensed higher molecular weight compounds, mostly insoluble tannins. This results in the dense brown pigmentation observed in fermented beans (Wollgast and Anklam, 2000). After centrifugation of the salting-out extracts of deshelled beans, four distinct layers are observed viz: supernatant acetonitrile layer; cocoa solids; cocoa fat; and salts at the bottom of the tube. In almost all cases, the layers of cocoa solids and cocoa fats had similar heights suggesting the high levels of fat (≈ 50%) often found in cocoa matrixes. The pigments together with the high fat content of the beans make the cocoa matrix quite complex, hence the need for an effective extraction and clean-up procedure. The QuEChERS procedure was adopted in this quest. The procedure involved an examination of the effects of different salts, sorbents, buffers and sample-to-solvent ratios on the extraction of analytes from the matrix.

3.3.2 Salting-out Extraction

The addition of salts under the original QuEChERS procedure was primarily to ensure phase separation. However influence of pH on analytes in resulting extracts has been utilized in current modifications of the original method, involving the use of buffering salts to control pH and ionic strength of matrixes, particularly for pH sensitive analytes. In our study, all three salting out procedures commonly used under the QuEChERS methodology were examined for their effectiveness in analyte extraction with minimal co-extractives. Pulverized samples were extracted with salts of sodium acetate (acetate buffered procedure), sodium citrate (citrate buffered procedure) and sodium chloride (non-buffered/original procedure) to produce

approximate matrix pH values of 7.2; 4.1; and 4.9 respectively. It was observed that, the acetate buffered extracts showed significantly cleaner acetonitrile layers compared to the other two salt extracts (Figure 10). Pigments appeared to be concentrated in the lower fat layer leading to a visibly cleaner acetonitrile upper layer. Further test involving the addition of varying amounts of $NaHCO_3$ during the salting out procedure showed the dependence of this occurrence on the pH of the matrix. This apparent "de-pigmentation" of the acetonitrile layer upon increasing the pH of the matrix was observed for all salting-out procedures. In effect, increasing pH of the extraction medium seemed to produce cleaner extracts.

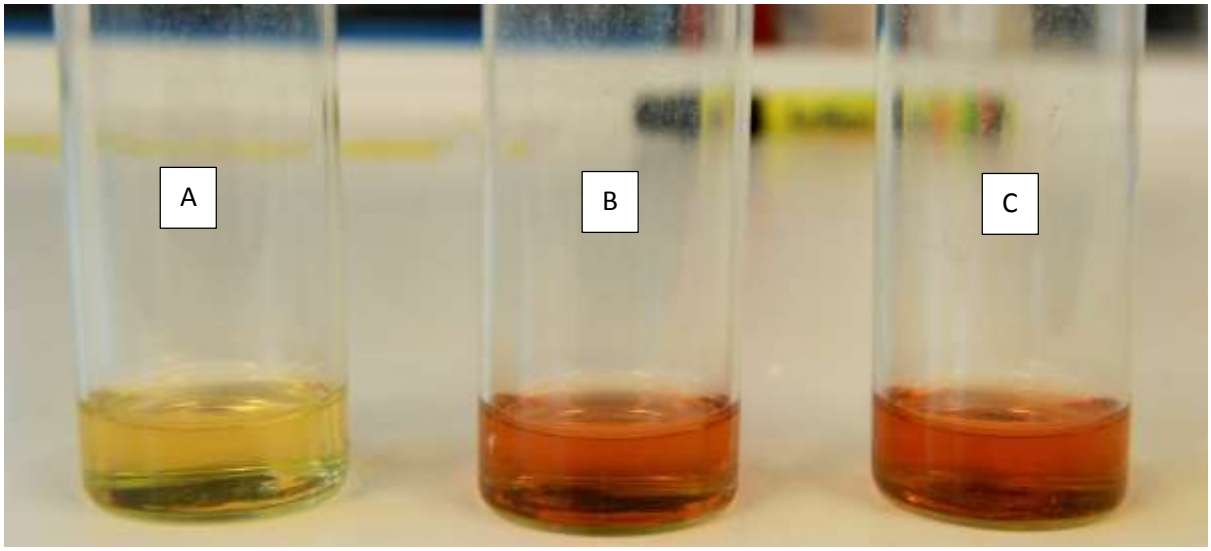

Figure 10. Visual appearance of extracts produced from acetate (A), unbuffered (B) and citrate (C) salting out procedures.

3.3.3 Matrix co-extractives

To ascertain the visually observed differences in matrix composition of extracts from the three salting out procedures, matrix co-extracts were examined using gravimetric measurements (Anastassiades et al., 2003; Lehotay et al., 2010). Five millilitres of extract from each procedure was measured into pre-weighed glass tubes and extracts dried using a gentle stream of nitrogen

gas and then heated in an oven at 105 °C for one hour. The new weight of the glass tubes was measured. The weight of co-extractives was estimated from the difference in weights (Figure 11). From the results obtained, the acetate buffered procedure showed markedly lower matrix co-extractives compared to both the citrate and the unbuffered method, confirming the visual observation.

At high pH, cocoa polyphenols are readily transformed and polymerize into higher molecular weight insoluble brown pigments (Li et al., 2013). This transformation is observed during alkalization (Dutching) of cocoa as often occurs during industrial processing (Miller et al., 2008).

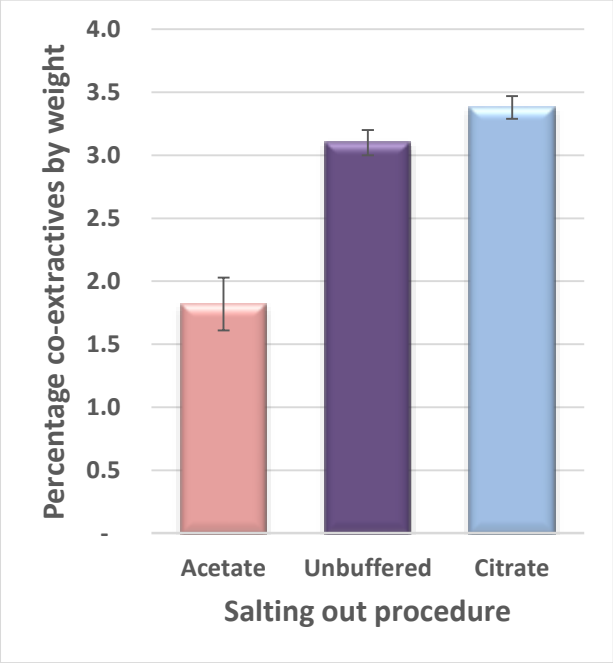

Figure 11. Percentage co-extractives by weight of sample based on the three QuEChERS salting out procedures, reported with standard error. Two replicates were performed for each procedure.

In this study, the extent of polymerization appears to increase with increasing pH of the medium. The newly formed high molecular weight pigments then precipitate out of solution and fall to the lower layers (consisting of cocoa fat and cocoa solids), thereby resulting in a cleaner upper acetonitrile layer with less co-extractives. Other studies have also observed a

decrease in fatty acid co-extractives upon increasing pH of the extraction matrix (Anastassiades et al., 2003). Nonetheless, the decreased stability of some pesticides at higher pH warrants the need for a good balance between matrix co-extractives and analyte stability. In our study, we observed that the extraction of analytes with 1% acetic acid in acetonitrile resulted in a reduction in pH values of both acetate and unbuffered extracts by approximately 1 unit each, with a corresponding increase in matrix co-extractives of about 14%. The effect on citrate buffered extracts was negligible. However, the stability of neonicotinoids over the pH range being investigated did not warrant the addition of acids (Guzsvány et al., 2006). The acetate buffered procedure in acetonitrile seemed to provide the right balance for the analytes and matrix under study.

3.3.4 Clean-up Procedure

One of the advantages of using acetonitrile under the QuEChERS procedure in the extraction of analytes from high fat matrixes is the relatively low matrix co-extraction due to low solubility of lipids in acetonitrile as compared to other solvents (Anastassiades et al., 2003; Koesukwiwat et al., 2010). That notwithstanding, the use of a clean-up procedure is essential to ensure the removal of the inevitable lipid co-extracts in high fat matrixes such as cocoa beans. The high pigmentation of the cocoa matrix adds to the complexity and contributes to the greater need for a clean-up step.

In this study, the ability of PSA; PSA+C18; PSA+C18+GCB and Z-Sep+ sorbents in removing matrix co-extracts during the dispersive solid-phase clean-up step without significant effects on analyte recovery was compared. Five millilitres of extracts from the various clean-up sorbents was used in gravimetric measurements as already described. The results suggest PSA+C18+GCB as the most effective sorbents/sorbent mixture for matrix clean-up based on visual inspection (Figure 12) and gravimetric measurements (Figure 13). While PSA is important in the retention of fatty acids and other polar components, C-18 is essential for the

removal of lipids and non-polar co-extracts particularly in high fat matrixes. In our study, GCB was found to be crucial for the total removal of visible pigments, and resulted in significantly lower co-extractives with no observed effects on analyte recovery.

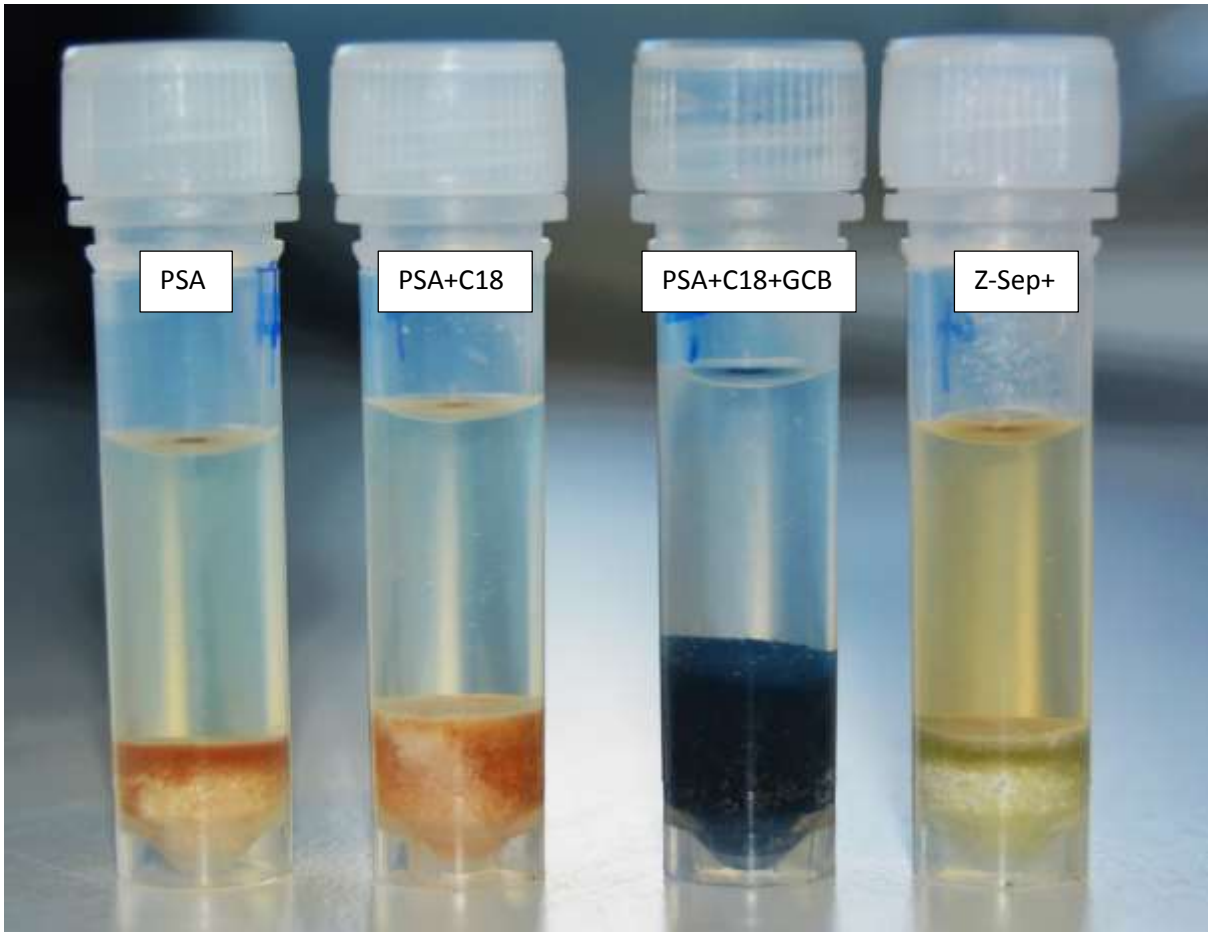

Figure 12. Extracts obtained after dispersive solid-phase clean-up of salting out extracts using various sorbents. Only extracts from the acetate salting out procedure are shown.

GCB has a strong affinity for pigments such as polyphenols, carotenoids and chlorophyll often found in food matrixes (Anastassiades et al., 2003). Moreover, its affinity for planar molecules including pesticides was not observed in this study. In the matrix under study, it was observed that, GCB could be avoided when higher solvent-to-sample ratios (≥ 8:1) or higher matrix pH were employed. Z-Sep+, a relatively new sorbent marketed for its ability to remove fat in high

fat matrixes appeared to be least effective at removal of co-extractives in the matrix under study, which appeared to be predominantly polyphenols (pigments).

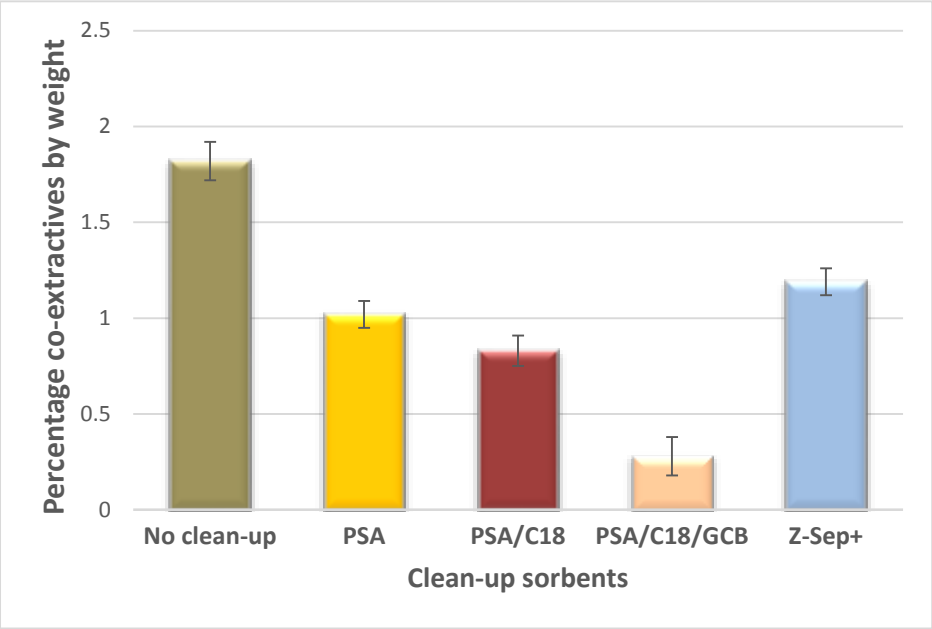

Figure 13. Effect of different sorbent on clean-up efficiency of cocoa matrix. Extract was obtained from the acetate buffered procedure. Two replicates each was performed for each study, reported with their standard error.

3.3.5 Optimum QuEChERS procedure

Based on the nature of samples employed in this work, it was evident that the degree of pigmentation in the cocoa matrix varied widely, perhaps due to the variety or pattern of fermentation of the beans. As such, samples showed different shades of pigmentation ranging from light brown to deep brown coloration. In the development of an optimum procedure for extraction and clean-up of analytes, it was ensured that the most deeply pigmented samples were employed to adequately address the issue of pigmentation. As such results obtained in this study may represent some of the highest level of interference that may arise from the cocoa matrix.

Despite the high fat content of the matrix studied, it was quite clear that, the majority of fat was excluded from the extract, based on very low solubility in acetonitrile. The high polarity of

analytes studied also ensured their effective partitioning in the acetonitrile layer and ensured high recovery. Pigmentation of the matrix appeared to have a major influence on extracts and was addressed by the control of pH and use of sorbents particularly GCB. Ultimately, a choice of acetate buffered salting out procedure in acetonitrile and clean-up using a mixture of PSA+C18+GCB sorbent was made as it better reflected accuracy, precision and cleaner chromatograms for all analytes.

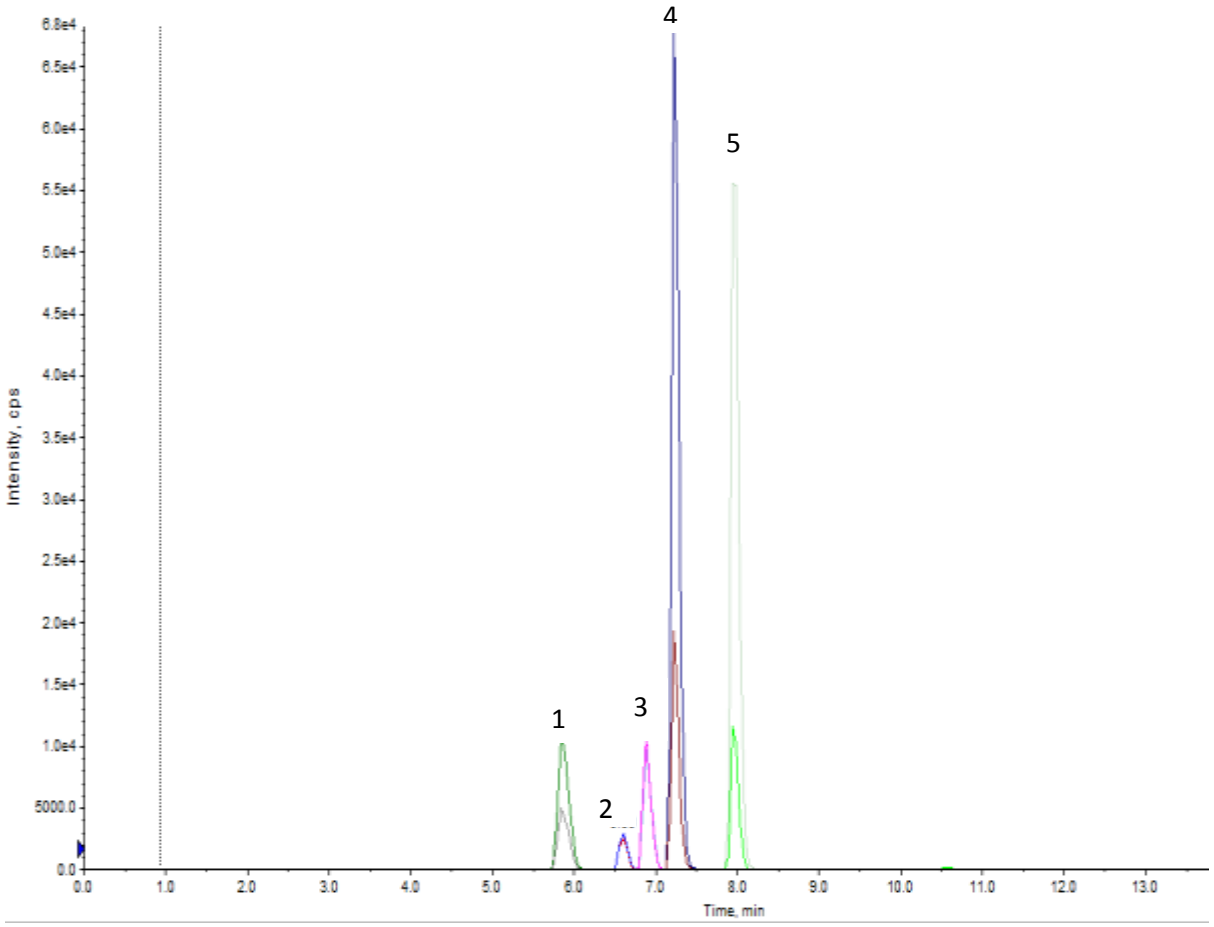

Figure 14. A chromatogram of a blank cocoa sample spiked at a concentration of 100 µg/kg. 1 - Thiamethoxam; 2 - Clothianidin; 3 -Imidacloprid; 4 -Acetamiprid; 5 –Thiacloprid

3.3.6 Validation of QuEChERS procedure

The performance of the chosen procedure was assessed by evaluating parameters including accuracy, precision, linearity, matrix effects and LOQ. Limit of quantitation (LOQ) was

calculated as 10 times the signal-to-noise ratio of analytes using matrix-matched standards. In all analytes, very good linearity ($R^2 \geq 0.998$) were observed using matrix-matched calibration standard solutions of concentrations ranging from 1.56 to 800 µg L^{-1}. Multiple recovery experiments were conducted at several levels of fortification by spiking of samples at different concentrations of analytes. Five replicates were employed at each level of fortification for all analytes. As shown in Table 14, good recoveries (92-111%) for all analytes and at all levels of fortification with good precision (2≤ RSD ≤16) were obtained. As expected, precision was higher at higher levels of fortification.

Table 14. Average percentage recovery of analytes reported with their RSDs at 4 levels of fortification. Samples were extracted using NaOAc and $MgSO_4$ and clean-up using a mixture of PSA+C18+GCB. Five replicates were analysed at each level of fortification.

Analyte	Level of fortification (µg/kg)			
	10	50	100	200
Acetamiprid	93 ± 7	96 ± 4	96 ± 3	95 ± 2
Clothianidin	92 ± 15	98 ± 9	95 ± 6	95 ± 6
Imidacloprid	111 ± 10	101 ± 6	95 ± 6	97 ± 4
Thiacloprid	97 ± 7	100 ± 4	92 ± 5	92 ± 3
Thiamethoxam	95 ± 16	103 ± 7	100 ± 5	97 ± 2

Matrix influence in the chosen procedure was estimated by comparing the slopes of matrix-matched standards and solvent standards (Romero-González et al., 2011). The effect of matrix was found to be minimal for all analytes studied (Table 15). During method development, significant matrix influence was observed at the sample-solvent ratio of 1:1 commonly used in the QuEChERS procedure, albeit at high water content of samples (≥ 80%). Matrix influence was characterized by > 100% recoveries and low precision in most analytes (Figure 15). This influence was observed in all salting-out extracts in spite of clean-up procedures, except when Z-Sep+ sorbents were used. The Z-Sep+ sorbents produced good recoveries albeit in a dirty

(highly pigmented) matrix, suggesting the greater influence of lipid co-extractives in the MS. With increased solvent-sample ratios (≥ 5:1) matrix influence were significantly reduced with cleaner chromatograms and good recoveries as shown in Table 15. The advantages of increased solvent-sample ratios has already been observed by Chamkasem et al, (2013) (Chamkasem et al., 2013). In this study, a solvent-sample ratio of 5:1 was found to be optimum in ensuring clean matrixes and good efficiency in solvent usage. Notwithstanding the low matrix influence as a results of higher solvent-sample ratio, all quantitation were performed in matrix-matched standards and with the help of internal standards. Nevertheless all quantitation were performed in matrix-matched standards and with the help of internal standards.

Table 15. Retention time, linearity, matrix effects and limit of quantitation of analytes.

Analyte	RT (min)	Linearity (R^2)	Matrix effect*	LOQ (µg/kg)
Acetamiprid	7.56	0.999	0.97	< 5
Clothianidin	6.85	0.999	0.97	10
Imidacloprid	7.16	0.999	0.96	10
Thiacloprid	8.26	0.999	0.98	< 5
Thiamethoxam	6.13	0.998	0.96	10

* Matrix effect expressed as a ratio of the slopes of the calibration curves obtained from solvent and matrix-matched standards

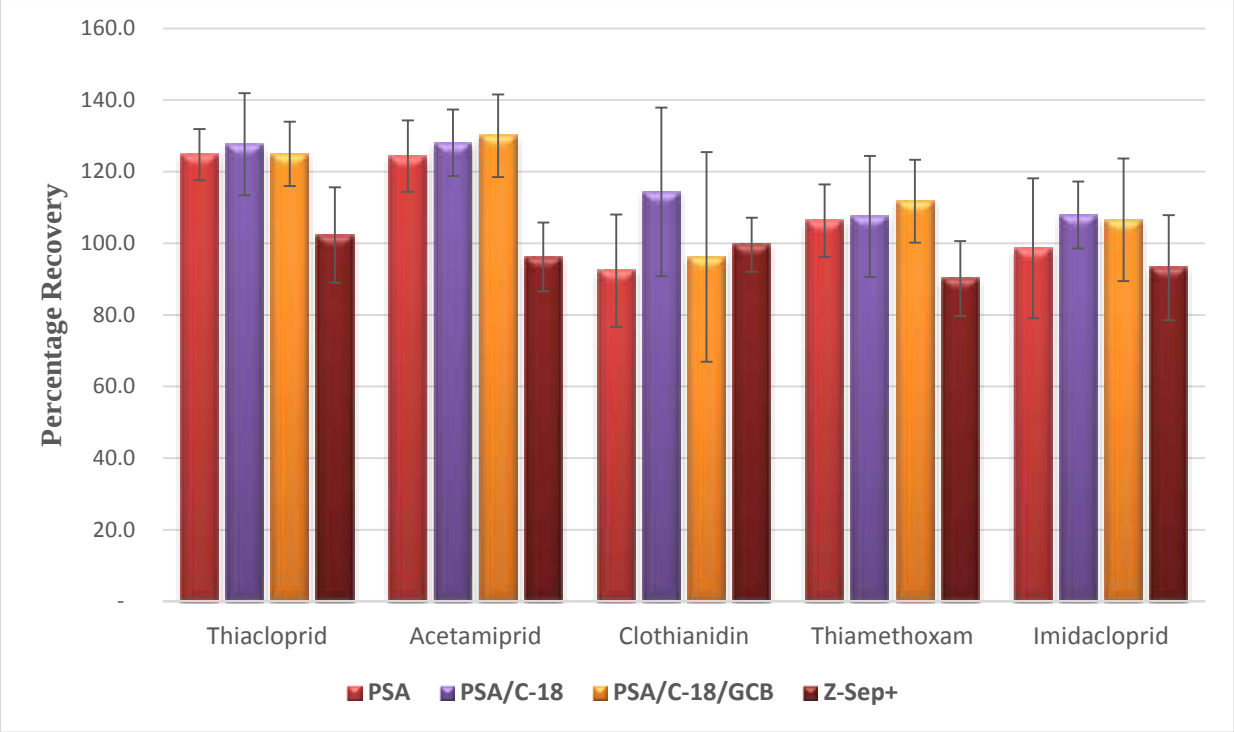

Figure 15. Influence of matrix at low solvent-sample ratio (1:1). Analytes from acetate extracts (5 replicates) are shown. Error bars represent RSD. Results are characterized by low precision (high RSDs) and over-recoveries (> 120%) particularly in thiacloprid and acetamiprid

3.3.7 Application of procedure to neonicotinoids in cocoa beans and shells

A total of 86 samples from all cocoa-producing regions of Ghana were extracted in acetonitrile with NaOAc & $MgSO_4$, cleaned-up using PSA+C18+GCB and quantified using the LC-MS/MS. Shells and deshelled beans (hereafter referred to as nibs) from the same sampling lot were analyzed separately to examine the distribution of neonicotinoids in both food parts. From the results (Table 16), imidacloprid was found to be present in a greater number of samples and in higher concentrations compared to all other insecticides studied. It was quantified in more than 10% of nibs and 30% of shell samples studied with concentrations of up to 35.6 and 214 µg/kg respectively. The high frequency may reflect its high popularity and usage among farmers in the country compared to other insecticides. Acetamiprid was quantified in one sample of cocoa nib and 2 samples of shell, being present in both nib and shells from Achimfo.

Thiamethoxam was quantified in only two shell samples, whereas clothianidin and thiacloprid were both below the limits of quantification in all samples studied.

Table 16. Concentrations (µg/kg) of neonicotinoids present in deshelled beans (cocoa nib) and cocoa shell samples. Only samples with concentrations above the limit of quantification (LOQ) have been presented.

	Imidacloprid		Thiamethoxam		Acetamiprid	
Sampling town	Nibs	shells	Nibs	Shells	Nibs	Shells
Abochia	16.1	29.4	-	-	-	-
Achimfo	-	15.9	-		12.4	31.1
Agona Amenfi	-	23.7	-	-	-	-
Anwiaso	-	13.4	-	-	-	-
Anyinam	-	11.8	-	-	-	-
Anyinasuso	15.9	30.6	-	-	-	-
Asankragua	-	14.5	-	-	-	-
Asawinso	16.7	28.9	-	-	-	-
Dadieso	-	21.1	-	-	-	-
Dunkwa	-	22.7	-	-	-	-
Enchi A	35.6	214.0	-	-	-	12.9
Enchi B	-	22.7	-	-	-	-
Hohoe	24.7	55.4	-	-	-	-
Insu-Bogosu	-	12.1	-	-	-	-
Juaso	11.5	26.6	-	24.7	-	-
Manso Amenfi	17.2	89.6	-	-	-	-
Nkawie	-	16.5	-	-	-	-
Nkawkaw	-	12.8	-	-	-	-
Oda	-	13.4	-	-	-	-
Samreboi A	-	15.2	-	-	-	-
Samreboi B	-	22.6	-	39.6	-	-
Sehwi Bekwai	-	15.0	-	-	-	-
Simpa	-	20.8	-	-	-	-
Tarkwa	-	24.3	-	-	-	-
Wassa Akropong A	17.9	30.5	-	-	-	-
Wassa Asikuma	17.1	29.4	-	-	-	-

From the results obtained, the levels of neonicotinoids in the two cocoa parts suggest a selective accumulation in shells compared to nibs. All neonicotinoids that were quantified showed this tendency. Of the 10 samples where a particular neonicotinoid was present in both nib and shell,

levels in shells were 1.7 to 6 times the levels in nibs. For instance, imidacloprid appeared to be quantified (≥ 10 µg/kg) in nibs only when levels present in shells were in excess of about 26 µg/kg. Moreover, thiamethoxam was present only in shell samples (2) and despite relatively high concentrations of 24.7 and 39.6 µg/kg, concentrations in deshelled beans were below LOQ (10 µg/kg). Thus the deshelling of beans during processing may not only enhance the properties of processed cocoa but may also serve as a good decontamination measure.

In the cocoa processing industry, restrictions of up to 1.75% (by weight) of shells allowed in cocoa nibs have been set in the USA (Code of Federal Regulation, CFR 21, sec. 163.110) and by Codex (codex stan 141-1983, rev. 2001, Amen 2014). In the EU however, former limit of up to 5% shell content has not been retained in new regulations (2000/36/EC). That notwithstanding, it is evident from our study that, knowledge of insecticide residue levels in cocoa shells may give a good indication of the extent of application of chemicals and potential contamination of the main edible portion (nibs) of the beans.

The selective distribution of pesticides in skin and pulp of food crops has been observed in literature (Placido et al., 2013; Teixeira et al., 2004; Xu et al., 2012). However, the relevance or otherwise of cocoa shells for nutritional and toxicological purposes is underscored in differences in policy formulation on pesticide residue levels among various countries. In the EU and the USA, residue or tolerance limits refer to deshelled beans ("edible portion"), whereas in Japan and Australia, limits are applicable to whole bean.

In spite of the relatively low frequency and levels of neonicotinoids in cocoa nibs, our study suggests that neonicotinoids may accumulate to relatively high levels in cocoa beans. This perhaps may be due to the widespread and intensive application rates of these insecticides in cocoa farms across the country. From this study however, knowledge of neonicotinoid application levels and rates on farms, and their corresponding levels in cocoa samples could

not be directly established since a particular sample may comprise beans from multiple farms and multiple farmers in the various towns and villages.

Over the past few decades, organochlorines have been the major insecticides used in cocoa farms in Ghana (Frimpong et al., 2012; Owusu-Ansah et al., 2010). In recent years however, the use of newer pesticides particularly neonicotinoids with perceived less detrimental effects have been highly endorsed and widely used without knowledge of their fate in food and the environment. Currently, imidacloprid, thiamethoxam and thiacloprid are approved for use in cocoa plantations and are recommended to be applied four times each year during the months of August, September, October and December when the mirid population is believed to be particularly high.

Until now, knowledge of their concentrations in cocoa beans did not exist. In general, available literature on the levels of pesticide residues in cocoa beans remain low. In a recent study by Zainudin et al (2015) on pesticide levels in whole beans from different countries including Ghana, concentrations of 3 pesticides (chlorpyrifos, amethryn and metalaxyl) out of 12 studied ranged from 10-200 µg/kg (Zainudin et al., 2015). In a similar study of synthetic pyrethroids and organophosporus pesticide residue levels in whole beans from Ghana, concentrations ranging from 5.0 – 105.0 and 5.0 – 133.0 µg/kg respectively have been reported (Frimpong et al., 2012b, 2012c). These relatively high concentrations of insecticides suggest an increased tendency of build-up in cocoa beans.

Globally, neonicotinoids are registered in more than 120 countries and are currently the most widely used class of insecticides (Jeschke et al., 2011). In Ghana's cocoa production, neonicotinoids are widely applied in cocoa plantations for the treatment of mirids. The widespread usage is as a result of a free insecticide application policy by the government under which neonicotinoids particularly imidacloprid is not for sale but given to cocoa farmers at no

cost or directly applied on farms. Currently, imidacloprid, thiamethoxam and thiacloprid are approved for use in cocoa plantations and are recommended to be applied four times each year during the months of August, September, October and December when the mirid population is believed to be particularly high. According to Adu-Acheampong et al (Adu-Acheampong et al., 2014) however, these recommendations are largely based on research performed several decades ago with less current usefulness. In a recent study on soils from cocoa farms in Ghana, it was evident that, neonicotinoids were prevalent in soils several months after their application (Dankyi et al., 2014). While the levels in the soils studied may be due to persistence, repeated application in cocoa farms may play a major role and ultimately in their levels in cocoa beans and shells.

Neonicotinoids have broad spectrum activity including contact. However, they act mainly as systemic insecticides in plants based on their favorable physicochemical properties (Tomizawa and Casida, 2005). Once applied, they may be absorbed into plant tissues through the leaves, roots and other plant parts and tend to provide long-term systemic activity against piercing and sucking insects such as mirids (Buchholz and Nauen, 2002; Shi et al., 2011). The presence and levels of neonicotinoid residues obtained in the cocoa beans studied may suggest their presence in other plant parts such as the stem and leaves, and hence protection through systemic activity. Thus it is suggested that, repeated monthly application of neonicotinoid insecticides as currently recommended in cocoa farming in Ghana may not be very efficient.

In the EU, current maximum residue levels for all neonicotinoids studied in fermented cocoa beans (deshelled or edible part) is 50 µg/kg ("EU Pesticides database," n.d.). The limit for thiamethoxam and clothianidin (a metabolite of thiamethoxam) are expressed together as thiamethoxam with same residue limit of 50 µg/kg. Despite the lower concentrations of

residues in deshelled beans found in this study, the need for greater efficiency in the application and management of neonicotinoid insecticides in cocoa farming is evident.

3.5 Conclusion to chapter 3

The QuEChERS procedure has been successfully optimized and applied in the extraction and clean-up of neonicotinoid insecticides from cocoa beans, a complex matrix with high fat and high pigments. Sample extraction in acetonitrile using a solvent sample ratio of 5:1 and clean-up using a sorbent mixture of PSA/C18/GCB were found to be the optimum conditions for the matrix. The availability of different salting out/buffering procedures shows that, the procedure can be adapted to other analytes based on the pH sensitivity. While clean-up with PSA/C18/GCB ensured good matrix cleanliness and low interference, the use of Z-Sep+ appears promising for high fat matrixes. The chosen procedure enables low detection and quantitation (5-10 µg/kg) limits for neonicotinoids in the cocoa matrix using LC-MS/MS. The current study has demonstrated the likelihood of accumulation of neonicotinoid insecticide residues to relatively high levels in cocoa shells. This may be due to intense application. Based on this study, greater efficiency in neonicotinoid application is recommended in order to avoid the possible build-up of these insecticides to unsafe levels in cocoa beans.

4. Soil dissipation of neonicotinoid insecticides

4.1 Introduction to chapter 4

This chapter discusses the dissipation of imidacloprid and thiamethoxam, the two most widely used neonicotinoids, in soil. Dissipation half-lives (DT_{50}) of both compounds and their deuterated isotopes were determined. Finally, the formation of possible metabolites in soils was examined.

4.1.1 Dissipation of neonicotinoids in soils

On the one hand, pesticides are aimed at being stable enough in the environment so as to reach and control the target pest. On the other hand, their effect should preferably be easily removed from the environment and non-target organisms immediately after their action on pests. Meeting this high selectivity and environmental toxicity requirements presents a huge challenge to the pesticide industry whenever new active compounds are developed. The introduction of neonicotinoids appeared to have gone a long way in addressing this challenge. In the development of imidacloprid, the first neonicotinoid, its precursor (2E)-2-(nitromethylidene)-1,3-thiazinane (commercial name: Nithiazine) was found to exhibit high potency, good plant systemic activity and low toxicity to mammals, but also showed substantial photolytic and hydrolytic instability under field conditions (Jeschke and Nauen, 2008). Together with other structural changes, the introduction of an N-nitro-imino chromophore with lower light absorption led to the discovery of imidacloprid, which had improved field stability and enhanced activity. Similar to the creation of imidacloprid, further chemical adaptations of the chromophore based on light absorption led to the discovery of thiacloprid, creating a new era of insecticides.

Under field conditions however, the complex environment-pesticide interactions often lead to variability in field persistence and degradation rates. In general, variability in dissipation rates

of pesticides is expected due to factors including geography, climate differences and soil-pesticide interactions. Ford & Casida (2008) have observed that, the fate of a pesticide is dependent on its chemical and physical properties and the environment to which it is exposed (Ford and Casida, 2008a). Factors that influence persistence can be considered to belong to four overall categories: The intrinsic properties of the chemical compound, the climate, the soil and its properties, the method and the amount of chemical compound applied (Børgesen et al., 2015). In neonicotinoids, evidence points to a huge variability making estimations and prediction of persistence either difficult or inclusive (Goulson, 2013). As such, neonicotinoids have been labelled as environmentally persistent (PMRA, 2001). In general, persistence is controlled by degradation which may be chemical, biological or photo-induced, but also influenced by environmental mobility involving leaching, sorption, run-off, volatilization and uptake by plants. In most published reports, the rate of dissipation of parent pesticide is often used as a non-specific measure of persistence.

An area of increasing concern and research has been degradation or transformation of pesticides into new products in the environment. The interest has been due to the fact that these products may accumulate to relatively high levels, and while generally of lower toxicity to biota, evidence have shown equal or greater toxicity of some degradation products compared to the parent pesticide. A typical example is clothianidin insecticide which is a known metabolite of thiamethoxam (precursor) (Nauen et al., 2003). In imidacloprid, a number of metabolites including the olefin, nitroso and hydroxyl have been found to exhibit good bio-efficacy against insect species including Myzus persicae and Aphis gossypii (Homoptera:Aphididae) (Nauen et al., 1998). For instance, the olefin metabolites were observed to be 10 times more active than the parent imidacloprid against cotton whitefly Bemisia tabaci in oral ingestion bio-assays (Nauen et al., 1999). As a result, the toxicology of neonicotinoid insecticides in mammalian or insect systems are found to be a summation of that

of the pesticide and its metabolites (Ford and Casida, 2008b). Consequently, these metabolites may ensure long-term protection of plants in spite of decreasing concentrations of the parent insecticide itself.

For almost all neonicotinoids, metabolic pathways in plants, animal systems and the environment may include nitro reduction, cyano hydrolysis, demethylation, sulfoxidation, imidazolidine and thiazolidine hydroxylation and olefin formation, oxadiazine hydroxylation and ring opening, and chloropyridinyl dechlorination (Figures 16 & 17) (Ford and Casida, 2008b). This multiplicity and complexity of transformation products of diverse physical and chemical properties and hence differences in environmental behavior is of even greater concern (Casida, 2011). Owing to possible differences in toxicity compared to the parent pesticide and the possible implications for non-target organisms in the environment, there is a greater need for a deeper knowledge of transformation products.

In recent years, degradation of neonicotinoids by microorganism has been of special interest due in parts to reported persistence and the implications of microorganisms for environmental safety and bioremediation of contaminated soils (Anhalt et al., 2007; Liu et al., 2011). Recent reports have suggested significant degradation of neonicotinoids by microorganisms including bacteria and fungi. Sabourmoghaddam et al, (2014) have reported over 25-45% decrease in imidacloprid concentrations over 25 days in tropical soils from Cameron (Sabourmoghaddam et al., 2014). In a similar study under tropical conditions (30 °C), isolated strains of bacteria have been found to degrade imidacloprid up to 78% over 7 days (Phugare et al., 2013).

Largely, the focus has been on the transformation of the parent insecticide with little focus on metabolites and their subsequent transformation by organisms. Nonetheless, microbial transformation of neonicotinoid is seen as key in ensuring remediation of neonicotinoids in soils particularly due to reported high persistence in various soils.

While a great deal of work has been undertaken on dissipation of neonicotinoids in soils, no generalized conclusions has been possible due to huge variability in rates. For a deeper understanding of their fate, a larger and wider assessment of these insecticides under different climatic conditions and different soils types is needed. This may enable better extrapolation and prediction to a higher degree of accuracy and ensure the safe use of these insecticides. In the current study, dissipation of imidacloprid and thiamethoxam in tropical soils from Ghana were studied to estimate half-lives and possible metabolites.

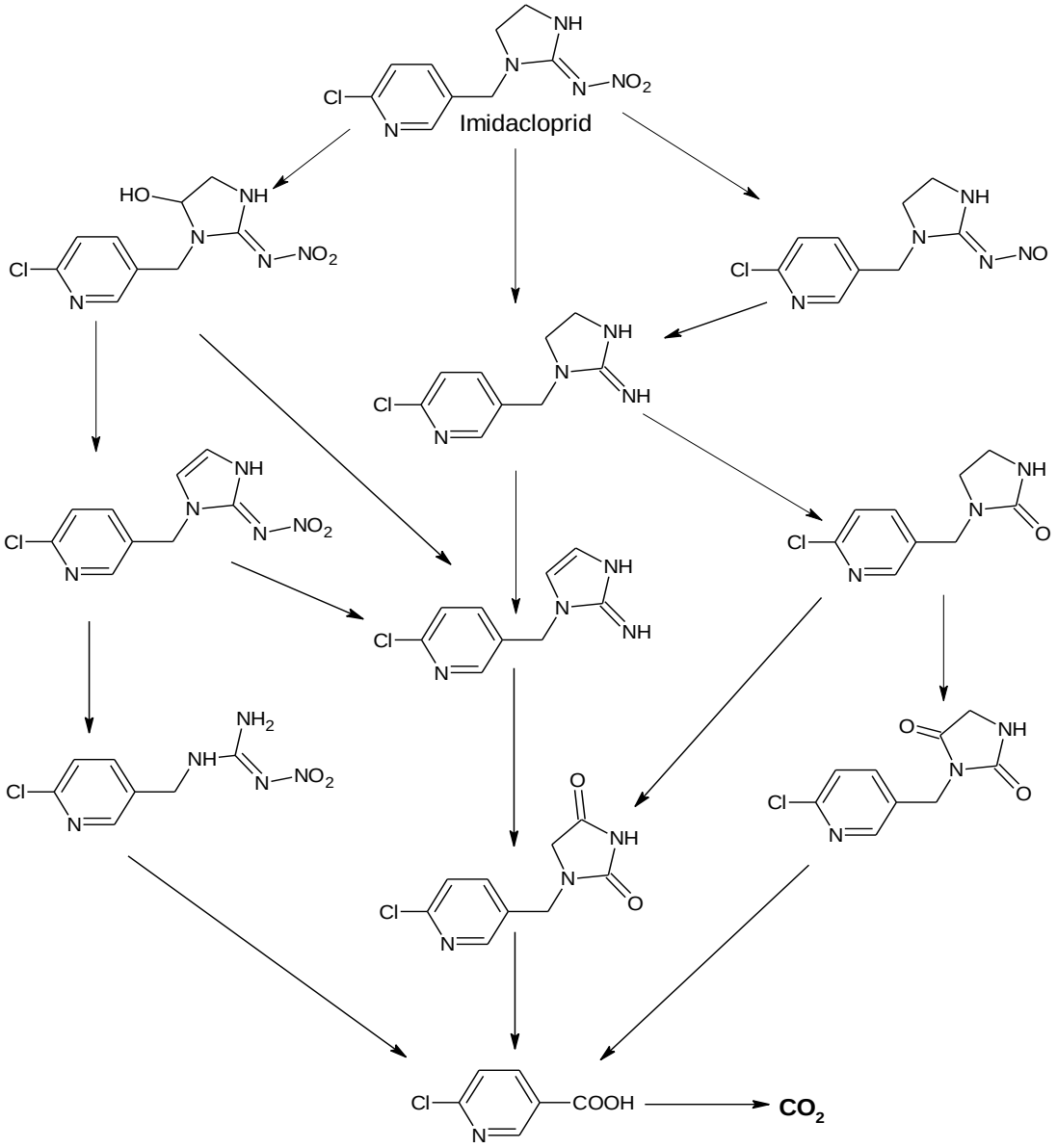

Figure 16. Proposed degradation of imidacloprid in soil (Fossen, 2006)

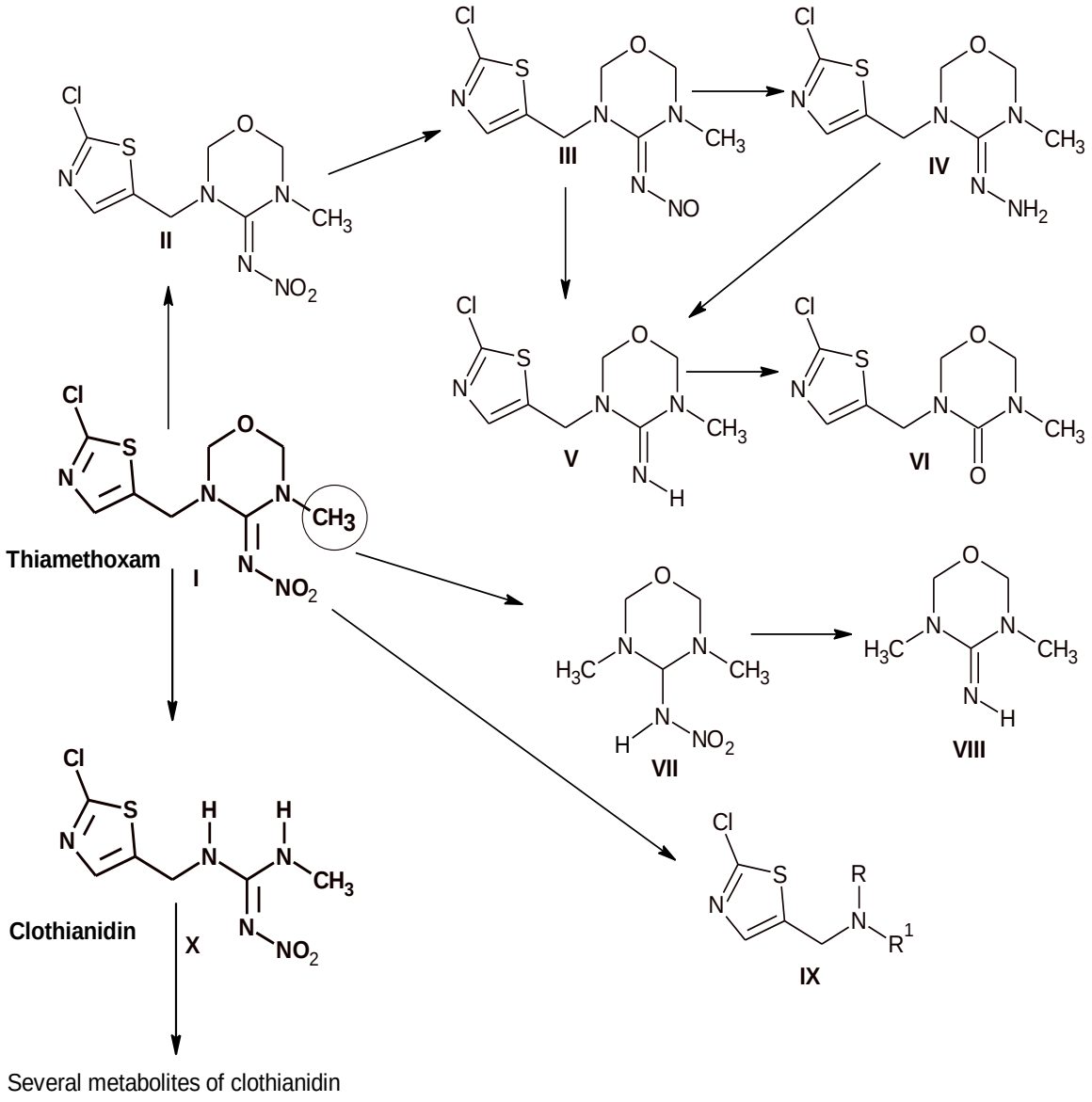

Figure 17. Proposed partial metabolite pathway of thiamethoxam (and clothianidin) in mice. Redrawn from Ford and Casida (2006) (Casida, 2011).

4.2 Materials and Methods

4.2.1 Reagents and chemicals

Much of the materials used for this experiment has already been described in materials and methods in chapter 3. Insecticide standards of thiamethoxam (99.6%) and imidacloprid (99.9%) as well as deuterium labelled standards of imidacloprid-d_4 (99.9%) and thiamethoxam-d_3 (98%) were all purchased from Sigma-Aldrich (Steinheim, Germany).

Pre-packaged 12 mL tubes containing 4 g of $MgSO_4$ & 1 g of NaCl was purchased from Supel QuE product lines, Sigma-Aldrich. Inert Ottawa sand (particle size: 20-30 mesh) was obtained from Fischer Scientific (Leicestershire, UK). All solvents used were of HPLC grade. Acetonitrile were obtained from Rathburn (Walkerburn, Scotland). Deionized water was prepared using a Merck Millipore Milli-Q advantage A10 ultrapure water purification system (Darmstadt, Germany).

4.2.2 LC-MS/MS instrumentation

Instrumentation is as similarly described in previous chapters with only minor changes. Chromatographic separation was performed using a BDS Hypersil reversed-phase C-18 column (250 mm x 2.1 mm; 5 µm) (Thermo Electron Co., UK) at a temperature of 30°C. The mobile phase consisted of 5 mM ammonium acetate and 95% acetonitrile in 5 mM ammonium acetate for solvents A and B respectively. The gradient was run at 300 µL min^{-1} for 12 minutes as follows: 20% B increased linearly to 100% in 5 minutes; held constant for 2 minutes; decreased to 20% B in 1 minute; and maintained for an equilibration time of 4 minutes. The injection volume was 10 µL. The mass spectrometer used was a 3200 QTRAP (AB Sciex, Forest City, CA, USA) equipped with electrospray ionization (ESI). The MS determination of all four analytes was performed in positive mode with multiple reaction monitoring (MRM) of the two most intense precursor-product ion transitions for each analyte. Optimized values of compound dependent parameters: declustering potential (DP), exit potential (EP), collision energy (CE) and collision cell entrance potential (CEP) are listed in Table 17. Data obtained was processed using the Analyst software (version 1.6.2).

Table 17. Instrument conditions and MRM transitions of precursor/product ions of analytes.

Analyte	Ion transition (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)
Imidacloprid	256.10 → 209.10[q]	41.0	5.0	18.0	17.0
	256.10 → 175.10	41.0	5.0	18.0	21.0
Imidacloprid-d_4	260.03 → 213.00[q]	51.0	6.5	24.0	23.0
	260.03 → 179.00	51.0	6.5	24.0	19.0
Thiamethoxam	291.95 → 211.00[q]	31.0	6.0	20.0	19.0
	291.95 → 181.00	31.0	6.0	20.0	27.0
Thiamethoxam-d_3	294.95 → 213.90[q]	31.0	6.5	16.0	19.0
	294.95 → 184.10	31.0	6.5	16.0	27.0

[q] Transitions used for quantification

4.2.3 Sample pre-treatment

A representative soil sample based on a large variety of tropical soils studied in chapter 2 was used for the study. Soils were sampled from a depth of 0-20 cm, air dried and passed through a 2mm sieve prior to analysis. Using a paper cone riffle splitting technique (Gerlach et al., 2002), about 4 kg soil sample was split into 256 different subsamples of approximately 15 g each. Splitting of samples into various subsamples was performed to ensure sample homogeneity in all the subsamples being studied. Each splitting produced 8 subsamples. Samples used for analysis were obtained from 3 consecutive splitting. After the second split, 64 subsamples obtained were combined to obtain 32 samples and further split into 8 each to obtain the 256 subsamples used for the experiment. Nonetheless, homogeneity of samples were tested using replicates of samples on specific dates.

Figure 18. A paper cone riffle splitter used in generating subsamples. Each sample splitting produces 8 subsamples (splits).

4.2.4 Incubation of samples

Five grams of inert Ottawa sand was measured into an Erlenmeyer flask and spiked with 1.0 mL of 10 µg/mL standard solution of insecticide in acetonitrile. After complete evaporation of solvent in the fume hood, 10 g of soil samples was measured into the flasks containing the sand and the insecticides. 2 ml of 0.01 M $CaCl_2$ solution was added to moisturize the soil sample and to attain field capacity. The flasks were sealed with rubber stoppers with holes clogged with cotton to ensure aeration of the samples. Each flask with its content was incubated at 25 °C (± 2). To ensure a uniform water content, periodic addition of measured volumes of 0.01M $CaCl_2$ solution were added to samples based on difference in weight due to loss of moisture. Samples were monitored at designated time intervals between 0 and 340 days. Each insecticide was analysed together with its deuterated compound in separate flasks. Blank samples without pesticides were analysed concurrently.

4.2.5 QuEChERS extraction of samples

At specific time intervals (days), samples were removed from the incubator and quantitatively transferred into 50 ml Falcon tubes with the aid of 8 ml of water. 15ml of acetonitrile was then added and the resulting mixture shaken by hand for about 1 minute. 4 g of $MgSO_4$ and 1 g of NaCl salts were then added and the mixture further shaken for another 1 minute. The resulting mixture was centrifuged at 3000 RPM for 5 minutes. Aliquots of the supernatant were measured, filtered through a 0.22 µm PTFE filter and diluted with an equal volume of water prior to injection onto the LCMS. When not being directly analysed, samples from the incubator were placed in a refrigerator at -18 ° C prior to extraction and analysis.

4.3 Results & Discussion

4.3.1 Soil properties

A representative soil type from the various regions of the country was used for the study. Properties of the soil used include: pH of 6.9; clay content of 21.4 %; soil organic carbon (SOC) of 3.5 %; cation exchange capacity (CEC) of 14.7 $cmol^+/kg$; and electrical conductivity (EC) of 240 µS/cm. This soil is classified as sandy-clay-loam based on the USEPA soil texture classification triangle.

4.3.2 Dissipation of neonicotinoids

The QuEChERS-LC-MS procedure used has been described in chapter 2 with minor changes to ensure better efficiency. The instruments' response over the range of concentration studied (1.56 – 800 µg L^{-1}) was linear with good correlation for all analytes ($r^2 \geq 0.998$). LOD and LOQ for both insecticides were estimated at 2 and 5 µg/kg respectively. Dissipation of both insecticides and their deuterated pairs were studied over 340 days as indicated in Table 18. Samples were spiked at a concentration of 1000 µg/kg of dried soil, which was four times the highest concentration found under field conditions as indicated in chapter 2 (Dankyi et al.,

2014). While the amount of insecticide used may be considered relatively high, it was essential for the purpose of identification of possible metabolites, which is often found in very low concentrations in the matrix.

Table 18. Persistence of imidacloprid and thiamethoxam in soils. Percentages were calculated based on an initial fortification of 1000 µg/kg.

Percentage remaining			Percentage remaining		
Day	Imidacloprid	Imidacloprid-d_4	Day	Thiamethoxam	Thiamethoxam-d_3
0	101.4	99	0	102.3	101.7
1	100.5	99.3	3	99.3	101.1
3	96	97.2	5	98.1	99.9
5	95.4	96	9	95.7	99.3
9	95.1	97.2	11	98.1	99
12	89.7	95.4	15	96.3	99.3
17	90.3	91.8	18	93.3	92.4
21	87.9	91.2	21	91.2	89.4
30	85.2	87	25	91.5	90
45	78	79.5	31	87.9	85.8
60	76.2	78	56	78.3	80.7
90	75.9	82.2	70	76.8	78.9
120	76.5	72.3	90	81.3	75
185	42.6	42.3	120	78.9	72.6
240	35.4	37.8	185	51.3	56.7
290	20.8	24.6	240	21.9	30.6
340	8.4	10.9	325	6.2	7.8

Degradation kinetics of neonicotinoids were studied based on approaches proposed by the European FOCUS working group (FOCUS, 2006). For many decades, single first order kinetics has been the preferred model for describing pesticide degradation and in the estimation of their DT_{50} and DT_{90}. Among other factors, the simplicity of SFO and the prevalence of many environmental processes that occur through this order have enabled its common application. The model assumes that the number of pesticide molecules is relatively small compared to the number of degrading microorganisms/enzymes or the number of water molecules in the case

of hydrolysis (FOCUS, 2006). Consequently, time-related decline in pesticide concentration is directly proportional to the actual concentration remaining in the system.

The model is expressed as:

$$C = C_0 e^{-kt} \text{ (Integrated form)}$$

Where C is the amount (%) of pesticide present at time t (days), C_0 is the amount (%) of pesticide at time 0, and k ($days^{-1}$) is the rate constant for the degradation process.

However, not all degradation processes can be well described with SFO. In reality, some processes may be more complex and may involve more components and phases in spite of having the first order process as its basis. Two alternative models have been proposed for use by the FOCUS expert group which addresses the limitations of SFO model in describing more complex processes. These are First Order Multi Component (FOMC) and Double First Order in Parallel (DFOP) models.

The FOMC model is expressed as:

$$C = C_0 (\frac{t}{\beta} + 1)^{-\alpha}$$

Where C is the amount of pesticide present at time t (days), C_0 is the amount (%) of pesticide at time 0, β is a parameter determined by the variation in k values and α is a position (location) parameter. This model was originally proposed by Gustafson and Holden (1990) although changes to the original model have been made (Gustafson and Holden, 1990). The model assumes that, soil is a heterogeneous medium with sub-compartments where degradation occurs with different first-order degradation rate constant (FOCUS, 2006).

DFOP regards the pesticide degradation process as being distributed between two different phases: A rapid degradation phase by one part of the pesticide molecules and a second slower phase dominated by sorption of molecules on soil surfaces.

The model is expressed as:

$$C = C_1 e^{-k_1 t} + C_2 e^{-k_2 t}$$

Where C is the amount (%) of pesticide present at time t (days), C_1 and C_2 are the amounts (%) of pesticide present at time 0 in the first and second compartments respectively; k_1 and k_2 are the rate constants for the degradation in first and second compartments respectively.

In this study, all three models were tested in the description of the degradation process for both neonicotinoids and their deuterated counterparts. However, dissipation of all compounds were best described by the SFO model based on visual inspection and distribution of residuals. A relatively good fits of $r^2 \geq 0.92$ was obtained in all compounds.

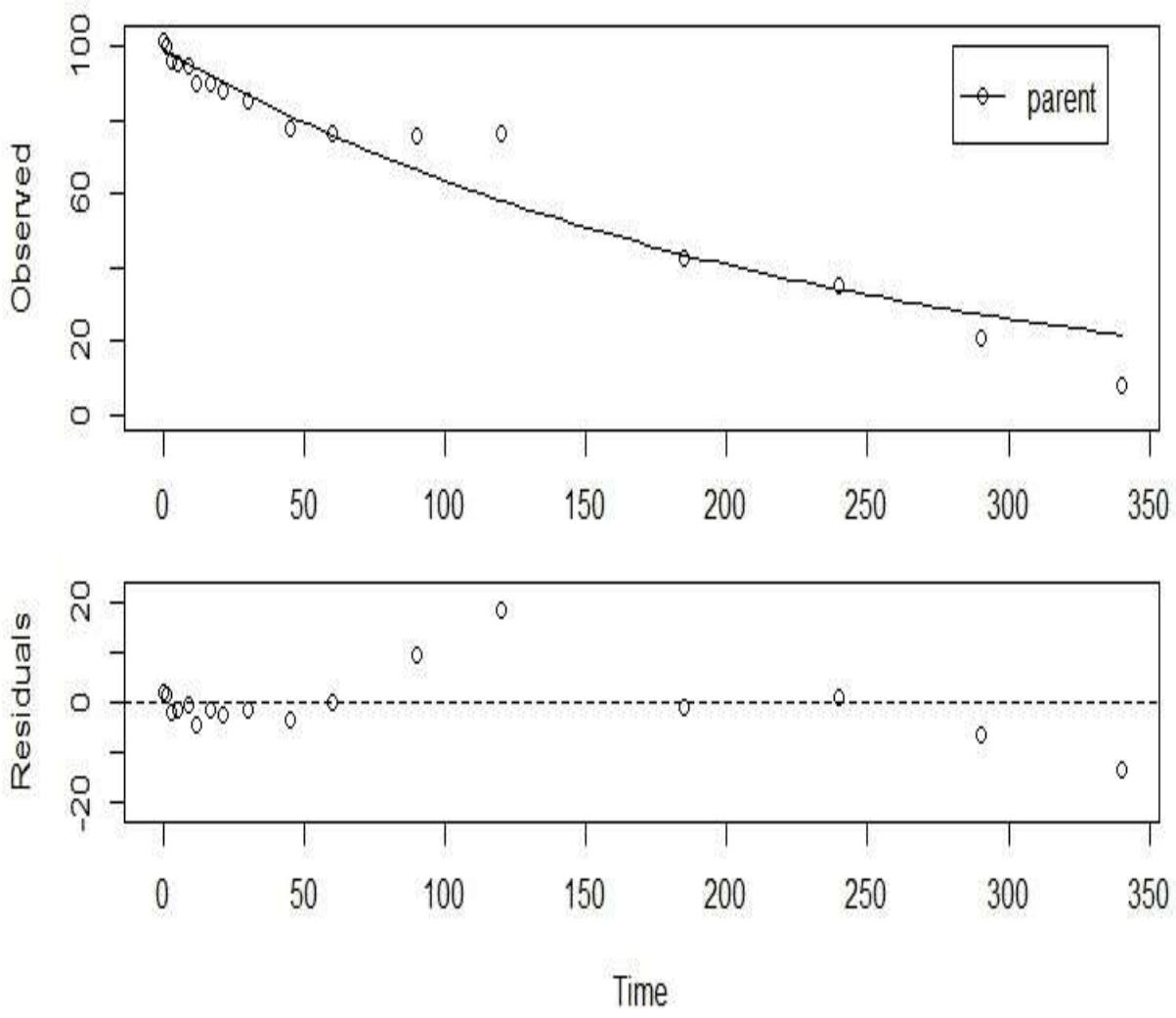

Figure 19. Dissipation of imidacloprid. Percentage of parent pesticide remaining is plotted over time (days). Dots are data points, solid line is the model

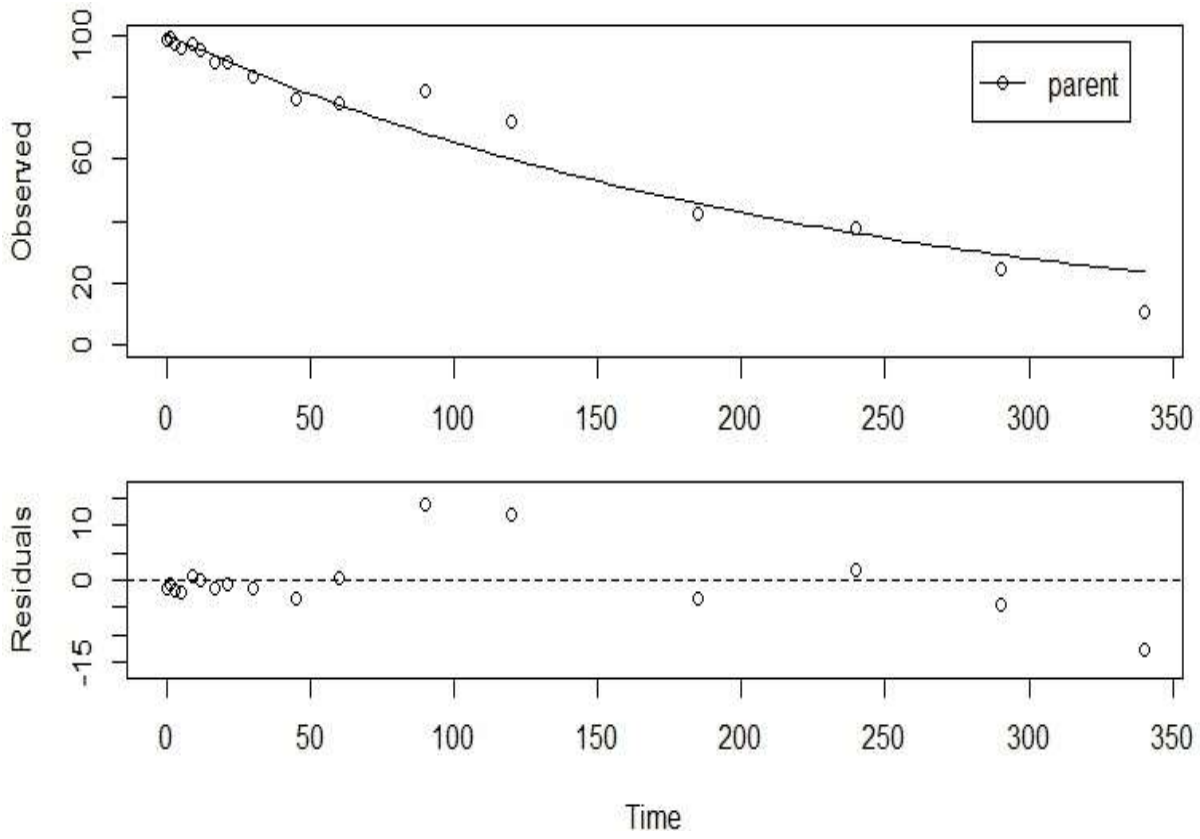

Figure 20. Dissipation of imidacloprid-d4. Percentage of imidacloprid remaining is plotted over time (days).

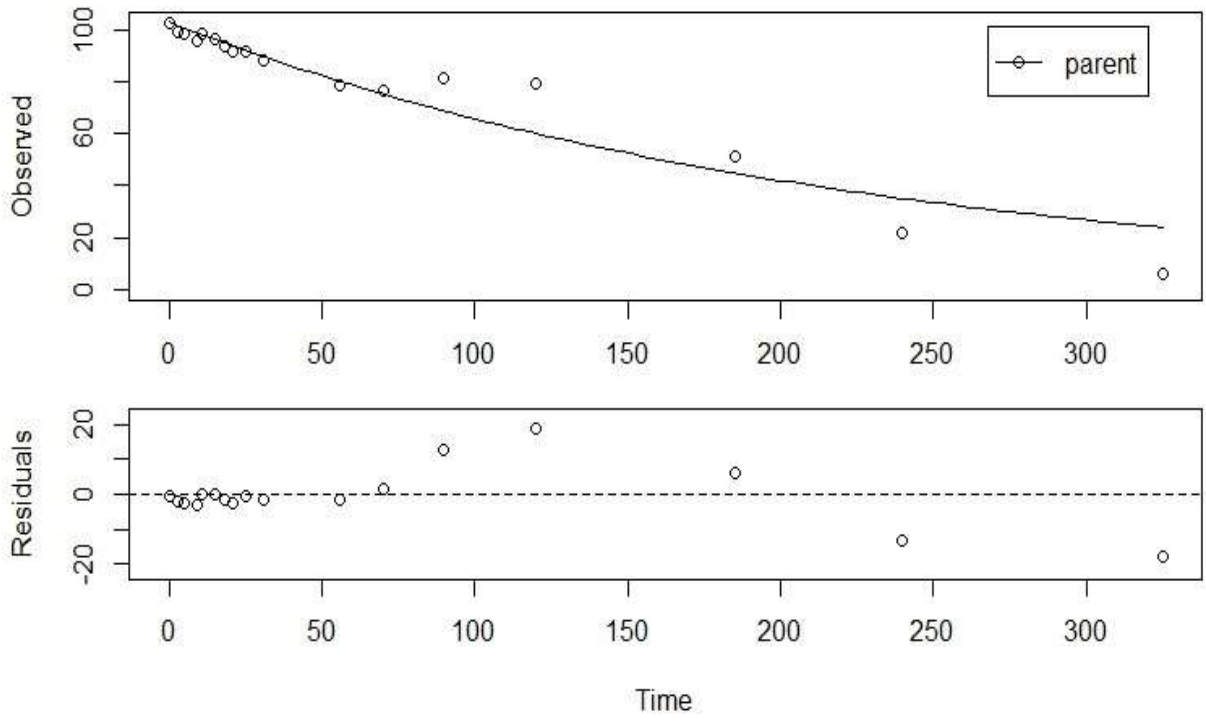

Figure 21. Dissipation of thiamethoxam. Percentage of imidacloprid remaining is plotted over time (days).

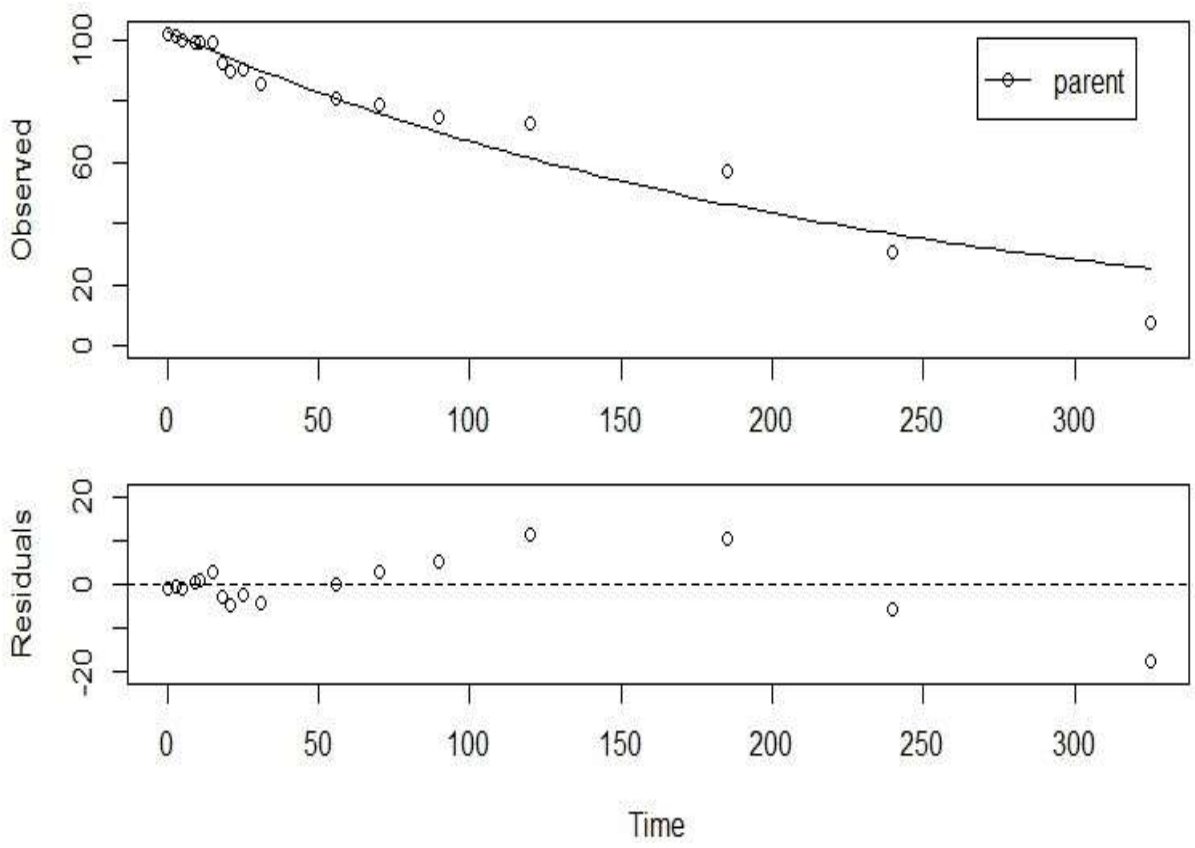

Figure 22. Dissipation of thiamethoxam-d3. Percentage of imidacloprid remaining is plotted over time (days).

Dissipation rates of each analyte in soil have been estimated in Figures 19-22 by plotting the percentage of fortified neonicotinoid remaining, as a function of time. The R statistical software (version 3.1.0) was used in developing the models. The results suggest an initial slow phase of dissipation followed by a more rapid one during the later stages as commonly observed in the dissipation of many pesticides. This initial lag phase is often attributable to the adaptation of microbial population. From the results obtained in the current study however, the long lag phase observed may also be attributed to experimental artefacts such as excessive air-drying leading to changes in microbial activity. Although slight changes in environmental and experimental conditions may not have significant effects on dissipation rates, larger and longer variations may significantly affect dissipation rates. As a consequence of the long lag phase, degradation appears to be bi-phasic, although the behavior is not well explained by both bi-

phasic models. As observed, the SFO models obtained for all compounds showed substantial deviations from data points due to the observed variation in dissipation rates between the two stages of dissipation.

Moreover the high initial concentration of pesticides used may have led to slower dissipation as a results of saturation of soil surfaces and low relative populations of microorganisms. In general, when the pesticide concentration is the only factor that determines the rate model (microorganisms and enzymes being present in "unlimited" amounts or already adapted) kinetics proceed in the first order. However when the concentration of the pesticide is too high relative to microbial population and adaptability, a lag phase (with implication for growth of microorganisms) is often seen in the best fitting kinetic model. This was seen in the mineralization studies of mecoprop by Helweg et al (1998) where 5 µg/g showed growth kinetics while 0.5 µg/g showed normal first-order kinetics (Helweg et al., 1998). Similarly Fomsgaard et al (1997) have shown that, degradation kinetics in plough layer at concentrations of 0.04-0.08 µg/g follow normal first order kinetics whereas kinetics in subsoils, often characterised by low presence of microorganisms showed growth kinetics (Fomsgaard, 1997). However, a similar "pseudo" bi-phasic mechanism has been reported for neonicotinoids albeit with contrasting kinetics from that obtained in this study. Gupta et al, (2008) have observed a fast initial dissipation of thiamethoxam followed by a slower second phase, explained by an initial slow adsorption and later fast adsorption, leading to the variation in dissipation rates (Gupta et al., 2008).

Table 19. Dissipation rates, confidence interval, DT_{50}, DT_{90} and correlation coefficients of analytes

Neonicotinoid	k	CI	DT_{50} (days)	DT_{90} (days)	r^2
Thiamethoxam	0.0045	0.0035- 0.0058	154.7	514	0.92
Thiamethoxam-d_3	0.0043	0.0035-0.0053	160.551	533.34	0.93
Imidacloprid	0.0044	0.0036-0.0054	156.33	519.34	0.95
Imidacloprid-d_4	0.0042	0.0036-0.0051	162.49	539.78	0.96

k – Dissipation rate; CI – Confidence interval of k

In the study, dissipation half-lives (DT_{50}) of both imidacloprid and thiamethoxam were estimated at $\ln {}^{2}/_{k}$ and were similarly high, confirmed by their deuterated pairs (Table 19). From the models, DT_{50} for imidacloprid and thiamethoxam were 156 and 155 days respectively. Deuterated counterparts showed DT_{50} values of 162 and 161 days for imidacloprid-d_4 and thiamethoxam-d_3 respectively. Within the interval of confidence and the values of k obtained, dissipation half-lives were similar for each insecticide and deuterated pair.

With reported wide variability in dissipation rates of neonicotinoids, comparison of current results obtained with literature does not appear meaningful. From this study however, the degradation kinetics of both neonicotinoids suggest the possibility of a considerable degree of persistence in the conditions and soils studied. Longer half-lives have been reported in literature under laboratory conditions (Gupta et al., 2008). In general, dissipation of many pesticides including neonicotinoids tend to be longer under laboratory conditions (Goulson, 2013; Gupta et al., 2008; Sarkar et al., 2001). Reasons such as drying up of soils, and mortality of microorganism due to lack of sufficient food have been suggested for the longer dissipation half-lives.

On the contrary, rapid degradation rates have been reported in field studies. Very rapid dissipations (≤ 2 to 9 days) have been reported for a number of neonicotinoids (Omirou et al., 2009; Wang et al., 2011). In general, field dissipation appears to occur at faster rates than laboratory studies for almost all neonicotinoids, with reported half-lives of < 20 days (Karmakar and Kulshrestha, 2009; Sharma and Parihar, 2013; Wang et al., 2011; X. Wang et al., 2013; Wu et al., 2012). The variations in individual rates of neonicotinoids and among study types are explained by several factors including soil type, moisture, temperature, pH, and microbial activity. However, despite the significant role played by these climatic and edaphic factors in the dissipation of neonicotinoids, their high water solubility and leaching potential may have a major influence in the observed differences in laboratory and field dissipation rates.

Under tropical conditions as prevails in Ghana, temperature and water content may be the most important climatic factors influencing dissipation rates of pesticides. The exact role of temperature in dissipation of pesticides is not quite clear, although many degradation reactions are believed to be endothermic and hence increase in temperature may promote rapid dissipation and shorter half-lives. However, under tropical conditions, the higher temperatures may result in more rapid evaporation of water. Gupta et al, (2008) have shown that, dissipation rates may be significantly reduced (about 4 times) under dry conditions in comparison with wet conditions, hence promoting persistence (Gupta et al., 2008). This is believed to be as a result of lower degradation of adsorbed compounds compared to those in the liquid phase, hence increasing persistence (Lei Guo et al., 2000).

Given the relatively high organic carbon content for most soils from the study areas (Average, 2.94), the possibility of adsorption is high, with an increased tendency for persistence. Likewise, high average rainfall in these regions coupled with good solubility of neonicotinoids

in water may promote degradation and leaching behavior. Under field conditions, the extent of persistence will be based on the relative importance of these factors together with microbial activity in the soils.

4.3.3 Soil metabolites of thiamethoxam and imidacloprid

Degradation products of both imidacloprid and thiamethoxam were studied to better understand their behavior in soils. To this end, the use of deuterated compounds of both insecticides was essential in the identification and confirmation of possible metabolites in soils. From the study of thiamethoxam, clothianidin which is an insecticide and a known metabolite of thiamethoxam was identified and quantified by use of its pure standard. Clothianidin concentration was quantified from day 11 (5.7 µg/kg) until day 240 (7.8 µg/kg), peaking on day 90 with a concentration of 33.9 µg/kg (Figure 23).

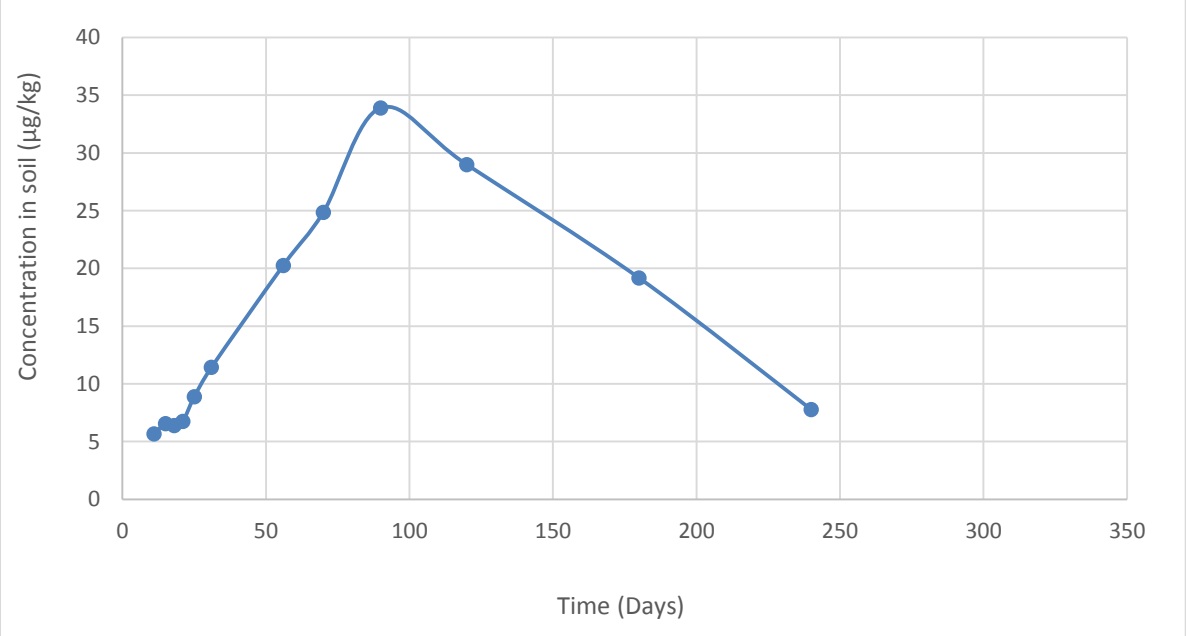

Figure 23. Concentration of thiamethoxam metabolite- clothianidin over time

With the help of LightSight® software (ABSciex, versions 2.3), several possible degradation products of imidacloprid and thiamethoxam (Figure 16 and 17) have been identified based on

their mass spectra and pattern of fragmentation, and aided by their deuterated counterparts, although yet to be verified. The software enables the easy and effective identification of metabolites and transformation products using compound specific predicted MRM-IDA methods. Here, metabolites are identified by comparing their mass spectral data with that of parent compounds as shown in Figure 24 for thiamethoxam (parent) and clothianidin (metabolite). Often, background noise from the hundreds of compounds normally associated with environmental matrixes may present a challenge of interference, and are reduced by adjusting noise levels as relevant or differentiated using an overlay of extracted ion chromatographs (XICs) of sample and control. While pure standards of metabolites are often needed for confirmation, their general lack of availability, as well as the multiplicity of metabolites possible, makes the current approach useful, at least as a basis for identification of metabolites.

In literature, several metabolic pathways for imidacloprid and thiamethoxam have been proposed (Figure 16 and 17) under various conditions through several degradation processes with consequent high diversity of possible metabolites (de Urzedo et al., 2007; Lopes et al., 2008) (Karmakar et al., 2009). The fate and behavior of imidacloprid is the most studied of all neonicotinoids and has served as a guide for the study of other neonicotinoids (Liu et al., 2011). Largely, research on metabolic products of neonicotinoids have been focused on animal systems. Few have been performed in food crops and even less so in soils. For imidacloprid, three major metabolites: imidacloprid urea, 6-chloronicotinic acid and 6-hydroxynicotinic acid have been identified in soil by use of standard compounds (Rouchaud et al., 1996). The need for identification of possible metabolites of neonicotinoids is an important step in the understanding of their behavior in the environment and in soils. In the current study, identification of metabolites are on-going in several samples representing several periods of degradation to provide a holistic picture of pesticide degradation in both compounds studied.

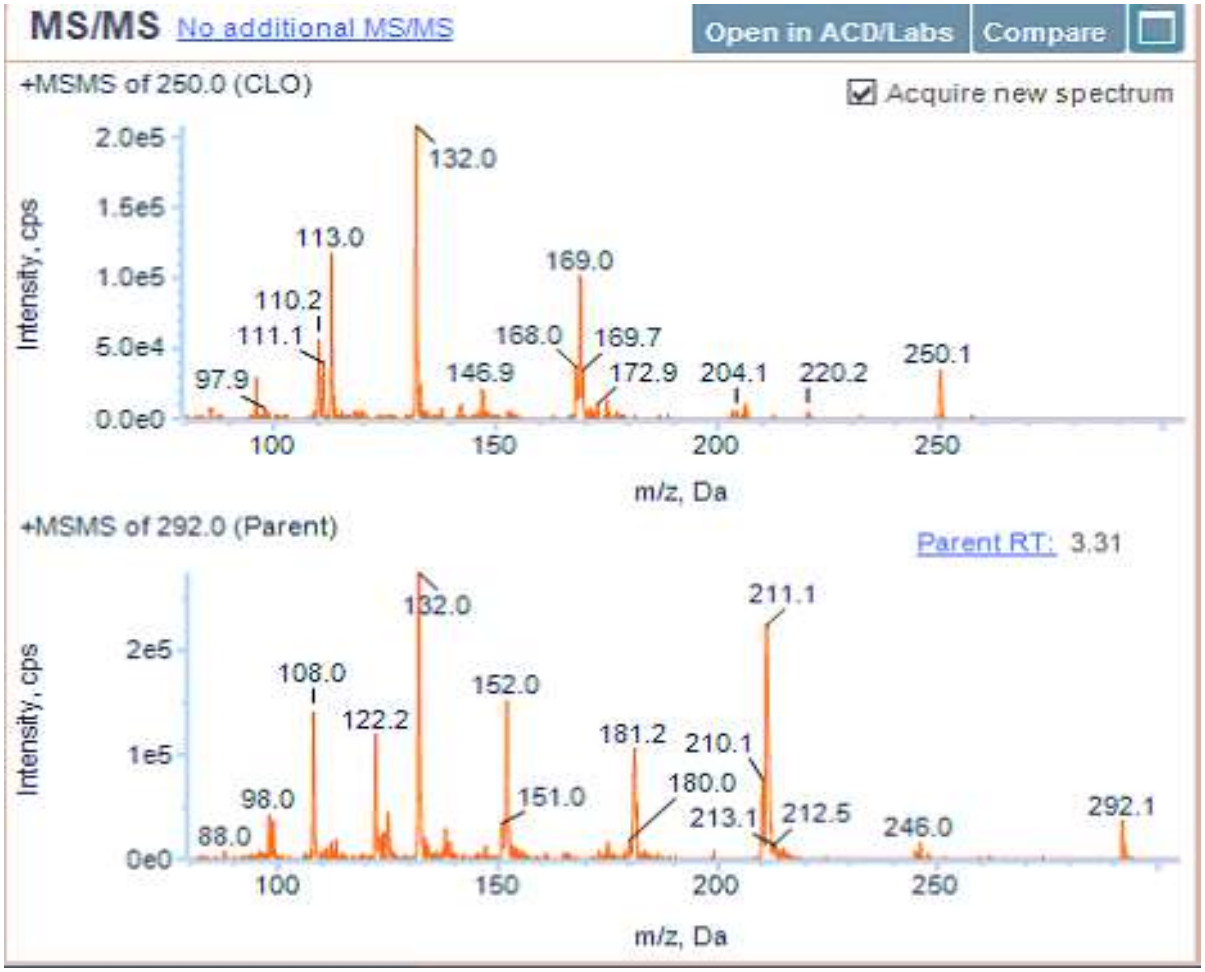

Figure 24. Comparison of MS/MS data of parent compound (thiamethoxam) and probable metabolite (clothianidin) for possible identification in Lightsight®.

4.4 Conclusion on chapter four

Despite the lack of a perfect model, dissipation behavior of imidacloprid and thiamethoxam have been described in Ghanaian soils. The findings suggest the persistence of both insecticides in the tropical soils studied, with half-lives > 150 days. From the study, influence of soil properties and climatic conditions in dissipation behavior of both insecticides is not clear, although these conditions may play a role in determining the dissipation rates and behavior in soils studied.

5. Sorption of neonicotinoid insecticides to agricultural soils

5.1 Introduction to chapter 5

This chapter examines the adsorption and desorption behavior of neonicotinoid insecticides in representative soil types from Ghana. The batch equilibrium procedure was used and has been described. The potential mobility of neonicotinoids in the soil ecosystem based on sorption/desorption (leaching) behavior has been examined. A manuscript of the work is being prepared for submission to Journal of Environmental Science and Health Part B, Pesticides, Food Contaminants, and Agricultural Wastes.

5.1.1 Sorption behavior of pesticides in soils

The importance of the soil compartment as a sink for applied pesticides has been highlighted in previous chapters. Once in the soil, pesticides may enter the aqueous phase or may be sorbed onto organic matter or mineral surfaces depending on the properties of both soil and pesticides. The type and extent of partitioning may not only play a major role in determining the fate and behavior of the pesticide but has a huge influence in its mobility and transformation. Together with degradation, sorption is one of the most crucial phenomenon in determining the fate, persistence and behavior of applied pesticides in soils. This is because the partitioning of pesticides between the aqueous and solid phases creates an adsorption-desorption equilibrium which controls bioavailability, leaching, mobility, degradation and their general behavior in soils.

In general, the rate of adsorption is controlled by the properties of the pesticide and soil. Several studies have shown the significant roles played by soil properties such as organic matter, pH and clay minerals in sorption mechanism. In particular organic matter has been found to play a crucial role in sorption processes of many pesticides (Alfaoui et al., 2012; Rodríguez-Liébana et al., 2013). Humic substances including fulvic and humic acids tend to provide organic matter

with chemically reactive functional groups, hydrophilic and hydrophobic sites which enable various forms of interactions with chemical compounds (Senesi, 1992). However the type of interactions occurring is dependent on the physical properties of the chemical and may include covalent, ionic and hydrogen bonding, ligand exchange, Van der Waals forces, and hydrophobic interactions.

While organic matter is often seen as the dominant factor influencing adsorption of most pesticides, mineral surfaces particularly from clay fractions play an important role (Liu et al., 2002). Pusino et al, (1992) have observed that, there is competing dominance of organic matter over clay minerals in soils (Pusino et al., 1992). Thus the influence of clay minerals in sorption is particularly dominant at lower depth where organic matter content is low. Also important in the sorption-desorption behavior of pesticides in soil is the influence of pH. This is particularly in the case of polar chemicals where pH may influence protonation or deprotonation of compounds. pH may also indirectly influence pesticide-soils interactions by affecting humic substances and clay mineral surfaces.

Sorption is commonly studied using the Batch equilibrium method, where the distribution of compounds between soil and soil water is determined. An important parameter of the process is the sorption coefficient, K_d, which is a measure of the strength of sorption of a chemical to soil, and hence its mobility and leaching potential (Weber et al., 2004). K_d (mL/g) is given by the ratio between the concentration of sorbed compound on the soil and the concentration in soil water and expressed as:

$$K_d = {}^{q}/_{C_e} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(i)}$$

Where q is the amount (µg/g) of pesticide adsorbed per unit mass of soil and C_e is the pesticide concentration (µg/mL) in solution at equilibrium.

Based on several measurements of K_d in soils, a high correlation between organic carbon content of soils and K_d has been observed. As a results, K_d is often normalized to organic carbon for easy comparison of compounds independent of the type of soil used. Soil organic carbon sorption coefficient K_{oc} is measured by dividing the measured K_d value in soil by the organic carbon fraction, OC of that soil:

$$K_{oc} = {}^{K_d}/_{OC} \quad \text{..... (ii)}$$

Whereas K_{oc} helps in the prediction of binding capacity of a chemical to soil, its use implies that soil organic matter is the only sorbing material in the solid phase. However, the sorption process may be far more complex, exhibiting varying kinetics and interactions with various sorbents in soils, with influence from conditions such as temperature, pH and ionic strength. In most sorption studies, K_d tends to decrease with increasing concentration of pesticides, resulting in a non-linear isotherm (from equation i). This sorption processes may be fitted to the Freundlich model which is given by:

$$q = K_f . C_e^{\,n} \quad \text{..... (iii)}$$

Where K_f is the Freundlich sorption coefficient and n is an empirical constant.

The model has been successfully applied in the description of several pesticide-soil sorption data due to its flexibility in application and ease of comparison with different soils (Banerjee et al., 2008; Broznić and Milin, 2012; Campbell et al., 2005).

Literature is replete with the description of sorption processes for several chemicals including neonicotinoids in varying soil types. However, different and often conflicting results have been observed even for one particular compound due to the multiplicity of factors and their complex relations to soils and chemical behavior. In neonicotinoids, sorption behavior of imidacloprid has been particularly studied by several researchers, with reported differences in behavior and fate. For instance, while organic matter has been reported to be important in the sorption

behavior of a number of neonicotinoids (Banerjee et al., 2008; Kandil et al., 2015), other reports have found no correlation between neonicotinoid sorption and organic matter content (Carbo et al., 2007; Fernandez-Bayo et al., 2008; Oliver et al., 2005). Other factors that have been suggested to be important in the sorption behavior of neonicotinoids include clay minerals, temperature and pH (Broznić and Milin, 2012; Kandil et al., 2015).

Of greater concern in the sorption behavior of neonicotinoids is their potential for leaching due to their high solubility in water. Several studies have reported low sorption coefficients for neonicotinoids in soils with a high possibility for runoff and leaching into surface and underground water (Broznić et al., 2012; Kurwadkar et al., 2014). In general, adsorption-desorption behavior of neonicotinoids have been observed to be concentration dependent, with greater percentage adsorption reported at lower concentrations. Desorption hysteresis is said to occur in several soil types particularly for imidacloprid with consequences for environmental mobility and fate (Broznić et al., 2012; Cox et al., 1997; Kandil et al., 2015).

Notwithstanding the extensive research into sorption behavior of many pesticides in soils, the diversity of pesticides and soils, coupled with the complexity of soil-pesticide interactions have made prediction and generalization quite difficult. Thus, much research is still needed in the accurate prediction of sorption behavior of new chemicals in different soils and environments. This is particularly important in the West African environment and soils as almost all studies on sorption behavior were conducted outside of this region. The current study is aimed at predicting the sorption behavior of five neonicotinoids commonly used or found in the Ghanaian environment.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

Thiamethoxam (99.6%), clothianidin (99.9%) and imidacloprid (99.9%) were purchased from Sigma-Aldrich (Steinheim, Germany) while acetamiprid (98.1%) and thiacloprid (98.0%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Pesticide working solutions were prepared by diluting stock solutions with 0.01 M $CaCl_2$ solution to maintain ionic strength.

Tubes containing 4 g of $MgSO_4$ & 1 g of NaCl were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate (NH_4Ac) was obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile obtained from Rathburn (Walkerburn, Scotland) was used in the study. Deionized water was prepared using a Merck Millipore Milli-Q advantage A10 ultrapure water purification system (Darmstadt, Germany).

5.2.2 LC-MS/MS instrumentation

LC-MS/MS instrumentation used is as similarly described in previous chapters (3 & 4) with the exception of changes to analytes being studied. Here, all five neonicotinoids were studied as done in chapter 3. Chromatographic conditions as well as MS parameters for the various analytes are as already described in chapter 3 of this thesis.

5.2.3 Soil samples

Soils used in this study were obtained from the upper 20 cm depth in cocoa farms in Ghana. For this study, four different soil types were chosen from the larger number of soils already described in chapter 2. Soils were selected based on differences in properties such as pH, OC and clay content (Table 20). Samples were air dried and passed through a 2 mm mesh sieve prior to use. To avoid degradation of compounds by microorganisms, samples were irradiated with 10 kGy gamma radiation for 1769 minutes.

Table 20. Physical and chemical properties of soils used in sorption studies

Property	SL1	SL2	SL3	SL4
pH ($CaCl_2$)	6.5	5.5	7.5	5.9
OC (%)	1.6	3.2	2.6	4.8
CEC ($cmol^+/kg$)	11.7	13.4	18.2	16.0
Clay (%)	27	15	42	19
Silt (%)	13	31	14	36
Sand (%)	59	54	44	45
Texture class	Sandy Clay Loam	Sandy Loam	Clay loam	Loam

5.2.4 Sorption-desorption experiments

Sorption of insecticides to soils were studied using the standard batch equilibrium method (OECD, 2000). 5 g of soil sample was equilibrated in 25 mL of 0.01M $CaCl_2$ aqueous solution of insecticide. In all the studies, a concentration of 200 µg/kg of insecticide in soil was used. The mixture was shaken on an Elmi intelli-mixer (Riga, Latvia) to establish equilibrium. Kinetic studies were first conducted for each insecticide in concentrations of 200 µg kg^{-1} over a period of up to 72 hours of sorption. Subsequently the sorption isotherm was determined. Five different concentrations: 0.1, 0.5, 1, 5, 10 mg/L of each insecticide was prepared in 0.01M $CaCl_2$ for the study of sorption behavior. Samples were centrifuged at 4500 RPM for 10 minutes at 20 °C. Aliquots of the supernatant formed were filtered using a 0.22 µm filter and analyzed using LC-MS. Samples were prepared in duplicates. Blank samples of each insecticide without soils were prepared to account for possible volatilization and adsorption onto walls of container. Finally, desorption kinetics was determined by equilibrating the sorbed soils with fresh solutions of equal volumes of 0.01M $CaCl_2$ solution without insecticide and as done in the case of sorption, desorption was studied over time intervals using aliquots of the supernatant following centrifugation.

5.3 Results & Discussion

5.3.1 Properties of neonicotinoids

Water solubility and Log P values (estimation of lipophilicity/hydrophobicity) are shown in table 3 in chapter 1. In the current study, sorption behavior of neonicotinoids was observed to increase in the order: TXM < ACE < CLO < IMI < TIA and correlated well with reported water solubility. Similarly, significant correlations between sorption coefficient (K_d) and water solubility or Log P (hydrophobicity) of neonicotinoids have been reported (Kurwadkar et al., 2014; Nemeth-Konda et al., 2002).

5.3.2 Sorption kinetics

The time taken for adsorption equilibrium to be established for each neonicotinoid was studied over a 48 or 72 hour period depending on how quickly equilibrium is established (Figure 25). For thiacloprid, clothianidin and acetamiprid equilibrium was reached in 24 hours. A longer duration of 48 hours was required in both imidacloprid and thiamethoxam.

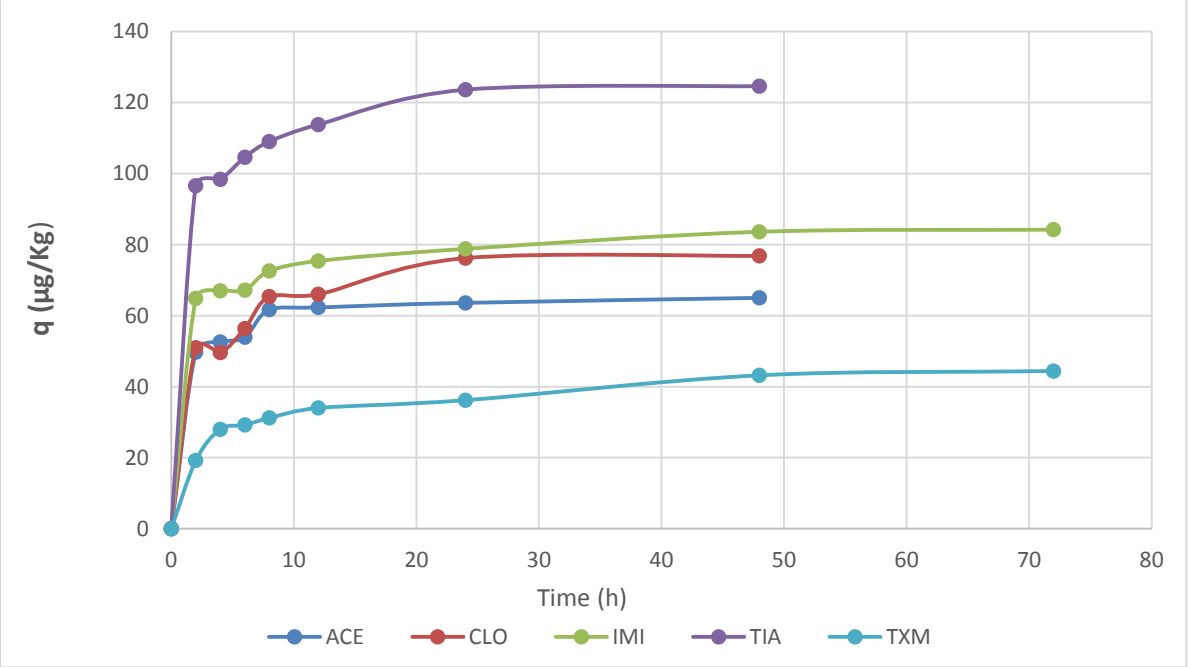

Figure 25. Time dependent sorption of neonicotinoids in soil.

From the study, sorption behavior appeared to be rapid at the onset and was nearly complete within the first 24 hours for all neonicotinoids. Similar findings have been reported in literature (Broznić and Milin, 2012; Nemeth-Konda et al., 2002). The rapid initial sorption is believed to be as a results of a rapid occupation of vacant sites in soil surfaces followed by a slow migration and diffusion into organic matter and mineral matrixes (Gao et al., 1998). In the current study, distinct sorption behaviors were observed for neonicotinoids in spite of similarities in structure and functional groups. From the trend observed in Figure 25, sorption in thiacloprid was more pronounced in contrast to other neonicotinoids particularly thiamethoxam. Percentage sorption increased in the order: TXM < ACE < CLO < IMI < TIA and ranged from approximately 30 to 70%. The generalization observed in literature indicates that the percentage sorption of neonicotinoids are low, although subtle variations may result from differences in soil types and properties, and relative amounts of soil, water and pesticide used in a study (Kurwadkar et al., 2013b).

Similar to the initial adsorption mechanism, desorption was almost instantaneous (Figure 26), and hysteresis was observed in all neonicotinoids studied, but was most prominent in thiacloprid where about 70% of the amount adsorbed was retained. In literature, hysteretic effects have also been reported for a number of neonicotinoids, with an index of < 1 reported in most soils (Broznić et al., 2012). This incomplete reversibility of sorption may be due to strong and stable binding interactions with soils and appeared to increase in the order TMX < ACE < IMI < CLO < TIA. Retention of sorbed compounds was particularly high in thiacloprid, where more approximately 70% of its concentration was retained, in contrast to thiamethoxam (≈ 30%). While the extent of binding interactions to soil may have a major impact on retention, the high water solubility of neonicotinoids may have an influence, and may help account for the trend observed. In general compounds that exhibit low water

solubility and high octanol-water partition coefficients (Pow) tend to be more strongly sorbed by soil and vice versa (Nemeth-Konda et al., 2002).

Even though desorption of most pesticides are rapid and often reversible, prolonged contact with soil material may result in further interactions which are often more stable and irreversible. In many cases, organic chemicals may form covalent bonds or be irreversibly trapped in soil matrixes (Senesi, 1992). However, prolonged desorption have also been found to occur in imidacloprid over several days (Broznić and Milin, 2012).

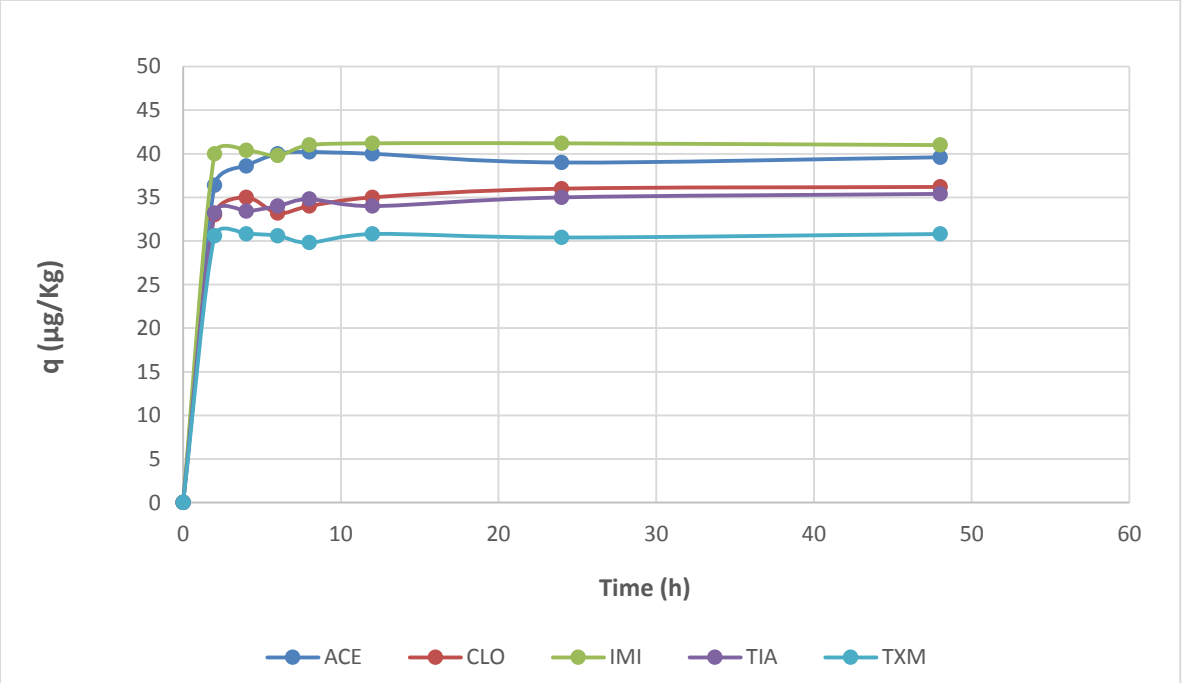

Figure 26. Time dependent desorption of neonicotinoids from varying adsorbed amounts.

5.3.3 Sorption Isotherms

The sorption behavior of the five insecticides in the various soils investigated are shown in Figure 27–31. Slopes obtained were generally non-linear, suggesting a decrease in percentage adsorption with increasing concentration of neonicotinoid. Under this condition, potential leaching of neonicotinoids is high, particularly at high application rates. The average values of K_d obtained from a linear range of sorption in the various soils are presented in Table 21 and

appear to be well correlated with organic matter and clay content irrespective of neonicotinoid used.

Table 21. Sorption parameters of neonicotinoid insecticides in the various soils studied.

Analyte	Parameter	SL1	SL2	SL3	SL4
Acetamiprid	K_d	2.4	4.3	4.4	7.7
	K_{oc}	150.0	122.9	169.2	160.4
	n	0.99	0.89	0.82	0.82
	K_f	2.98	6.98	10.74	19.80
	K_{f-oc}	186.3	218.1	413.1	412.5
Clothianidin	K_d	1.6	3.3	3.7	6.6
	K_{oc}	100.0	94.3	142.3	137.5
	n	0.76	0.71	0.79	0.75
	K_f	4.25	14.61	9.68	27.57
	K_{f-oc}	265.6	456.3	372.3	574.4
Imidacloprid	K_d	6.1	8.1	9.3	15.3
	K_{oc}	381.2	231.4	357.7	318.8
	n	0.84	0.86	0.84	0.79
	K_f	13.27	17.70	22.33	52.14
	K_{f-oc}	829.0	553.1	858.8	1086.3
Thiacloprid	K_d	10.6	15.2	18.4	28.1
	K_{oc}	662.5	434.3	707.7	585.4
	n	0.77	0.76	0.74	0.76
	K_f	34.46	66.56	93.93	129.99
	K_{f-oc}	2153.8	2080.0	3611.5	2708.0
Thiamethoxam	K_d	1.2	2.2	2.8	4.4
	K_{oc}	75.0	62.9	107.7	91.7
	n	1.13	1.12	0.98	0.93
	K_f	1.67	2.43	5.59	12.48
	K_{f-oc}	104.4	75.9	215.0	260.0

The data produced from all analytes were well described by the Freundlich equation ($r^2 \geq 0.988$). In general, n values were < 1, and ranged from 0.71-1.13 for all neonicotinoids whereas K_f ranged from 1.16 – 123.0 $\mu g^{1-1/n}$ mL $^{1/n}$ g^{-1} (Table 21). In general, sorption parameters obtained in this study were comparable to similar studies on neonicotinoids in tropical soils available in literature (Carbo et al., 2007). Calculated values of K_f and K_d for neonicotinoids

increased in the order: SL1 < SL2 < SL3 < SL4. This trend seemed to be well explained by organic carbon content, which appeared to play an important role in the sorption process of all neonicotinoids. Thus K_f and K_d values were normalized to organic carbon for easy comparison. Values for K_{oc} ranged from 62.9 – 707.7 mL/g for all neonicotinoids and reflected the important influence of organic carbon content in the soils studied.

In literature, K_{oc} values of 104-2877 and 155.1-413.3 mL/g have been reported for thiamethoxam (Carbo et al., 2007). Similarly, reported values of K_{oc} for acetamiprid ranges from 98 – 3235 mL/g (Banerjee et al., 2008). In the current study, sorption intensity was highest for thiacloprid and lowest for thiamethoxam. The contrast in the sorption behaviour of the pair may be explained by the type of interactions with soil as well as their relative disparity in water solubility.

The important influence of organic carbon in sorption processes is generally agreed, although its mechanisms of interaction in the sorption process is not clearly established in spite of several studies. This is due to the complex structural features and properties of organic matter which may also be influenced by sample history and age. As a result, the influence of organic matter on a particular pesticide may vary. For instance, Banerjee et al (2008), have identified soil organic fraction as the most importance influence on sorption behavior in thiamethoxam (Banerjee et al., 2008). However, Carbo et al (2007) have observed no significant correlation between sorption coefficients of thiamethoxam and organic carbon content of soils (Carbo et al., 2007).

Soils from the study area (cocoa farms) in Ghana generally possess high organic carbon content due to continuous influx of litter. In literature, a number of reports have indicated the ability of organic matter to explain sorption behavior in different types of pesticides including neonicotinoids (Alfaoui et al., 2012; Banerjee et al., 2008; Kandil et al., 2015; Rodríguez-

Liébana et al., 2013). However, this relationship is often complex and may be influenced by other soils properties (Kah et al., 2007b). In the current study, clay minerals and CEC may have played a role in the sorption behavior of neonicotinoids, although the limited number of samples, together with the often complex soil-pesticide interactions, does not allow for a meaningful conclusion.

The findings from this study suggest that, in general low sorption behavior occurs in neonicotinoids. Sorption coefficients increased in the order: TXM < CLO < ACE < IMI < TIA regardless of soil type used. As a result of the concentration dependence of sorption-desorption behavior of neonicotinoids, a greater percentage of sorption occurs at lower concentrations, which is further enhanced by the presence of soil organic carbon. Hysteresis is pronounced under such circumstances which may encourage persistence in soils (Cox et al., 1997).

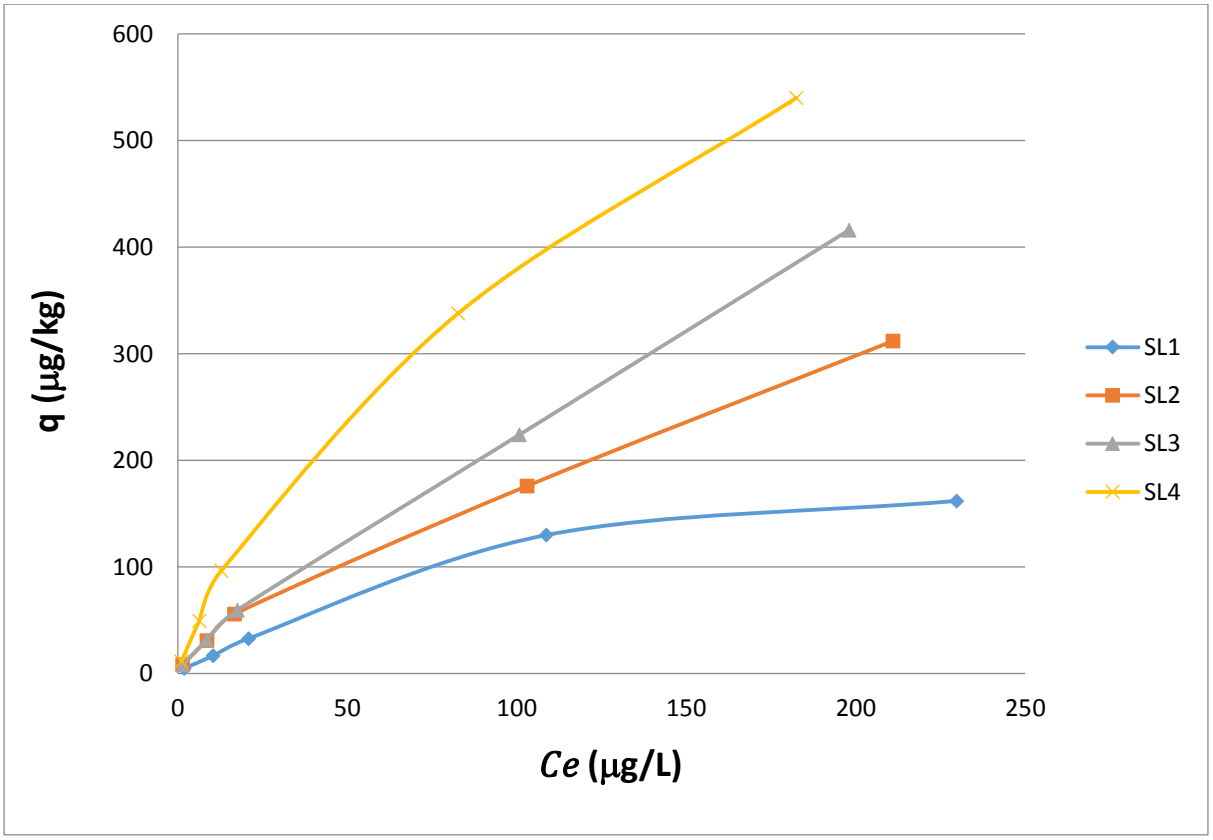

Figure 27. Sorption isotherm for clothianidin in the four soils studied.

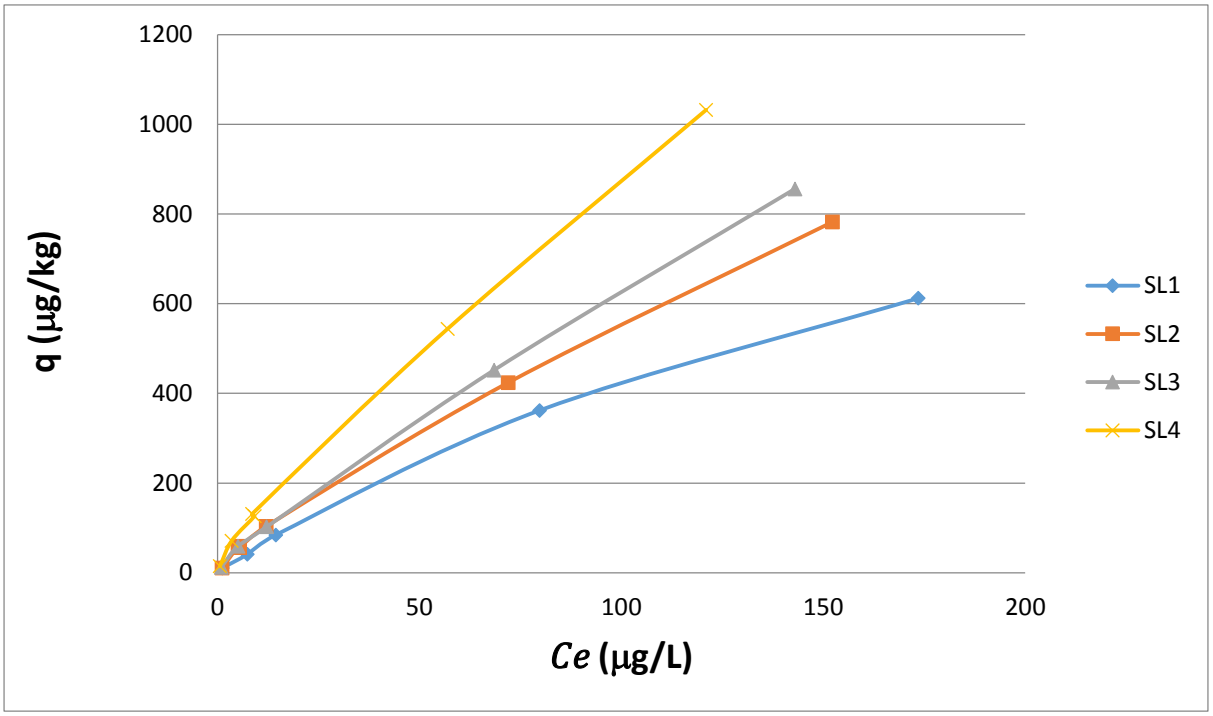

Figure 28. Sorption isotherm for imidacloprid in the four soils studied.

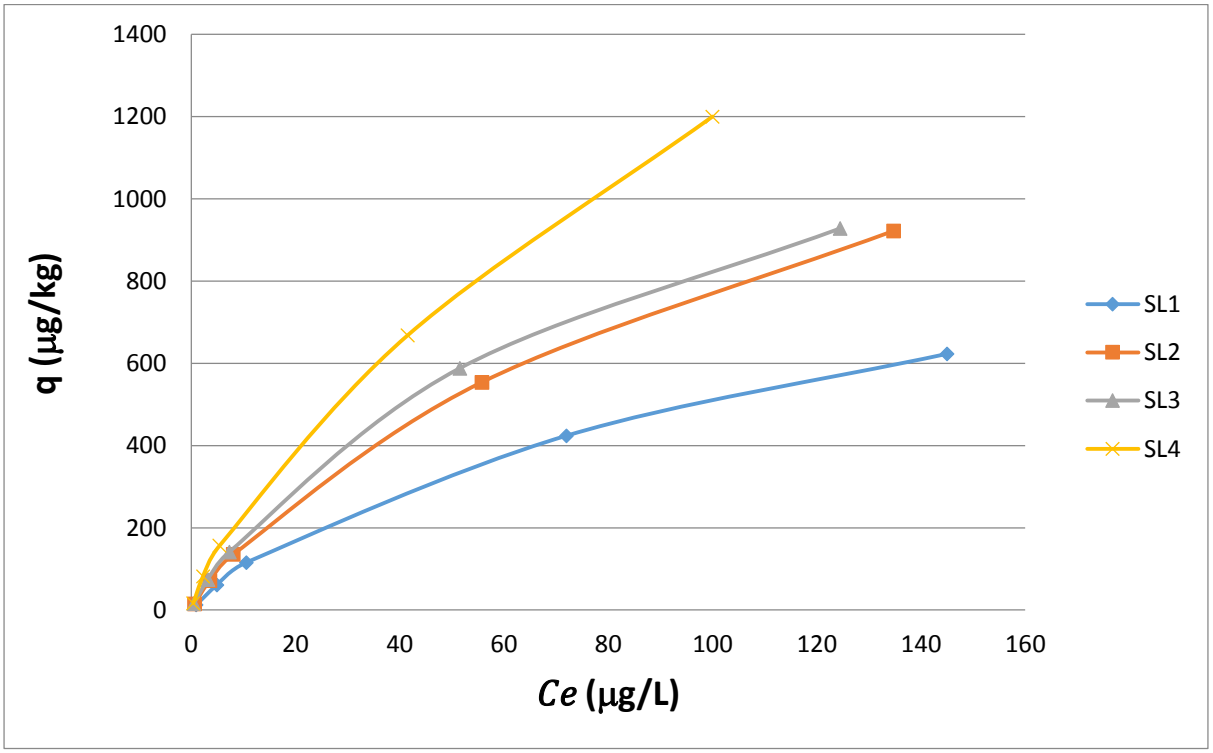

Figure 29. Sorption isotherm for thiacloprid in the four soils studied.

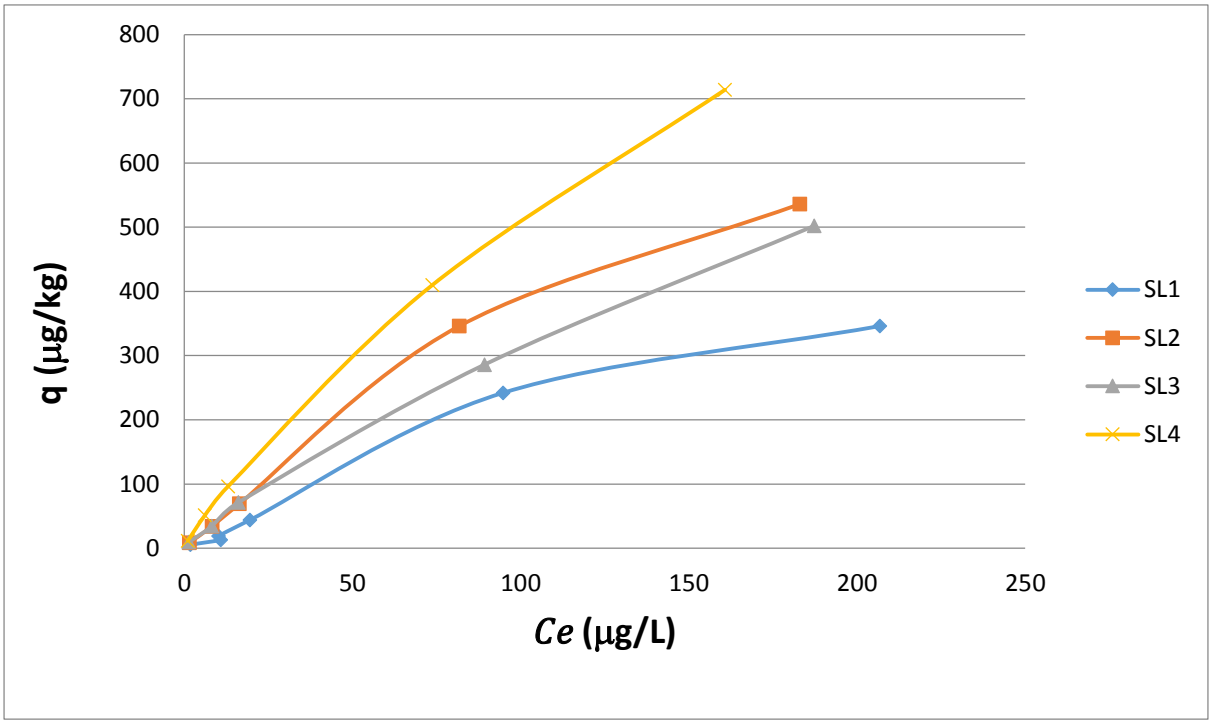

Figure 30. Sorption isotherm for acetamiprid in the four soils studied.

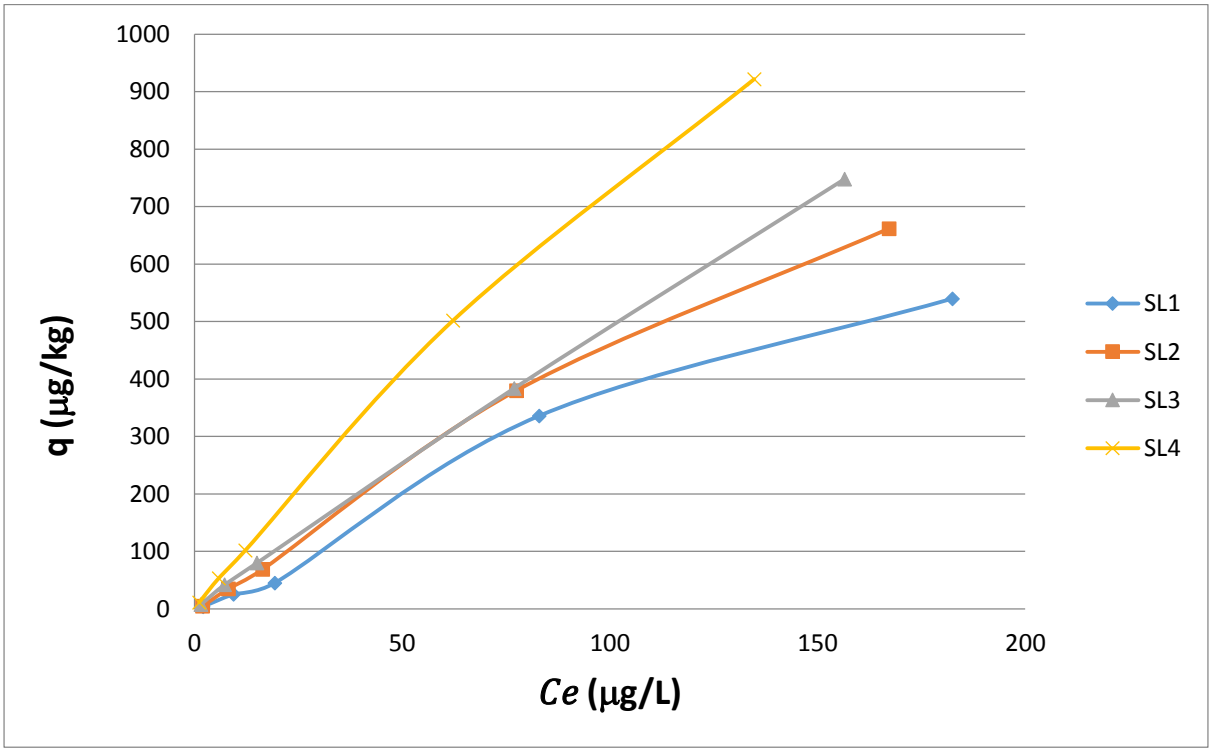

Figure 31. Sorption isotherm for thiamethoxam in the four soils studied

5.4 Conclusion to chapter five

In this study, sorption-desorption of neonicotinoids were examined in order to understand their behavior in Ghanaian soils and the possible influence of soil properties in controlling their fate. The findings from this study suggest that, neonicotinoids may exhibit generally low sorption behavior, with a huge influence of soil properties particularly organic carbon. Hysteretic effects observed in neonicotinoids suggest the binding of these insecticides to soils is not truly reversible. However, the high solubility of neonicotinoids coupled with decreasing percentage sorption at higher concentrations, enhances their potential mobility and leaching behavior. This buttresses the need for efficient use of these pesticides in crop production.

6. Conclusions and perspectives

6.1 Conclusions

The important role of neonicotinoids in world food production is not in doubt given their widespread application worldwide. Their key characteristics include: high pest toxicity, low toxicity to mammals, multiplicity of application methods, and excellent systemic properties. These properties have encouraged their usage on many crops for the control of a number of pests worldwide. In recent years however, reported harmful effects on non-target and beneficial organisms have resulted in restrictions in their usage in parts of the world. Understanding of their concentrations and behavior in the environment is key in the assessment of their effects on non-target organism. This served as the basis for the current work in Ghana, where neonicotinoids are widely used in cocoa production. The major findings from the study suggest a high possibility for mobility and persistence of neonicotinoids in soils, and accumulation in food crops.

6.1.1 The QuEChERS-LC-MS/MS procedure

The flexibility of the QuEChERS procedure enabled its application in the extraction and clean-up of samples from three different matrixes: soil, cocoa beans and water. This is as a result of the existence of diverse salting-out and clean-up conditions under the QuEChERS procedure. In each case, the procedure enabled the determination of analytes from clean matrixes, aided with the use of dispersive clean-up sorbents. The LC-MS/MS methods developed were rapid, sensitive and had low limits of detection and quantitation. This enabled the detection of analytes in diverse and difficult matrixes at very low concentrations.

6.1.2 Fate and behavior of neonicotinoids in soils

Analysis of soils across the various cocoa growing regions of the country has revealed a substantial level of persistence of neonicotinoids. These findings are in good agreement with several studies on persistence of neonicotinoids (Goulson, 2013). As such some neonicotinoids have been classified as persistent (PMRA, 2001). Following at least four month to over two years of application of neonicotinoids in cocoa farms, the results showed the presence of residues in about 75% of samples studied. Imidacloprid was not only the present in the highest concentrations but was also the most frequently encountered neonicotinoids. The observed high concentration and frequency of occurrence of imidacloprid in the soils studied is in good agreement with its usage as the predominant neonicotinoid insecticide in cocoa production in the country.

While the prevalence of neonicotinoids in the soils studied may be due to high application rates and dosages, sorption may also have contributed to the level of persistence. Although sorption was generally low, considerable increase in sorption occurred based on type of neonicotinoid being sorbed and soil properties, particularly organic matter. In a comparative study of sorption behavior of the five neonicotinoids under study in four soils of contrasting properties, the findings showed generally low sorption behavior, demonstrated by the relatively low values of sorption parameters including K_d, K_{oc} and K_f. (chapter 5). Sorption behavior of all neonicotinoids correlated well with soil organic carbon content in soils studied, and hence sorption was normalized to organic matter. K_{oc} produced as a result is considered an intrinsic property of the chemical and enables comparison with other chemicals, irrespective of the soils used. Sorption parameters obtained in this study compares well with reported values in several studies in spite of the high influence of organic carbon, which were present in high quantities (Carbo et al., 2007; Kurwadkar et al., 2013a). In the soils studied, the high organic matter content observed may be due to the continuous addition of litter, as often occurs in forest

ecosystems. The average value of organic carbon in the soils studied was approximately 2.9% as indicated in chapter 2. In most farms, dense litter completely covers soil surfaces, often augmented by agronomic practices such as pruning and weeding. The warm prevailing temperatures and high rainfall patterns may also ensure faster decomposition and enrichment of soils with organic matter. Thus the influence of organic matter may be more pronounced over time, resulting in greater adsorption, ageing and hence persistence. In general sorption has been observed to restrict the degradation of pesticides by keeping compounds held up in the solid phase and limiting their bioavailability (L. Guo et al., 2000).

The sorption-desorption studies from the current work revealed that, binding of neonicotinoids to soils was not completely reversible, perhaps due to the formation of strong and stable bonds with soil components. This is consistent with the soils containing relatively high organic matter content which usually have huge influence on strong pesticide-soil interactions (Senesi, 1992) . Consequently, the lower percentage desorption further enhances the tendency for persistence as degradation is reduced in adsorbed components. Although retention of neonicotinoids in soils may control mobility, the current study on sorption behavior revealed a decrease in percentage adsorption of insecticides at higher concentrations. Under this condition (of high concentration), leaching of neonicotinoids into surface and underground water may be pronounced. Some studies suggest that, the batch equilibrium method as used in the current study, tends to overestimate sorption in comparison to other methods such as centrifugation (Yazgan et al., 2005). While this may present a challenge for the estimation of the fate of pesticides, it suggest an even greater potential for mobility and leaching of neonicotinoids into surface and groundwater. Nonetheless the merit or otherwise of the batch equilibrium over other methods are beyond the scope of this study.

The current study revealed relatively high DT_{50} values of > 150 days for both imidacloprid and thiamethoxam, the most widely used neonicotinoids in the country's cocoa production. Degradation of both insecticides was best modelled with simple first order kinetics. However deviations from the first order kinetics was seen by a slight increase in degradation rate at the end of the process preceded by a long initial lag phase. The generally low sorption values, may also support the logic against DFOP (biphasic) as the best fit model. This is because biphasic degradation is often seen when a substantial part of the pesticide is sorbed to soil particles.

The lag phase could be attributed to the adaptation of microorganisms, although other experimental factors including temperature and moisture conditions may have played a role. For instance, studies have reported large variations in dissipation rates of about 40 folds as a results of changes in environmental conditions such as temperature and moisture content of soils (Gupta et al., 2008). The high dissipation half-lives obtained in this study are in agreement with the reported levels of persistence in cocoa farms across the country (Dankyi et al., 2014).

Based on the findings from this study, it is quite clear that the persistence of neonicotinoids, coupled with their high water solubility and leaching potential presents a cause for environmental concern. In this regard, more efficient and controlled usage of these insecticides is needed to ensure environmental safety.

6.1.3 Accumulation of neonicotinoids in food crops

The low toxicity of neonicotinoids to mammals notwithstanding, low levels are expected in food. In the current study, neonicotinoid insecticide residue levels in cocoa beans from all the major cocoa growing regions of the country were assessed for their level of exposure. As expected, large variations in concentration levels were observed across samples, due to peculiar application patterns. Even though neonicotinoid levels were generally low in many samples, relatively high concentrations were obtained in some samples, particularly the shells, although

shells are usually removed during the processing of the beans. Quite clearly, the high levels of residues in cocoa shells may be due to high application rates and dosages. The systemic activity of neonicotinoids may also play a role in their buildup in plant parts. As systemic insecticides, neonicotinoids are taken up into plants once applied, and are translocated to the growing parts of the plants. Thus, at high dosage of application the tendency of buildup in growing parts of the plant is high. Given their high target-selectivity, neonicotinoid insecticides are generally safe to humans. Nonetheless, their presence and relatively high levels in some cocoa beans and shells is a cause for concern and will require controlled use of these insecticides and continuous monitoring to ensure food safety.

6.1.4 Insecticide policy, food and environmental safety

The important role of insecticides in crop production as well as the negative effects of their use is generally recognized. As such, considerable efforts have been made by countries, organizations and international agencies to ensure low levels of pesticides in the environment, food and non-target organism. In cocoa production in Ghana, great efforts have been put into ensuring good quality cocoa beans as well as improved yields. Among several interventions, the free insecticide application policy was commenced over a decade ago and has not only helped improve yields, but has also ensured the use of only approved and safer insecticides in cocoa production in the country.

However, as it often pertains to pesticide usage, concerns for food and environmental safety are important for sustainable usage, particularly given the intensive application rates and general abuse of pesticides, encouraged by the ease of access to approved chemicals from the Ghana cocoa board. Based on findings from this study, the high tendency for accumulation of neonicotinoids in cocoa beans coupled with their prevalence in soils, suggest a need for greater efficiency and control in their application. In this regards, the re-evaluation of the current policy

on free insecticides in cocoa production, as well as a review of current recommendations of repeated monthly application in farms may be important in order to ensure sustainable cocoa production and safety with minimal environmental impact.

6.2 Perspectives

Over the past few years, a great detail of attention has been placed on neonicotinoids due to their possible harmful effects on bees and other beneficial arthropods. While research outcome on actual relationship between these insecticides and arthropods is sought, the contrast in their effectiveness for pest management in diverse crop production remains. This may have resulted in the partial lifting of the ban on neonicotinoids in parts of Britain to enable farmers control flea beetle damage in oilseed rapes, following a second emergency call by farmers (BBC, 2015).

The current results on neonicotinoid fate are based on laboratory studies by simulation of field conditions, and give a good indication of their behavior in soils. However, research has observed that, sorption coefficients in tropical soils tend to change significantly between laboratory and field conditions, particularly for polar pesticides such as neonicotinoids (Laabs and Amelung, 2005). Thus field experiments may be important in providing more realistic long-term knowledge of neonicotinoid behavior in the soils studied and are recommended.

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Appendix A

A.1 Published Paper:

Quantification of neonicotinoid insecticide residues in soils from cocoa plantations using a QuEChERS extraction procedure and LC-MS/MS

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Quantification of neonicotinoid insecticide residues in soils from cocoa plantations using a QuEChERS extraction procedure and LC-MS/MS

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HIGHLIGHTS

- First published data of neonicotinoid insecticide levels in West African soils.
- Extraction was performed using a QuEChERS procedure optimized for soils.
- Significant amount of imidacloprid present in soils several months after application.

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ABSTRACT

The use of neonicotinoids as an insecticide group in Ghana has been quite significant particularly in cocoa production. The high usage has been mainly as a result of a government policy of free insecticide spraying on cocoa farms, in an effort to curb declining yields caused by pests and diseases and to prevent the use of unapproved or banned insecticides on cocoa farms. However the scale of cocoa farming, the frequency and intensity of usage coupled with the mode of application may result in large physical volumes of insecticides in the environment. This makes the knowledge of the concentration and fate of neonicotinoids in the environment extremely important. The present study was aimed at assessing the levels of five major neonicotinoids in soils from cocoa farmlands in Ghana. Extraction and cleanup of analytes were performed by use of a method based on the original QuEChERS procedure after optimizing salts, sorbents and instrumental conditions. Analyte extraction with NaCl and $MgSO_4$ in acidified acetonitrile followed by cleanup with primary secondary amine (PSA) presented the optimum conditions for extraction. Quantification was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI). Validation of the procedure showed average recoveries ranging from 72.0 to 104.8% for all analytes at all fortification levels with relative standard deviation (RSD) $\leq$ 15.0. Limits of quantitation were $< 10\ \mu g\ kg^{-1}$ for all neonicotinoids studied. The results obtained from the analysis of 52 samples from cocoa farms revealed imidacloprid as the predominant neonicotinoid with concentrations ranging from 4.3 to 251.4 $\mu g\ kg^{-1}$ in > 50% of samples analyzed.

1. Introduction

The demand for higher food production has often been addressed by an increase in usage of agrochemicals such as pesticides and fertilizers. This is particularly the case in moist tropical environments where the prevailing warm and humid conditions tend to promote the growth and reproduction of insect pests. In Ghana, pests and diseases have been recognized as a major cause of declining yields in cocoa production (COCOBOD, 1995), a situation that prompted an upsurge in use of insecticides in general and neonicotinoids in particular. However, the active use of insecticides has often been associated with unintended environmental and human health consequences. In Africa and in most developing parts of the world, the consequences have often been severe due to misuse and overuse of chemicals instigated by ignorance or a lack of safety concerns, as well as a general lack of effective regulations on chemical usage (Ondieki, 1996; Fianko et al., 2011).

Pesticide use in Ghana is considered to be generally low albeit intensive in areas where usage occurs (Gerken et al., 2001). Application is concentrated in cocoa, vegetable and fruit production with reported cases of overuse and misuse (Ntow et al., 2006). Current usage of neonicotinoid insecticides is quite substantial in crop production in Ghana. Based on the country's latest data from the EPA pesticide registry, neonicotinoids are present in a greater number of formulations than any other class of insecticide. They are currently applied on cocoa farms in Ghana at no cost to farmers, or given free to farmers for self-application. In cocoa plantations, the insecticides are applied to the

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leaves and branches of trees by use of back-pack manual sprayers or motorized mist blowers. The tendency by farmers to misapply the insecticide is not only enhanced by the minimal financial burden but also the tacit approval by the Cocoa Research Institute (Tafo) in Ghana.

Studies have shown that significant amounts of applied neonicotinoids will end up in the soil (Sur and Stork, 2003). The fate of these compounds in soil is however uncertain, with a wide range of dissipation rates reported in literature (El-Hamady et al., 2008; Juraske et al., 2009; Pitam et al., 2013; Ramasubramanian, 2013). For instance in a review by Goulson (Goulson, 2013), imidacloprid and thiamethoxam are reported to have half-lives ranging from 28–1250 days and 7–301 days respectively. Though no systematic reviews exist on root causes of these wide variations, they are believed to be as a result of differing soil types, soil physico-chemical properties and environmental conditions.

It is quite obvious that useful decision-making regarding the safety and management of pesticide use in developing countries needs to be based on reliable qualitative and quantitative assessment of these compounds in the environment. This will require the use of simple, inexpensive and effective techniques in the extraction, separation and detection of analytes.

The QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) procedure developed by Anastassiades et al. (2003) has been shown to offer several of these advantages over other conventional and more sophisticated extraction methods (Lesueur et al., 2008; Prestes et al., 2012). The technique, which is based on a salting-out extraction of analytes in acetonitrile, followed by a dispersive solid phase extraction (d-SPE) clean-up procedure has been developed and optimized for a wide range of pesticides in food, mainly fruits and vegetables. In recent years, the technique has been applied to other food and environmental matrixes including animal products, water, sediments and soil (Xia et al., 2010; Angioni et al., 2011; Peña et al., 2011; Martins et al., 2013).

Several QuEChERS procedures exist including three extensively used salting out and sorbent clean-up procedures each. The existence of these diverse sorbents and salting out conditions has given much room for flexibility in utilizing any combination of salts–sorbents for multi-residue analysis often with good recoveries for most analytes. This flexibility is evident in the diversity of QuEChERS extraction and clean-up procedures that have been applied to one particular matrix such as soil (Lesueur et al., 2008; Dong et al., 2009; Drozdzyński and Kowalska, 2009; Asensio-Ramos et al., 2010; Chen et al., 2010; Rashid et al., 2010; Shi et al., 2010; Yang et al., 2010a; Caldas et al., 2011).

In nearly all cases of the application of these methods originally designed for food matrixes to other matrixes, no optimization has been done. This may be as a result of the good recoveries associated with the QuEChERS procedures as well as the application of the method mainly to multi-class pesticide analysis and hence the lack of need to optimize for one particular class of analytes. Lehotay et al. (2010) have shown that, although some differences may exist in recoveries of analytes from some matrixes, salting-out or sorbent conditions, in general differences are often subtle and insignificant when different QuEChERS procedures are employed for multi-class pesticides analysis in different food matrixes.

However, the absence of extensively researched data in the soil matrix particularly the soils being investigated, coupled with the availability of several QuEChERS procedures and no clearly defined procedures for soils make the need for optimization useful for the class of insecticides being investigated in this study. The purpose of the study therefore, was to determine the concentration levels of five neonicotinoid insecticides (Fig. 1) in tropical soils after extraction with an optimized QuEChERS procedure.

2. Materials and methods

2.1. Soil sampling

Cocoa production in Ghana is mainly concentrated in the forest regions, which covers the south-western parts of the country and comprises six out of the ten political regions (Fig. 2). Soil samples were obtained from cocoa farms across all the major cocoa producing regions of the country at a depth of 0–20 cm. In each farm, samples were obtained from at least five different locations 50 to 100 m apart. Soils obtained from a minimum of three to six different farms formed an aggregate sample representing a village/town. In all, 52 aggregate samples were obtained from the six major cocoa regions. After careful removal of litter, roots, stones and other large exogenous objects, samples were air-dried at room temperature and sieved using a 2 mm mesh and stored at ambient temperature prior to analysis. Samples obtained from forests and abandoned farms were used in method development and validation.

2.2. Reagents and chemicals

HPLC grade acetonitrile and methanol from Rathburn (Walkerburn, Scotland) and optima grade acetic acid (Fisher, Canada) were purchased for this study. Ammonium acetate and sodium chloride were purchased from Merck (Darmstadt, Germany). Sodium acetate, sodium citrate dihydrate (SCTD) and sodium citrate dibasic sesquihydrate (SCDS) were obtained from Sigma-Aldrich (Germany). Magnesium sulfate was purchased from BDH (Leuven, Belgium). Sorbent tubes (2 ml) containing magnesium sulfate with combinations of primary secondary amine (PSA), octadecyl C-18 and graphitized carbon black (GCB) were obtained from Phenomenex (CA, USA).

Analytical standards (purity > 99%) of acetamiprid, imidacloprid, thiacloprid, clothianidin and thiamethoxam were obtained from Fluka (Germany). Deionized water was prepared using a Millipore Milli-Q purification system (Millipore, USA).

2.3. Standard solutions and calibration curves

Stock solutions of each insecticide were prepared by dissolving an exact amount of each solid compound in acetonitrile. A stock mixture of insecticides of same concentration was obtained from individual

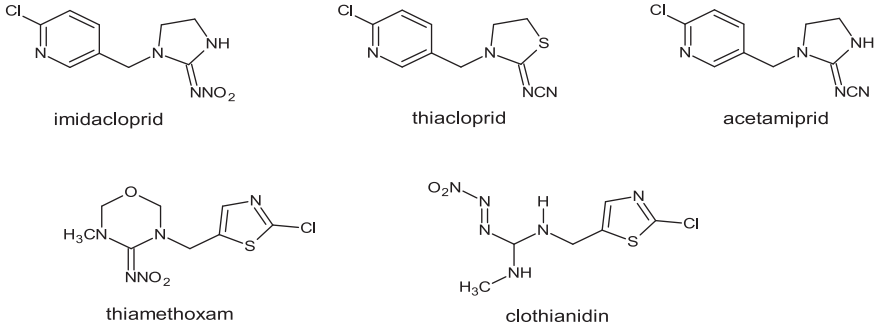

Fig. 1. Chemical structures of neonicotinoids investigated.

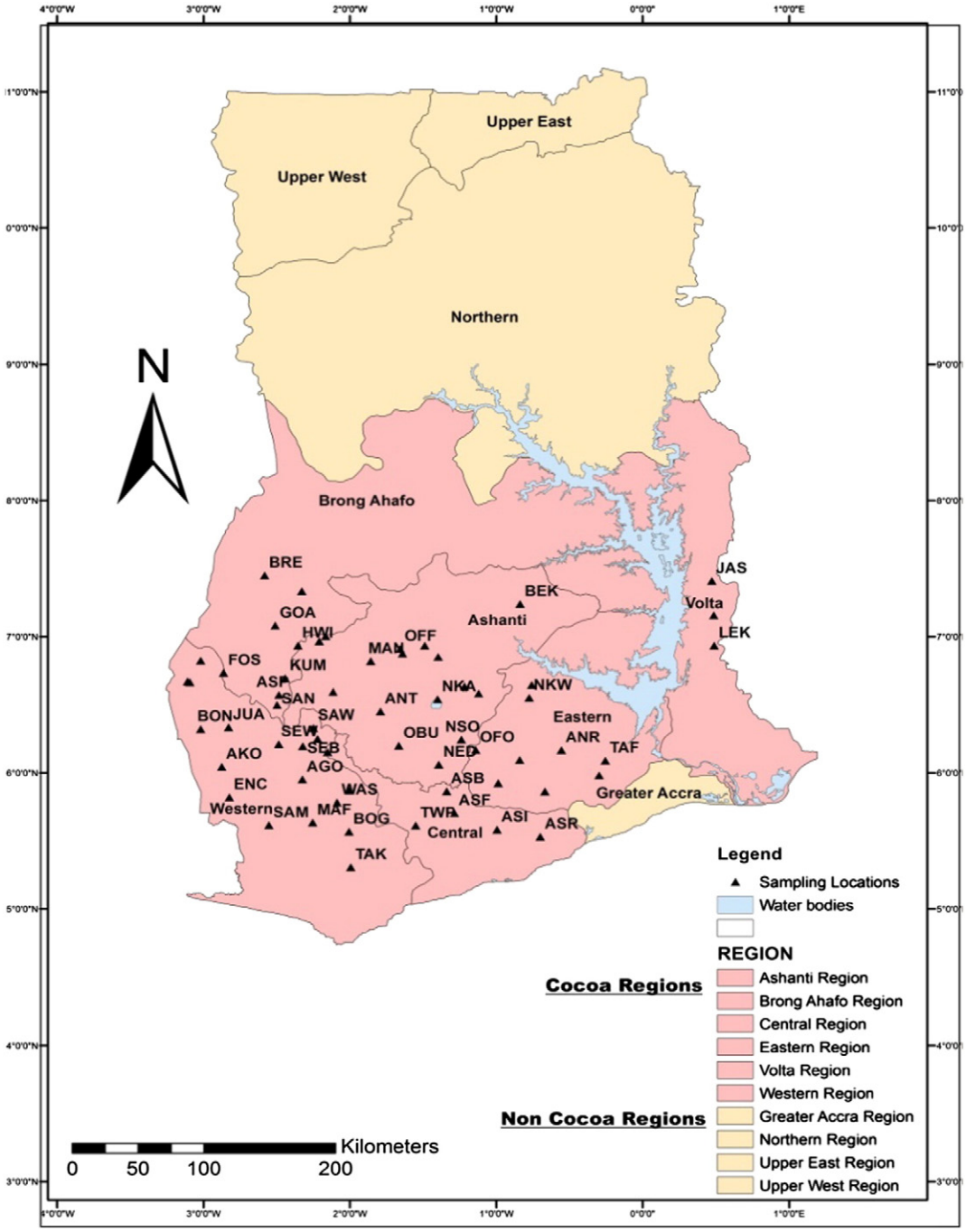

Fig. 2. A map of GPS locations of sampling in the major cocoa-growing regions of Ghana.

stock solutions by measuring and combining the desired volumes. An aliquot of this mixture was then diluted in acetonitrile to obtain concentrations of 0.78, 1.56, 3.125, 6.25, 12.50, 25, 50, 100, 200, and 400 μg L^{-1} used for calibration. Standard solutions were all stored at < 4 °C prior to use. Concentrations of neonicotinoids (μg L^{-1}) in all sample extracts mentioned in the subsequent sections were quantified on the basis of the standard curves. Subsequently the amount of soil was taken into consideration for the final calculation of neonicotinoids in μg kg^{-1}.

2.4. LC-MS instrumentation

Chromatographic separation of analytes was performed on an Agilent 1200 HPLC system (Santa Clara, CA, USA) coupled with an AB Sciex (Forest City, CA) 3200 QTRAP mass spectrometer (MS). The MS was equipped with electrospray ionization (ESI). Multiple reaction monitoring (MRM) data were acquired and processed for all analytes in positive ion mode. Optimized values of declustering potential (DP), exit potential (EP), collision energy (CE) and collision cell entrance potential (CEP) are listed in Table 1. The selection and optimization of precursor ion and product ions for each analyte were carried out by direct injection of standards prepared in methanol: water (50:50 v/v) at a flow rate of 200 μL min^{-1}. For all compounds, the efficiency of ionization was best in positive mode. Optimal values of instrumental parameters were selected and applied to obtain the best MRM transition with the highest intensities possible. The two most intense precursor-to-product ion transitions were chosen for each compound: the most

Table 1
Instrument conditions and MRM transitions of precursor/product ions of analytes.

Analyte	Ion transition (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)
Imidacloprid	256.13 → 209.10*	31.0	4.5	17.3	27.0
	256.13 → 175.00	31.0	4.5	17.3	23.0
Acetamiprid	223.14 → 126.00*	41.0	2.0	16.4	29.0
	223.14 → 99.10	41.0	2.0	16.4	53.0
Thiacloprid	253.06 → 126.00*	51.0	2.5	17.2	29.0
	253.06 → 99.00	51.0	2.5	17.2	57.0
Thiamethoxam	292.08 → 211.20*	26.0	9.5	18.3	17.0
	292.08 → 181.00	26.0	9.5	18.3	31.0
Clothianidin	250.00 → 169.01*	26.0	8.0	17.1	17.0
	250.00 → 132.00	26.0	8.0	17.1	19.0

* Transitions used in quantitation.

intense being used for quantification and the other used for confirmation. This was particularly useful in the cases of imidacloprid and clothianidin, both of which produced two transitions of similarly high intensities. Data obtained were processed using the Analyst software (version 1.5.2).

The LC system was equipped with a BDS Hypersil reversed-phase C-18 column (250 mm × 2.1 mm; 5 μm) (Thermo Electron Co., UK), at a temperature of 30 °C. Mobile phase A and B consisted of 99% 10 nM ammonium acetate, with 1% methanol and 90% methanol with 10% 10 nM ammonium acetate respectively. The chromatographic method began with an initial mobile phase composition of 10% for solvent B, increased to 100% over 10 minutes, held constant for a further 10 minutes and decreased to 10% for 1 min. The injection volume of sample extracts or standards was 10 μL. The flow rate was 200 μL min^{-1}, and the total run time was 21 minutes. Optimization of chromatographic conditions involved the use of different eluents, varying gradients and injection volumes in order to obtain good chromatograms in the shortest time possible.

2.5. Extraction procedure

An assessment of various salting out procedures based on the QuEChERS technique was performed using 5 g of blank soil. Samples were placed into 50 ml Falcon tubes and spiked with known concentrations of analytes. After allowing to stand for about 45 minutes, 5 ml of distilled water was added and mixed with the sample. 10 ml of acetonitrile in 1% acetic acid was then added and the resulting mixture hand-shaken vigorously for one minute. Three different mixtures of salts commonly used in the QuEChERS procedure: (i) 4.0 g $MgSO_4$ & 1.5 g NaOAc (ii) 4.0 g $MgSO_4$ & 1.0 g NaCl (iii) 4.0 g $MgSO_4$, 1.0 g NaCl, 1.0 g SCTD & 0.5 g SCDS were assessed by adding each to separate blank samples. The resulting mixtures were hand-shaken vigorously for one minute and then centrifuged at 4000 RPM for 5 minutes. The supernatant was separated and used for the next step. Six replicates were prepared for each salting out procedure.

2.6. Clean-up procedure

Three of the most widely used solid-phase sorbents, primary secondary amine (PSA), C-18 and graphitized carbon black (GCB) were examined for their efficiency of clean-up of the matrix. The sorbents were added in the following compositions: (i) 25 mg PSA + 150 mg $MgSO_4$; (ii) 25 mg PSA + 25 mg C18 + 150 mg $MgSO_4$; (iii) 25 mg PSA + 7.5 mg GCB + 150 mg $MgSO_4$. Each sorbent/sorbent mixture was added to a 1 ml aliquot of salt extract, vortex-mixed for one minute and centrifuged at 4000 RPM for 3 minutes. The supernatant was separated and filtered through a 0.45 μm PTFE syringe filter. Aliquots of this solution were diluted with an equal volume of water prior to injection onto the LC column. To assess the need for a clean-up step, aliquots of the solution obtained from the salting-out step were injected directly onto the column after addition of 150 mg of $MgSO_4$, followed by filtration and dilution without the use of clean-up sorbents. The addition of the $MgSO_4$ was not only to ensure the removal of any remaining molecules of water but to ensure uniformity in the sample matrix such that any improvement in efficiency of recovery could only be attributable to the use of the sorbents which were all pre-packed with $MgSO_4$.

2.7. Method validation

The performance of the various salting out and clean-up procedures was assessed by evaluation of parameters including accuracy, precision, matrix effects, limit of detection (LOD) and limit of quantification (LOQ). For each procedure, the accuracy and precision was evaluated by estimating percentage recoveries and relative standard deviations (RSDs) at two fortification levels (8 and 80 μg kg^{-1}) for all neonicotinoids.

The linearity of the analytical procedures was studied using matrix-matched calibration solutions prepared in blank soil extracts, using the different extraction and clean-up procedures at a concentration range between 1.56 and 400 μgL^{-1}. LOD and LOQ were estimated by injection of matrix-matched standard solutions at the lowest concentration that yielded a signal to background noise (S/N) ratio of three and ten respectively. An assessment of the influence of matrix was done by comparing responses of matrix-matched and solvent standards (Pizzutti et al., 2007). The absolute matrix effect was estimated by comparing the slopes in calibration solutions prepared in matrix and in solvents.

3. Results and discussion

3.1. Soil physical and chemical properties

Despite the high rate of decomposition/depletion of organic matter often found in tropic soils, soils from cocoa farms were characterized by high organic carbon content perhaps due to the high influx of litter. In most farms, litter was greater than 5 cm in thickness completely covering the soil. Soil organic carbon content determined using the Walkley-Black method (Walkley and Black, 1934) is shown in Table 2. The measured pH (in calcium chloride) ranged from 4.46 to 7.54 (average, 6.20) and reflected the varying soil types and their associated physical and chemical properties in most regions of Ghana. Based on grain size, results indicate the cocoa soils as predominantly sandy–loam, loam or sand–clay–loam (Fig. 3) according to the United States Department of Agriculture (USDA) classification system (USDA, 1987). Texture analysis was performed using the hygrometer method. In general the content of sand was greater than 50% in the majority of samples analyzed and varied widely whereas clay content was lower than 20% in about half of the analyzed samples. The content of clay was less variable across farms in the various geographical location of the country as seen in Fig. 3. These soil physico-chemical properties may play an important role in determining the fate of neonicotinoids in the soils. From the results obtained, the low variability of important physico-chemical properties across cocoa-growing regions of the country may aid in the prediction of insecticide fate and management across the country.

Table 2
Soil organic carbon, pH and clay content of soils.

Sample	Farm (n = 52)	Blank[a]
SOC (%)	1.28–7.43 (2.94)	3.63
pH ($CaCl_2$)	4.46–7.54 (6.20)	6.52
Clay content (%)	12.4–49.5 (20.4)	27.3

[a] Properties of soil used in method development.

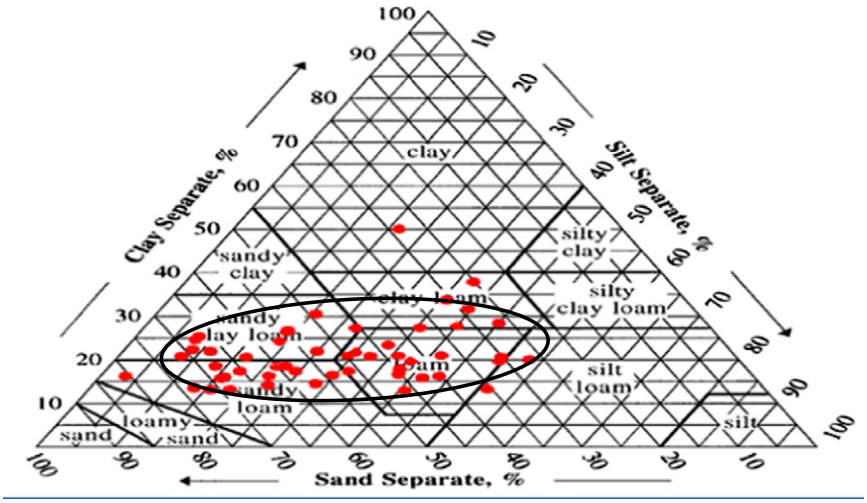

Fig. 3. Soil texture classification based on the USDA textural triangle.

3.2. Salting out extraction procedure

Average recoveries obtained from the extraction of neonicotinoids in soils with various salts are shown in Table 3. Good recoveries of analytes (77.5–96.8%; RSD ≤ 9.0) were obtained for all salts at the 80 μg kg^{-1} fortification level without the need for clean-up sorbents. However analyte recoveries at the 8 μg kg^{-1} fortification level were relatively low with higher relative standard deviations (48.6 to 87.4%; RSD ≤ 17.7) especially when citrate salts were used for extraction. Better yields were obtained from both NaCl & $MgSO_4$ (76.5–87.4; RSD ≤ 14.2) and NaOAc & $MgSO_4$ (64.9–86.3; RSD ≤16.2) extracts for all analytes. The low recoveries with high relative standard deviations for analytes at the lower fortification level may have been due to a higher influence of matrix at that low concentration of analytes.

Differences in recoveries for the three salts were minimal as shown in Fig. 4 for the 80 μg kg^{-1} level of fortification. However the differences were found to be statistically significant in almost all analytes except thiamethoxam, based on a one way analysis of variance (ANOVA) at a 95% confidence interval. Bonferroni post hoc comparisons suggest the choice of NaCl & $MgSO_4$ for extraction resulted in significantly higher yields in almost all analytes compared to the other two salt mixtures. The peculiarity in the results of thiamethoxam may have been due to the high variability among replicates which led to higher values of RSDs.

Besides inducing phase separation, the type of salts applied in extracting analytes under the QuEChERS procedure may impact pH, matrix polarity and matrix constitution thereby influencing recovery. Although the addition of the various salts impacted on the pH of the matrix (citrate, chloride and acetate salts in increasing order of pH), its influence on analytes as well as matrix components is unclear in the current study. It was however observed that soil pH had less of an influence on the final pH of the matrix after extraction.

In general, the important differences in the various salts used under the QuEChERS procedure are the resulting pH of the medium and the presence or absence of buffering. The original salting-out procedure employed the use of NaCl & $MgSO_4$ in acetonitrile (Anastassiades et al., 2003). However, low recoveries of some pH sensitive analytes prompted the need for a buffering medium. The result of the application of sodium acetate and sodium citrate in response to this need increased recoveries remarkably leading to the adoption of both as official methods (AOAC 2007.01 and EN 15662 respectively) (Lehotay et al., 2005).

The results obtained in this study however, do not suggest a need for a buffering medium for neonicotinoids in the matrix being studied. Despite the minimal differences, NaCl & $MgSO_4$ were chosen as optimum for extraction due to the higher yields and lower RSDs compared to the other salts. Shi et al. (2010) and Dong et al. (2009) have reported similar salt choices in acetonitrile extraction of oxadiargyl and metaflumizone respectively from soils without the need for buffering. While acetate salts have been used in some studies (Rashid et al., 2010), the use of citrate salts appears to be the most commonly reported in literature for soil matrix (Lesueur et al., 2008; Asensio-Ramos et al., 2010; Yang et al., 2010b; Qiao et al., 2011). It is quite obvious that the important considerations in the choice of a particular salting out procedure are largely based on the nature of analytes and matrix under study (Lehotay et al., 2005).

3.3. Clean-up procedure

Recovery of analytes following d-SPE ranged from 78.5 to 104.8% and 48.6 to 87.2% at 80 and 8 μg kg^{-1} fortification levels respectively (Table 4). Yields were improved after clean-up, in almost all analytes and sorbent types particularly at the 80 μg kg^{-1} level of fortification.

Table 3
Percentage recoveries of neonicotinoids with varying salting out extraction procedures at two fortification levels (μg kg^{-1}) (n = 6 for each treatment).

Salts	Fortification	Imidacloprid	Thiacloprid	Thiamethoxam	Acetamiprid	Clothianidin
NaOAc & $MgSO_4$	8	71.0 ± 9.6	64.9 ± 16.2	86.3 ± 10.9	72.0 ± 10.7	76.5 ± 11.4
	80	83.8 ± 3.3	80.8 ± 2.8	84.9 ± 6.7	92.4 ± 2.4	88.9 ± 1.3
NaCl & $MgSO_4$	8	77.0 ± 13.8	76.3 ± 8.9	87.4 ± 14.2	76.5 ± 6.0	77.3 ± 14.0
	80	91.2 ± 5.0	89.3 ± 3.5	86.1 ± 6.9	93.9 ± 4.2	96.8 ± 1.3
NaCitrate & $MgSO_4$	8	53.6 ± 17.7	48.3 ± 11.4	72.9 ± 8.6	55.0 ± 10.4	65.4 ± 14.4
	80	85.2 ± 2.7	77.5 ± 5.5	90.1 ± 9.0	86.7 ± 3.6	90.1 ± 8.0

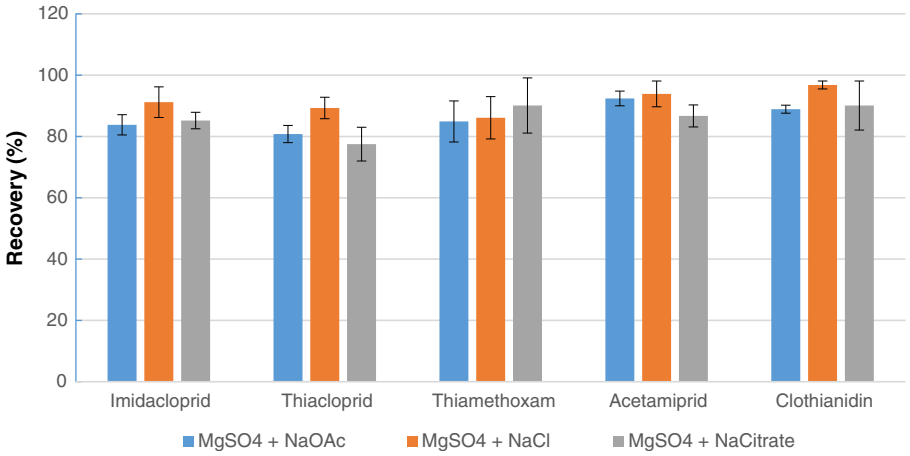

Fig. 4. Percentage recoveries of neonicotinoids showing RSDs after extraction with different salts at 80 μg kg^{-1} level of fortification (n = 6).

Recoveries were highest with PSA and appeared to decrease upon addition of C-18 and GCB. With the exception of thiamethoxam, analysis of variance indicated higher yields of statistical significance for all analytes upon PSA clean-up at the 80 μg kg^{-1} level of fortification. The apparent effectiveness in the use of PSA as sorbent was evident in all salt matrixes. However analytes appeared less amenable to d-SPE clean-up at the relatively low concentration of 8 μg kg^{-1} (Table 4). This may probably have been due to a relative increase in matrix influence at the low concentrations of analytes, closer to their limits of quantification as suggested in Section 3.2.

The QuEChERS procedure as originally developed by Anastassiades et al. (2003) comprises a salting out extraction followed by a d-SPE clean-up procedure. The addition of the clean-up step was important in removing most matrix co-extracts. Soil as a matrix is very complex and diverse. This complexity is further enhanced by high levels of organic matter which have been reported to decrease extraction efficiency (Bragança et al., 2012; Correia-Sá et al., 2012) often warranting the use of a clean-up procedure. PSA has been employed often in the removal of organic acids, fatty acids and sugars from diverse sample matrixes. The presence of primary and secondary amines in its structure helps in the retention of many polar compounds including sugars, organic acids and fatty acids which may have been components in the soil matrix studied. Unlike PSA, the addition of C-18 commonly used in the removal of fats and other non-polar compounds as well as GCB often used in the removal of pigments tends to decrease the recovery of analytes suggesting perhaps the absence of these as matrix components and a possible adsorption of analytes by these sorbents.

By far the choice of sorbent in clean-up extraction of various analytes from soils has been PSA (Lesueur et al., 2008; Dong et al., 2009; Asensio-Ramos et al., 2010; Yang et al., 2010b). Reported recoveries however vary, perhaps based on analytes and matrix composition. The use of PSA & C-18 as sorbents has been reported by Drozdzynski and Kowalska with recoveries ranging from 83–104% (Drozdzyński and Kowalska, 2009). However not all studies have employed sorbents. Shi et al. (2010) have reported recoveries ranging from 82.9 to 112% for oxadiargyl residues in soils after a salting out extraction with $MgSO_4$ and NaCl without the need for clean-up. Similarly Caldas et al. (2011) have reported recoveries of 70.3 to 120% in the QuEChERS extraction of multiple classes of pesticides from soils without clean-up sorbents. In this study, the importance of a clean-up procedure was particularly evident at higher concentrations (80 μgkg^{-1}) of analyte and less so at lower concentrations (8 μgkg^{-1}). Although PSA was found to be the best sorbent for the clean-up of analytes in the matrix studied, the choice of a clean-up procedure will be largely based on the degree of precision required. In the current study, a clean-up procedure using PSA as sorbent was chosen for the reasons stated above.

Table 4
Percentage recoveries of neonicotinoids from varying d-SPE clean-up conditions (n = 6 for each treatment).

Level	Salts	Sorbent	Imidacloprid	Thiacloprid	Thiamethoxam	Acetamiprid	Clothianidin
80 μg kg^{-1}	NaOAc & $MgSO_4$	PSA	97.8 ± 2.7	92.3 ± 1.2	100.5 ± 2.5	101.2 ± 2.4	100.0 ± 5.6
		PSA + C18	93.2 ± 3.5	89.3 ± 3.9	92.5 ± 9.3	96.7 ± 2.6	98.3 ± 1.8
		PSA + GCB	92.8 ± 2.7	84.5 ± 2.8	87.9 ± 12.9	94.3 ± 1.6	94.0 ± 1.9
	NaCl & $MgSO_4$	PSA	98.6 ± 3.1	97.1 ± 1.9	94.0 ± 9.0	102.9 ± 1.7	104.8 ± 2.1
		PSA + C18	96.3 ± 1.9	95.5 ± 3.5	92.4 ± 10.3	99.6 ± 3.9	98.6 ± 3.9
		PSA + GCB	94.5 ± 1.6	91.7 ± 3.3	85.3 ± 6.9	96.5 ± 1.1	97.3 ± 4.3
	NaCitrate & $MgSO_4$	PSA	89.8 ± 5.3	81.0 ± 6.9	92.2 ± 5.5	93.4 ± 4.5	89.3 ± 6.7
		PSA + C18	86.5 ± 3.7	80.3 ± 5.6	84.8 ± 11.7	89.6 ± 4.4	91.8 ± 2.8
		PSA + GCB	84.7 ± 3.7	78.5 ± 5.5	87.4 ± 9.6	88.9 ± 2.9	88.4 ± 9.5
8 μg kg^{-1}	NaOAc & $MgSO_4$	PSA	65.2 ± 13.5	63.8 ± 19.1	76.1 ± 14.1	72.0 ± 12.9	70.6 ± 13.7
		PSA + C18	67.0 ± 20.9	63.8 ± 16.5	80.2 ± 13.3	70.7 ± 13.0	74.1 ± 18.6
		PSA + GCB	60.2 ± 19.4	60.9 ± 17.2	82.3 ± 10.3	69.1 ± 12.4	68.0 ± 18.8
	NaCl & $MgSO_4$	PSA	79.3 ± 11.3	79.6 ± 8.7	85.8 ± 15.0	81.3 ± 4.8	72.0 ± 14.4
		PSA + C18	78.5 ± 17.2	77.4 ± 6.8	86.4 ± 9.1	76.7 ± 7.3	69.3 ± 13.9
		PSA + GCB	87.2 ± 7.5	74.2 ± 6.5	85.2 ± 11.0	75.3 ± 5.0	74.5 ± 13.2
	NaCitrate & $MgSO_4$	PSA	58.2 ± 13.4	51.0 ± 8.8	75.2 ± 5.9	56.8 ± 8.5	60.7 ± 6.3
		PSA + C18	62.5 ± 13.6	50.6 ± 11.1	74.6 ± 9.9	55.0 ± 9.1	63.9 ± 20.8
		PSA + GCB	48.6 ± 17.7	48.8 ± 8.6	75.4 ± 8.4	54.3 ± 6.1	49.8 ± 21.9

3.4. Validation of QuEChERS procedure

The performance of the chosen procedure was evaluated for linearity, precision, LOD and LOQ. Concentration of neonicotinoids was calculated from matrix-matched calibration standards using the peak area with a weighting of $^1/x$. Calibration curves were linear over a range of 1.56 to 400 μgL^{-1} for all neonicotinoids with correlation coefficients ≥ 0.9986 (Table 5). Percentage recoveries ranged from 72.0 to 104.8 with RSD ≤ 15.0 for all neonicotinoids at both 8 and 80 μgkg^{-1} fortification levels indicating good recovery and precision as recommended by the DG SANCO guidelines (SANCO/12571/2013). The limit of quantification (LOQ) was estimated between 2.0 and 9.0 $\mu g\ kg^{-1}$ based on analyte. Dong et al. (2009) have reported LOQ of 4.0 μgkg^{-1} in the analysis of metaflumizone from soil using a similar procedure on an UPLC-MS/MS. Ramasubramanian (2013) reports LOQ of 10 μgkg^{-1} using HPLC with diode array detection in clothianidin analysis employing a similar salting out procedure without further clean-up. Even lower LOQ of < 1 μgkg^{-1} has been reported for this procedure using UPCL-MSMS (Mei et al., 2011). In general the results obtained were comparable to results from similar procedures in literature despite the difference in analytes and some parameters.

Matrix influence was low particularly when sodium chloride and magnesium sulfate were used in extraction. The effects were more pronounced at lower concentration of analytes especially in the citrate salt extraction procedure. The low matrix influence may have been due to the use of clean-up sorbents as well as the highly selectivity of the LC-MSMS process. Nonetheless, all calibrations of analytes were performed in matrix-matched standards.

3.5. Application to soil samples from cocoa farms

The developed procedure was applied in the extraction and analysis of neonicotinoid insecticides in fifty two soil samples obtained from cocoa farmlands in Ghana. In all farms, the most current application of neonicotinoids had occurred at least four months prior to sampling with some cases beyond two years. Samples were analyzed on an LC-MS/MS instrument after extraction with magnesium sulfate and sodium chloride in acetonitrile with 1% acetic acid. Extracts were further cleaned up using PSA as sorbent. Quantities of salts, sorbents and solvents used were as described in the procedure development (Sections 2.5 and 2.6). Results obtained are shown in Table 6.

Acetamiprid, thiacloprid and thiamethoxam were not detected in any of the samples analyzed. While acetamiprid is not yet recommended for use in cocoa production, thiacloprid marketed as Proteus® (150 g/L thiacloprid + 20 g/L deltamethrin) and thiamethoxam marketed as Actara® are approved for cocoa production. However their use is quite limited based on responses from farmers and cocoa technical officers perhaps reflecting their apparent absence in cocoa soils sampled.

Table 5
Matrix effects and LOQ in optimized procedure: salting out extraction with NaCl & $MgSO_4$ and clean-up with PSA.

Analyte	Solvent (%)[a]	Matrix (%)[b]	Matrix effect[c]	R^2	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)
Imidacloprid	88.3	97.8	0.91	0.9998	2.0	4.0
Thiacloprid	94.6	97.1	1.01	0.9986	1.0	2.0
Thiamethoxam	96.8	94.0	1.01	0.9995	2.0	5.0
Acetamiprid	97.7	102.9	0.95	0.9992	1.0	2.0
Clothianidin	103.0	104.8	0.97	0.9999	3.0	9.0

[a] Analytes recovery using solvent calibrated standards.
[b] Analyte recovery using matrix-matched calibration standards.
[c] Matrix effect expressed as the ratio of the slopes of the calibration curves obtained from the matrix-matched standards and solvent standards.

Table 6
Concentration of neonicotinoids ($\mu g\ kg^{-1}$) in soil samples from cocoa farms in Ghana.

Sample	Imidacloprid	clothianidin	Sample	Imidacloprid	clothianidin
OBU	251.4	23.1	NKR	4.4	–
ASK	110.0	–	ASA	4.3	–
SAN	82.2	–	ASM	–	–
BRE	64.0	–	DUN	–	–
JUA	58.4	–	SAW	–	–
OFO	49.6	–	HWI	–	–
WAS	48.0	–	ASI	–	10.1
ASB	32.1	12.2	GOA	–	–
TWP	34.5	–	KAS	–	–
KPA	29.6	–	TWH	–	–
SEB	23.5	–	ANT	–	9.8
BEK	21.5	–	TAF	–	–
ANY	18.9	–	ENC	–	–
SAE	18.4	–	ASR	–	–
LEK	13.4	–	JAS	–	–
MIM	13.3	–	BON	–	–
DOR	11.0	–	KAJ	–	–
NSO	8.9	15.9	KON	–	–
ASU	8.4	–	MAN	–	–
FOS	8.0	–	SEW	–	–
SUN	7.2	–	NKA	–	–
BOG	6.6	–	NKW	–	–
KUM	6.3	–	ODA	–	–
AKO	6.2	–	OFF	–	–
TEP	5.7	–	ASF	–	–
AGO	5.1	–	NED	–	–

Thiaclorid, thiamethoxam and acetamiprid were below quantification limits in all samples.

Clothianidin levels were quantified in approximately 10% of samples analyzed. Values ranged from 9.8 to 23 $\mu g\ kg^{-1}$. The observed result is in spite of the absence of registration of the insecticide in any formulation in the country. However, aside being an insecticide itself, clothianidin is a known metabolite of thiamethoxam which has been applied in relatively limited quantities in some cocoa farms. Thus the likelihood of its transformation from thiamethoxam is high.

In contrast, imidacloprid was quantified in more than 50% of samples with values ranging from 4.3 to 251.4 $\mu g\ kg^{-1}$. Undoubtedly imidacloprid marketed as Confider® (200 g/L) is the most popular insecticide used. Together with bifenthrin (27 g/L), they are the most widely used insecticides under a free national cocoa spraying program in the country. The popularity of imidacloprid was evident in some farmers' perception of higher quality of beans following its usage. Its recommended rate of application on cocoa farms is four times in a year, mostly in the months of August, September, October and December. The rate of application is even higher in farms with less optimum agronomic practices.

The results obtained suggest that considerable levels of neonicotinoids may be found in soils months after application. Thus the tendency for non-point contamination is high under this circumstance. Although the variation in the levels of neonicotinoids may be due to the amount and frequency of application, interactions in soil often determined by the physical and chemical properties of both insecticide and soil may play a significant role in determining their fate. Processes such as sorption, leaching and degradation may be significant in this regard. While sorption of neonicotinoids has been found to increase with increasing soil organic carbon content (Cox et al., 1997), their high solubility in water coupled with the high composition of sand in the samples analyzed may increase the tendency of leaching also.

In the current study, the relationship between soil properties and concentrations of neonicotinoids in soils could not be established. This is due to the requirement of some equally important information such as amount of applied neonicotinoids per area of land, number and frequency of application as well as exact periods of application. A complete set of information was impossible to obtain in a number of farms visited at the time of sampling. In practice, complete information could only be

obtained with prior knowledge of farmers who were eager to give "suitable" information to researches rather than actual information that may not reflect positively on the farmer. Most farmers were quick to point to the use of recommended dosage on chemical containers, often with their own interpretation of the required dosage for application. Besides the recommended spraying periods of August, September, October and December, a number of farmers employed a "spray on sight" approach whereby insecticides were applied or re-applied whenever insects, particularly aphids were sighted. The inclination to increase the concentration of chemicals in an effort to reduce the frequency of spraying was also found to occur among farmers.

Although disputed, there is some awareness of the possible effects of neonicotinoids on bee health (Blacquière et al., 2012; Goulson, 2013). However, current knowledge on possible effects on other non-target organisms remains low and largely deduced (Miranda et al., 2011; Goulson, 2013). Knowledge of concentrations of these insecticides in various environmental media following their application will be important in providing a better understanding of their possible effects on non-target organisms.

4. Conclusions

The QuEChERS procedure has been adapted successfully in extracting neonicotinoids in tropical soils from cocoa plantations. Sample extraction with sodium chloride & magnesium sulfate in acidified acetonitrile and clean-up using PSA was found to be the optimum conditions for analysis of neonicotinoids in the soils studied. Yields for all analytes were high with low LOQs. The results suggest that imidacloprid may be found in soils several months after application. This knowledge will be essential in decision making regarding usage and environmental management of neonicotinoids in tropical environments. Further studies on fate of neonicotinoids in soil and the cocoa plant, particularly in cocoa beans. will be important in this regard.

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A.2 Revised Paper:

Application of the QuEChERS Procedure and LC-MS/MS for the Assessment of Neonicotinoid Insecticide Residues in Cocoa Beans and Shells

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Original Research Article

Application of the QuEChERS procedure and LC–MS/MS for the assessment of neonicotinoid insecticide residues in cocoa beans and shells

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ABSTRACT

The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure was applied and validated for the analysis of neonicotinoid insecticide residues in cocoa bean matrix with high fat and high pigments. Samples employed in the study were fermented and dried beans obtained from major cocoa producing regions in Ghana where neonicotinoids are extensively used. Shells covering the beans were removed and analyzed separately to examine insecticide distribution. Analytes in both matrices were extracted in acetonitrile with sodium acetate and magnesium sulfate salts, cleaned up using a sorbent mixture of primary secondary amine (PSA), C18 and graphitized carbon black (GCB), and quantified using liquid chromatography tandem mass spectrometry (LC–MS/MS). Average recoveries at four levels of fortification ranged from 92 to 111% with relative standard deviation of ≤16% for all analytes. Limits of quantification ranged from 3 to 10 μg/kg for all neonicotinoids. Imidacloprid was the most frequently encountered neonicotinoid and was quantified in more than 10% of deshelled bean and 30% of cocoa shell samples, with concentrations ranging from 11.5 to 35.6 μg/kg and 11.8 to 214 μg/kg in cocoa beans and shells, respectively. The findings from this study suggest a need for greater efficiency in neonicotinoid application, to avoid the build-up of these insecticides to unsafe levels in cocoa beans.

1. Introduction

Cocoa beans are the main raw material for cocoa products including chocolate. The cocoa tree is mainly grown in the tropics, particularly in West Africa where more than 60% of worldwide cocoa beans is produced (ICCO, 2014). Ghana is the second largest producer of cocoa beans and the industry is highly controlled by government regulations. This is because the cocoa sector contributes significantly to the country's gross domestic product, through export and provision of employment for farmers (Ghana Statistical Service, 2014; Global Agricultural Information Network, 2012). However, cocoa yields per hectare in Ghana are considerably lower than other top cocoa producing countries (Aneani and Ofori-Frimpong, 2013). Pest and diseases are said to contribute significantly to the low yields (Adu-Acheampong et al., 2014). According to the international cocoa organization (ICCO), up to 40% of global annual cocoa production is lost to insect pests and diseases (ICCO, 2013). In response, the Government of Ghana, through the Ghana Cocoa Board, introduced the Cocoa Diseases and Pests Control (CODAPEC) programme over a decade ago (COCOBOD, 2012). Under this initiative, mass application of insecticides is performed on cocoa farms across the country, up to four times in a year, at no financial cost to farmers. Neonicotinoids particularly imidacloprid are probably the most extensively applied insecticides under the programme and have contributed to significant increases in yield (COCOBOD, 2012).

However, a drawback to the mass application of insecticide programme is the tendency for crop and environmental

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contamination, due to the extensive use and multiple application rates. In previous studies, we have demonstrated that, applied neonicotinoids (in particular imidacloprid) in cocoa farms may enter and persist in soils for several months following application (Dankyi et al., 2014). Unfortunately the fate of these insecticides in cocoa beans, which is the main product consumed, is not yet known. In the literature, much of the current knowledge on residues of neonicotinoids has been restricted to honey and honey bees due to the perceived detrimental health effects of this class of insecticides on pollinators (Blacquière et al., 2012; Jovanov et al., 2015; Laycock et al., 2012; Tanner and Czerwenka, 2011). Although some research on fruit and vegetables exist, they are mainly restricted to periodic market surveys or the application of newly developed procedures, often in multi-class pesticides analysis (Bakırcı et al., 2014; Garrido Frenich et al., 2008; Gilbert-López et al., 2010; Obana et al., 2002; Wang et al., 2012; Xie et al., 2011; Zhang et al., 2012). A recent study by Chen et al. (2014) has demonstrated the widespread presence of neonicotinoid insecticide residues in foods, including fruit and vegetables (Chen et al., 2014). However knowledge in tropical foods, such as cocoa beans, and of actual sources of contamination remains low. Although neonicotinoids have been used extensively in the production of cocoa and other crops in Ghana and other West African countries for several years, their levels in food is not yet known.

Whereas some research exists on pesticide residues in cocoa beans, they have been restricted to other classes of pesticides, including organochlorines, organophosphates and carbamates, which are less readily used in cocoa production in recent years, particularly in Ghana (K.S. Frimpong et al., 2012a, 2012b; Frimpong et al., 2012; Owusu-Ansah et al., 2010). With the coming into force of market regulations seeking to limit pesticide usage in food crops, such as the new European Union (EU) Regulation 396/2005/EC on "maximum residue levels of pesticides in or on food and feed of plant and animal origin" (European Commission, 2008), the need for knowledge on pesticide levels in produce such as cocoa beans mainly produced for export to European and other markets cannot be overemphasized.

In literature, conventional methods that are widely used for the extraction of pesticides from fatty matrixes include solid-phase micro-extraction (SPME), matrix solid-phase extraction (MSPD, gel permeation chromatography (GPC) and supercritical fluid extraction (SFE) (Gilbert-López et al., 2009).

It is quite evident that the effective management of these insecticides in food from developing countries will require reliable quantitative and qualitative assessment based on simple, efficient and less expensive techniques to ensure food safety. The QuEChERS procedure does not only offer this simplicity, reliability and effectiveness but also a high flexibility for application to a wide range of analytes and matrixes (Lehotay et al., 2010).

However, the QuEChERS method was originally designed for low-fat food matrices; application to high fat matrices often presents a challenge due to high lipid co-extractives that may not only adversely affect extraction and chromatographic efficiency but also instrumentation (Chamkasem et al., 2013). In recent years, the method has been applied to matrices of medium (≈15%) to high fat (>40%) content, including nuts, avocado, fish and animal foods with varying degrees of success (Chamkasem et al., 2013; Choi et al., 2015; Koesukwiwat et al., 2010; Lozano et al., 2014; Luzardo et al., 2013; Rajski et al., 2013; Sobhanzadeh et al., 2012). Lehotay et al. (2005) have observed that the QuEChERS method compares favourably with established methods such as MSPD in the analysis of polar and semi-polar pesticides from low fat food matrices but performed poorly with nonpolar pesticides (Lehotay et al., 2005). Various modifications of the method have been performed to enhance the efficiency of QuEChERS extraction in high fat matrices, including the use of a freeze-out step (Koesukwiwat et al., 2010), higher solvent-sample ratios (Chamkasem et al., 2013), and zirconium sorbents (Lozano et al., 2014; Rajski et al., 2013; Sapozhnikova and Lehotay, 2013).

The challenge in the application of the QuEChERS method to the cocoa matrix is not only due to its high fat content (>40%) but also its highly pigmented nature (Torres-Moreno et al., 2015). In the current study, the QuEChERS procedure was explored in the extraction and clean-up of residues of five neonicotinoid insecticides: imidacloprid, acetamiprid, thiamethoxam, clothianidin and thiacloprid in cocoa beans and shells acquired from Ghana. An optimized procedure was then used to assess the levels of residues of neonicotinoid insecticides resulting from their use in the country's cocoa production. Cocoa shells comprise the thin outer covering (husk) of the beans, which are usually removed during the processing of cocoa. In our study, the shells were analyzed separately from the de-shelled beans (cocoa nibs) to examine insecticide distribution.

2. Materials and methods

2.1. Chemicals and reagents

Insecticide standards of thiamethoxam (99.6%), clothianidin (99.9%) and imidacloprid (99.9%) as well as labelled internal standards (IS) of imidacloprid-d_4 (99.9%) and thiamethoxam-d_3 (98%) were all purchased from Sigma–Aldrich (Steinheim, Germany). Acetamiprid (98.1%) and thiacloprid (98.0%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Prepackaged 12-mL tubes containing one of the following: (a) 4 g of $MgSO_4$ & 1 g of NaCl; (b) 6 g of $MgSO_4$ & 1.5 g of sodium acetate (NaOAc); (c) 4 g $MgSO_4$, 1 g of NaCl, 0.5 g sodium citrate dibasic sesquihydrate (SCDS), 1 g of sodium citrate tribasic dehydrate (SCTD) were purchased from Supel QuE product lines, Sigma–Aldrich. Dispersive solid phase clean-up sorbent tubes (2 mL) containing 150 mg of $MgSO_4$ together with one of the following sorbents: (a) 50 mg PSA only; (b) 50 mg PSA & 50 mg C-18; (c) 50 mg PSA, 50 mg C-18 & 50 mg GCB; (d) 50 mg Z-Sep+ were all purchased from Sigma–Aldrich. Sodium hydrogen carbonate ($NaHCO_3$) and ammonium acetate (NH_4Ac) were obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and glacial acetic acid were obtained from Rathburn (Walkerburn, Scotland) and VWR (Fontenay-sous-Bois, France), respectively. Deionized water was prepared using a Merck Millipore Milli-Q advantage A10 ultrapure water purification system (Darmstadt, Germany).

2.2. LC–MS/MS instrumentation

Chromatographic separation of analytes was performed on an Agilent 1260 Infinity HPLC system (Santa Clara, CA) equipped with a BDS Hypersil reversed-phase C-18 column (250 mm × 2.1 mm; 5 μm; Thermo Electron Co., Waltham, MA) at a temperature of 30 °C. Mobile phases **A** and **B** consisted of 5 mM ammonium acetate and 95% acetonitrile in 5 mM ammonium acetate, respectively. The gradient was run at 300 μL min^{-1} for 16 min as follows: 10% **B** increased linearly to 100% in 8 min; held constant for 2 min; decreased back to 10% **B** in 1 min; and maintained for an equilibration time of 5 min. The injection volume was 5 μL. The mass spectrometer used was a 3200 QTRAP (AB Sciex, Foster City, CA) equipped with electrospray ionization (ESI). The MS determination of all analytes was performed in positive mode with multiple reaction monitoring (MRM) of the two most intense precursor-product ion transitions for each analyte, one used for quantification and the other for confirmation. The source parameters employed were: curtain gas (CUR) of 20 psi, collision gas (CAD) of medium pressure, ion spray voltage of 4500 V, source

temperature of 475 °C and both nebulizer and heater gas (GS1 and GS2 respectively) pressures of 60 psi each. Optimized values of compound dependent parameters: declustering potential (DP), exit potential (EP), collision energy (CE) and collision cell entrance potential (CEP) are listed in Table 1. Data obtained were processed using the Analyst software (version 1.6.2). Analytes were quantified from matrix-matched standard curves using peak area and a weighting of 1/*x*.

2.3. Sampling

Fermented and dried cocoa beans were obtained from all the major cocoa growing regions of Ghana. Sampling locations are indicated in Fig. 1. Samples were collected from cocoa farmers, licensed buying centres and depots in the various regions, towns and villages. The number of samples acquired from each region was based on relative production of cocoa beans. In total, 86 samples from the 6 cocoa-growing regions of the country were collected for the study. Organic cocoa bean samples were obtained from farmers in parts of the Volta region of the country where no agrochemical was said to have been used. In all locations, only fermented and dried beans were sampled.

2.4. Sample preparation

The fermented and dried beans were manually de-shelled and homogenized. Shells were homogenized separately. All samples were again air-dried at room temperature to a moisture content of <5.0% to ensure uniformity of sample weight. When necessary, caked samples were disaggregated. The organic cocoa beans were used as blank matrix samples for matrix matching, method development and validation. Recoveries at different levels of fortification: 10; 50; 100 and 200 μg/kg were prepared by spiking 3 g of sample with 30 μL of 1, 5 and 10 μg/mL and 60 μL of 10 μg/mL concentration of insecticide solution, respectively. Samples were spiked with analytes and/or internal standards prior to acetonitrile extraction. For reagent blanks, 7 mL of deionized water were used without sample.

2.5. Sample extraction and clean-up

Different QuEChERS extraction procedures were experimented with to determine the optimum procedure for the matrix under study. A 3-g aliquot of sample was accurately weighed into a 50-mL Falcon tube and fortified with the appropriate standards for about 30 min; 7 mL of water were then added and allowed to soak up the matrix. Acetonitrile (15 mL) was added and the mixture shaken for 2 min on a Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ) at 1500 strokes/min. Different salting-out extraction procedures were performed on samples using (a) 4 g of $MgSO_4$ & 1 g of NaCl; (b) 6 g of $MgSO_4$ & 1.5 g of sodium acetate (NaOAc); (c) 4 g $MgSO_4$, 1 g of NaCl, 0.5 g sodium citrate dibasic sesquihydrate (SCDS), 1 g of sodium citrate tribasic dehydrate (SCTD). After addition of salts, the shaking process was repeated under the same conditions and the resulting mixture centrifuged at 4000 rpm for 5 min. Sample clean-up involved the addition of 1 mL of extract (from the acetonitrile layer) to various sorbent mixtures (50 mg PSA only; 50 mg PSA & 50 mg C-18; 50 mg PSA, 50 mg C-18 & 50 mg GCB; and 50 mg Z-Sep+) to determine the most appropriate. The resulting mixture was shaken on a vortex mixer (Velp Scientifica, Usmate Velate, Italy) at 3000 rpm for 30 s and centrifuged at 4000 rpm for 5 min. Extracts were diluted 1:1 with water and filtered using a 0.22-μm PTFE syringe filter prior to injection onto the LC–MS.

Table 1
Instrument conditions, retention time and MRM transitions of precursor/product ions of analytes.

Analyte	Ion transition (*m/z*)	DP (V)	EP (V)	CEP (V)	CE (V)
Acetamiprid	223.10 → 126.20[q]	46.0	5.0	20.0	27.0
	223.10 → 99.00	46.0	5.0	20.0	49.0
Clothianidin	250.00 → 169.00[q]	41.0	4.5	26.0	19.0
	250.00 → 132.00	41.0	4.5	26.0	19.0
Imidacloprid	256.10 → 209.10[q]	41.0	5.0	18.0	17.0
	256.10 → 175.10	41.0	5.0	18.0	21.0
Imidacloprid-d_4	260.03 → 213.00[q]	51.0	6.5	24.0	23.0
	260.03 → 179.00	51.0	6.5	24.0	19.0
Thiacloprid	252.98 → 126.10[q]	56.0	7.5	18.0	27.0
	252.98 → 99.10	56.0	7.5	18.0	55.0
Thiamethoxam	291.95 → 211.00[q]	31.0	6.0	20.0	19.0
	291.95 → 181.00	31.0	6.0	20.0	27.0
Thiamethoxam-d_3	294.95 → 213.90[q]	31.0	6.5	16.0	19.0
	294.95 → 184.10	31.0	6.5	16.0	27.0

[q] Transitions used for quantification.

2.6. Validation of procedure

Validation of an optimum QuEChERS procedure was performed, by evaluating parameters including accuracy, precision, matrix effects, limit of detection (LOD) and limit of quantification (LOQ) based on the DG SANCO guidelines (SANCO/12571/2013, 2013). For each procedure, the accuracy and precision was evaluated by estimating percentage recoveries and relative standard deviations (RSDs) at four different fortification levels for all analytes. The ruggedness of the procedures was assessed by examining the influence of various treatments, such as manual shaking and use of Geno grinder, use of varying sample-solvent ratios and influence of pH by addition of base or acid. The linearity of the analytical procedures was studied using matrix-matched calibration solutions prepared in blank cocoa extracts. Calibration curves were plotted at 10 levels of concentration ranging between 1.56 and 800 μg L^{-1}. LOD and LOQ was estimated using matrix-matched standard solutions at the lowest concentration of analytes that yielded a signal to background noise (S/N) ratio of three and ten respectively. Subsequently, actual values in μg/kg were determined by taking into consideration the amount of sample and total volume of extract. Matrix influence was assessed by comparing responses of matrix-matched and solvent standards (Pizzutti et al., 2007). Absolute matrix effects were estimated by comparing the slopes in calibration solutions prepared in matrix and in solvents.

3. Results and discussion

3.1. Cocoa bean matrix

Cocoa beans are rich in polyphenols, which are associated with colour and flavour of the beans and its products (Niemenak et al., 2006). During fermentation, cocoa polyphenols undergo oxidation and polymerization to form condensed higher molecular weight compounds, mostly insoluble tannins, leading to the dense brown pigmentation observed in fermented beans (Wollgast and Anklam, 2000). After centrifugation of salting-out extracts of deshelled beans, four distinct layers are observed: supernatant acetonitrile layer; cocoa solids; cocoa fat; and salts at the bottom of the tube. In almost all cases, the layers of cocoa solids and cocoa fats had similar heights suggesting the high levels of fat (≈50%) often found in cocoa matrices. The pigments together with the high fat content of the beans make the cocoa matrix quite complex; hence the need for an effective extraction and clean-up procedure. The QuEChERS procedure was used in this quest, involving an examination of the

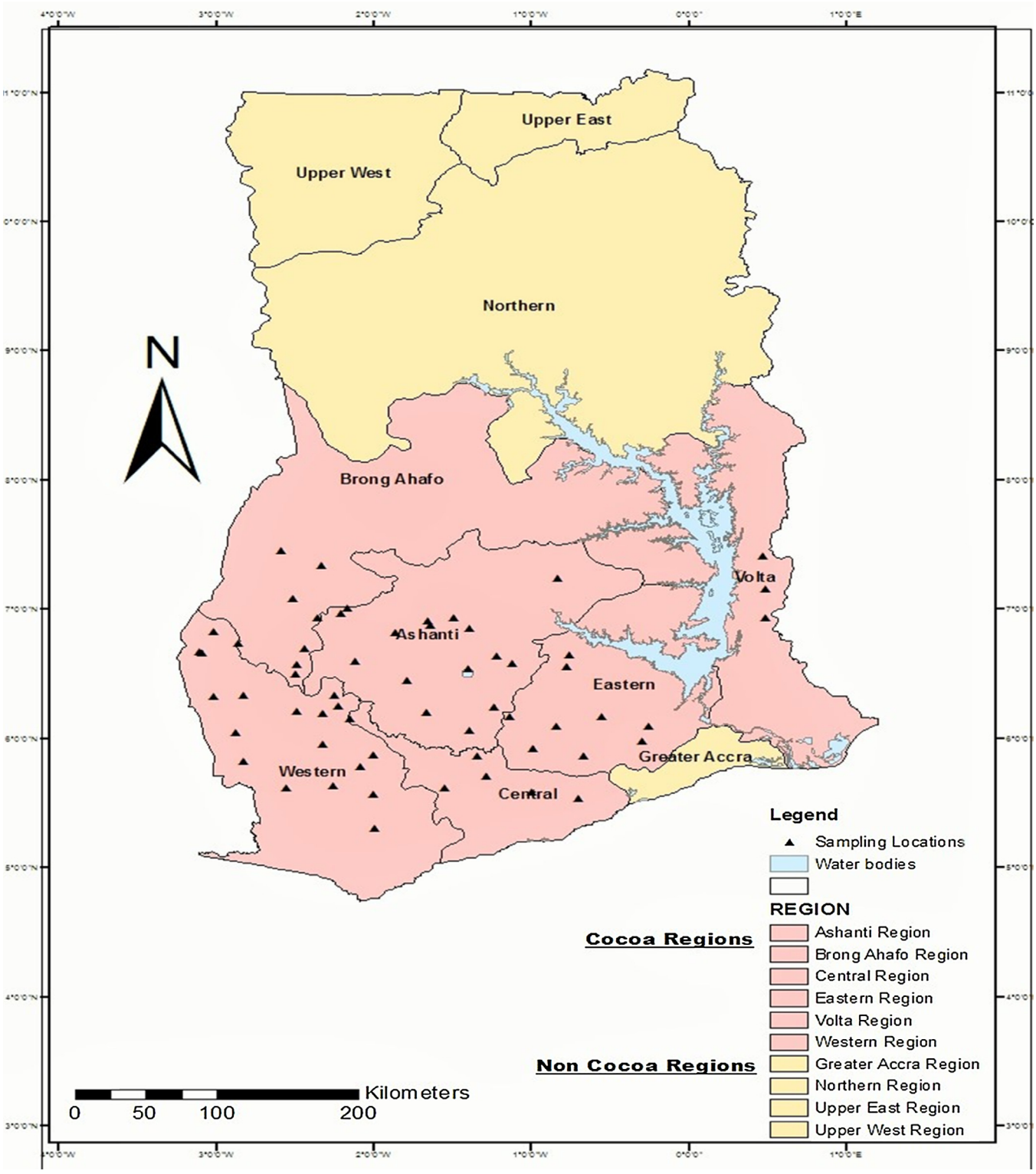

Fig. 1. A map of Ghana showing cocoa-growing regions and locations of sampling.

effects of different salts, sorbents, buffers and sample-to-solvent ratios on the extraction of analytes from the matrix.

3.2. Salting-out extraction

The addition of salts under the original QuEChERS procedure was primarily to ensure phase separation. However influence of pH on analytes in resulting extracts has been utilized in current modifications of the original method, involving the use of buffering salts to control pH and ionic strength of matrixes, particularly for pH-sensitive analytes. In our study, all three salting out procedures commonly used under the QuEChERS methodology were examined for their effectiveness in analyte extraction with minimal co-extractives. Pulverized samples were extracted with salts of sodium acetate (acetate buffered procedure), sodium citrate (citrate buffered procedure) and sodium chloride (non-buffered/

original procedure) in acetonitrile to produce approximate matrix pH values of 7.2; 4.1; and 4.9 respectively. It was observed that, the acetate buffered extracts showed significantly cleaner acetonitrile layers compared to the other two salt extracts. Pigments appeared to be concentrated in the lower fat layer leading to a visibly cleaner acetonitrile upper layer. Further tests involving the addition of varying amounts of $NaHCO_3$ during the salting out procedure showed the dependence of this occurrence on the pH of the matrix. This apparent "de-pigmentation" of the acetonitrile layer upon increasing the pH of the matrix was observed for all salting-out procedures. In effect, increasing pH of the extraction medium seemed to produce cleaner extracts.

3.3. Matrix co-extractives

To ascertain the visually observed differences in matrix composition of extracts from the three salting out procedures, matrix co-extracts were examined using gravimetric measurements (Anastassiades et al., 2003; Lehotay et al., 2010). Five millilitres of extract from each procedure were measured into pre-weighed glass tubes and extracts dried using a gentle stream of nitrogen gas and then heated in an oven at 105 °C for 1 h. The new weight of the glass tubes was measured. The weight of co-extractives was estimated from the difference in weights (Fig. 2). From the results obtained, the acetate buffered procedure showed markedly lower matrix co-extractives compared to both the citrate and the unbuffered method, confirming the visual observation.

At high pH, cocoa polyphenols are readily transformed and polymerize into higher molecular weight insoluble brown pigments (Li et al., 2013). This transformation is observed during alkalization (Dutching) of cocoa as often occurs during industrial processing (Miller et al., 2008). In this study, the extent of polymerization appears to increase with increasing pH of the medium. The newly formed high molecular weight pigments then precipitate out of solution and fall to the lower layers (of cocoa fat and cocoa solids), thereby resulting in a cleaner upper acetonitrile layer with less co-extractives. Other studies have also observed a decrease in fatty acid co-extractives upon increasing pH of the extraction matrix (Anastassiades et al., 2003). Nonetheless, the decreased stability of some pesticides at higher pH warrants the need for a good balance between matrix co-extractives and analyte stability. The acetate-buffered QuEChERS method (AOAC Official 2007.01) normally involves the extraction of analytes using acetonitrile with 1% acetic acid to ensure a good pH range, particularly for pH sensitive analytes. In our study, we observed that utilizing the acidified acetonitrile decreases the resulting pH of our matrix by approximately 1 unit, with a corresponding increase in matrix co-extractives of about 14%. However, the stability of neonicotinoids (Guzsvány et al., 2006) over the pH range being investigated did not warrant the addition of acid to acetonitrile in our study. Sample extraction using sodium acetate in acetonitrile seemed to provide the right balance in matrix cleanliness and compound stability for the analytes and matrix under study.

3.4. Clean-up procedure

One of the advantages of using acetonitrile under the QuEChERS procedure in the extraction of analytes from high fat matrixes is the relatively low matrix co-extraction due to low solubility of lipids in acetonitrile as compared to other solvents (Anastassiades et al., 2003; Koesukwiwat et al., 2010). That notwithstanding, the use of a clean-up procedure is essential to ensure the removal of the inevitable lipid co-extracts in high fat matrixes such as cocoa beans. The high pigmentation of the cocoa matrix adds to the complexity and contributes to the greater need for a clean-up step.

In our study, we compared the ability of PSA; PSA + C18; PSA + C18 + GCB and Z-Sep+ sorbents in removing matrix co-extracts during the dispersive solid-phase clean-up step without significant effects on analyte recovery. Aliquots of extracts (5 mL) from the various clean-up sorbents were used in gravimetric measurements as already described. Our results suggest PSA + C18 + GCB as the most effective sorbents/sorbent mixture for matrix clean-up based on visual inspection and gravimetric measurements (Fig. 3). While PSA is important in the retention of fatty acids and other polar components, C18 is essential for the removal of lipids and non-polar co-extractives particularly in high fat matrices. In our study, GCB was found to be crucial for the total removal of visible pigments, and resulted in significantly lower co-extractives with no observed effects on analyte recovery. GCB has a strong affinity for pigments such as polyphenols, carotenoids and chlorophyll often found in food matrices (Anastassiades et al., 2003). Moreover, its affinity for planar molecules including pesticides was not observed in this study. In the matrix under study, it was observed that, GCB could be avoided when higher solvent-to-sample ratios (≥8:1) or higher matrix pH were employed. Z-Sep+, a relatively new sorbent marketed for its ability to remove fat in high fat matrices appeared to be least effective at removal of co-extractives in the

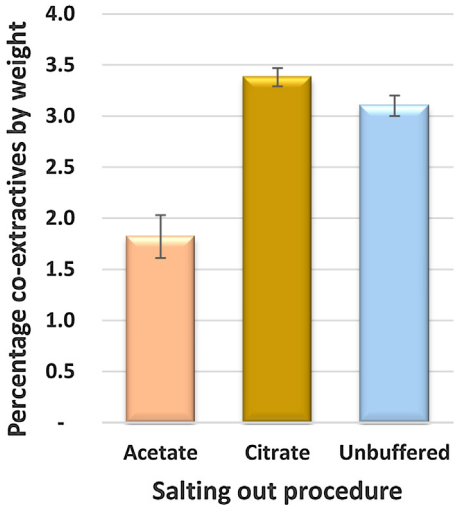

Fig. 2. Percentage co-extractives by weight of sample based on the three QuEChERS salting out procedures, reported with standard error. Two replicates were performed for each procedure.

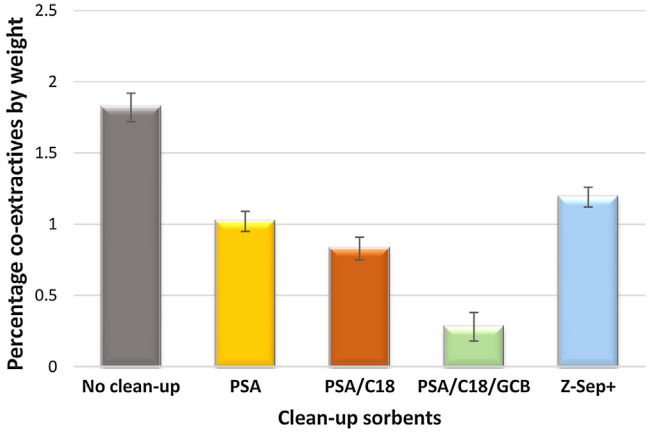

Fig. 3. Effect of different sorbent on clean-up efficiency of cocoa matrix. Extract was obtained from the acetate buffered procedure. Two replicates each was performed for each study, reported with their standard error.

matrix under study, which appeared to be predominantly polyphenols (pigments).

3.5. Optimum QuEChERS procedure

Based on the samples employed in this work, it was evident that the degree of pigmentation in the cocoa matrix varies widely, perhaps based on variety or method of fermentation of beans. As such, samples showed different shades of pigmentation ranging from light brown to deep brown colouration. In the development of an optimum procedure for extraction and clean-up of analytes, it was ensured that the most deeply pigmented samples were employed to adequately address the issue of pigmentation. As such, results obtained in this study may represent some of the highest level of interference that may arise from the cocoa matrix.

Despite the high fat content of the matrix studied, it was quite clear that, the majority of fat was excluded from the extract, based on very low solubility in acetonitrile. The high polarity of analytes studied also ensured their effective partitioning in the acetonitrile layer and ensured high recovery. Pigmentation of the matrix appeared to have a major influence on extracts and was addressed by the control of pH and use of sorbents particularly GCB. Ultimately, a choice of acetate-buffered salting out procedure in acetonitrile and clean-up using a mixture of PSA + C18 + GCB sorbent was made, as it better reflected accuracy, precision and cleaner chromatograms for all analytes.

3.6. Validation of QuEChERS procedure

The performance of the chosen procedure was assessed by evaluating parameters, including accuracy, precision, linearity, matrix effects, LOD and LOQ. LOQ and LOD were calculated as 10 and 3 times the signal-to-noise ratio of analytes respectively, using matrix-matched standards. In all analytes, very good linearity ($r^2 \geq 0.998$) was observed using matrix-matched calibration standard solutions of concentrations ranging from 1.56 to 800 μg L^{-1}. Multiple recovery experiments were conducted at several levels of fortification by spiking of samples with different concentrations of analytes. Five replicates were employed at each level of fortification for all analytes. As shown in Table 2, good recoveries (92–111%) for all analytes and at all levels of fortification with good precision ($2 \leq RSD \leq 16$) were obtained. As expected, precision was higher at higher levels of fortification. While the use of a Geno grinder ensured high sample throughput, percentage recoveries of spiked samples were comparable to conventional hand-shaking procedure.

Table 2
Average percentage recovery of analytes reported with RSDs at 4 levels of fortification. 5 replicates were analyzed at each level of fortification.

Analyte	Level of fortification (μg/kg)			
	10	50	100	200
Acetamiprid	93 ± 7	96 ± 4	96 ± 3	95 ± 2
Clothianidin	92 ± 15	98 ± 9	95 ± 6	95 ± 6
Imidacloprid	111 ± 10	101 ± 6	95 ± 6	97 ± 4
Thiacloprid	97 ± 7	100 ± 4	92 ± 5	92 ± 3
Thiamethoxam	95 ± 16	103 ± 7	100 ± 5	97 ± 2

Table 3
Retention time, linearity, matrix effects, LOD and LOQ of analytes.

Analyte	RT (min)	Linearity (r^2)	Matrix effect[a]	LOD (μg/kg)	LOQ (μg/kg)
Acetamiprid	7.56	0.999	0.97	2	3
Clothianidin	6.85	0.999	0.97	5	10
Imidacloprid	7.16	0.999	0.96	5	10
Thiacloprid	8.26	0.999	0.98	2	3
Thiamethoxam	6.13	0.998	0.96	5	10

[a] Matrix effect expressed as a ratio of the slopes of the calibration curves obtained from solvent and matrix-matched standards.

Matrix influence in the chosen procedure was estimated by comparing the slopes of matrix-matched standards and solvent standards (Romero-González et al., 2011). The effect of matrix was found to be minimal for all analytes studied (Table 3). During method development, significant matrix influences were observed at the sample-solvent ratio of 1:1 commonly used in the QuEChERS procedure, albeit at high water content of samples (≥80%). Matrix influence was characterized by recoveries >100% and low precision in most analytes (Fig. 4). This influence was observed in all salting-out extracts in spite of clean-up procedures, except when Z-Sep+ sorbents were used. Z-Sep+ sorbents produced good recoveries albeit in a dirty (highly pigmented) matrix, suggesting greater influence of lipid co-extractives in the MS. With increased solvent-sample ratios (≥5:1) matrix influence were significantly reduced with cleaner chromatograms and good recoveries as shown in Table 3. The advantages of increased solvent-sample ratios have already been observed (Chamkasem et al., 2013). In our study, a solvent-sample ratio of 5:1 was found to be optimum in ensuring clean matrices and good efficiency in solvent usage. Notwithstanding the low matrix influence, as a result of higher solvent-sample ratio, all quantitation was performed in matrix-matched standards and with the help of internal standards.

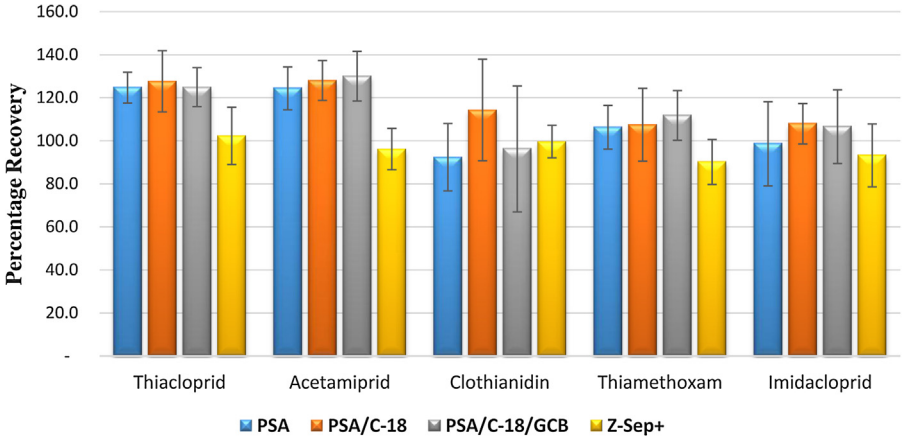

Fig. 4. Influence of matrix at low solvent-sample ratio (1:1). Analytes from acetate extracts (5 replicates) are shown. Error bars represent RSD. Results are characterized by low precision (high RSDs) and overestimated recoveries (>120%) particularly in thiacloprid and acetamiprid.

3.7. Application of procedure to neonicotinoids in cocoa beans and shells

A total of 86 samples from all cocoa-producing regions of Ghana were extracted, cleaned-up and quantified using the chosen QuEChERS procedure already described. Shells and deshelled beans (hereafter referred to as *nibs*) from the same sampling lot were analyzed separately to examine the distribution of neonicotinoids in both food parts. From the results (Table 4), imidacloprid was found to be present in a greater number of samples and in higher concentrations compared to all other insecticides studied. It was quantified in more than 10% of nib and 30% of shell samples studied with concentrations up to 35.6 and 214 μg/kg, respectively. The high frequency may reflect its high popularity and usage among farmers in the country compared to other insecticides. Acetamiprid was quantified in one sample of cocoa nib and 2 samples of shell, being present in both nib and shells from Achimfo. Thiamethoxam was quantified in only two shell samples, whereas clothianidin and thiacloprid were both below the limits of quantification in all samples studied.

From the results obtained, the levels of neonicotinoids in the two cocoa parts suggest a selective accumulation in shells compared to nibs. All neonicotinoids that were quantified showed this tendency. Of the 10 samples where a particular neonicotinoid was present in both nib and shell, levels in shells were 1.7 to 6 times the levels in nibs. For instance, imidacloprid appeared to be quantified (≥10 μg/kg) in nibs only when levels present in shells were in excess of about 26 μg/kg. Moreover, thiamethoxam was present only in shell samples (2) and despite relatively high concentrations of 24.7 and 39.6 μg/kg, concentrations in deshelled beans were below LOQ (10 μg/kg). Thus the deshelling of beans during processing may not only enhance the properties of processed cocoa but may also serve as a good decontamination measure. In the cocoa processing industry, restrictions of up to 1.75% (by weight) of shells allowed in cocoa nibs have been set in the USA (Code of Federal Regulation, CFR 21, section 163.110) and by Codex (Codex Std 141-1983, rev. 2001, Amen 2014). In the EU however, a former limit of up to 5% shell content has not been retained in new regulations (2000/36/EC). That notwithstanding, it is evident from our study that, knowledge of insecticide residue levels in cocoa shells may give a good indication of the extent of application of chemicals and potential contamination of the main edible portion (nibs) of the beans.

The selective distribution of pesticides in skin and pulp of food crops has been observed in the literature (Placido et al., 2013; Teixeira et al., 2004; Xu et al., 2012). However, the relevance or otherwise of cocoa shells for nutritional and toxicological purposes is underscored in differences in policy formulation on pesticide residue levels among various countries. In the EU and the USA, residue or tolerance limits refer to deshelled beans (“edible portion”), whereas in Japan and Australia, limits are applicable to the whole bean.

In spite of the relatively low frequency and levels of neonicotinoids in cocoa nibs, our study suggests that neonicotinoids may accumulate to relatively high levels in cocoa beans. This perhaps may be due to the widespread and intensive application rates of these insecticides in cocoa farms across the country. From this study however, knowledge of neonicotinoid application levels and rates on farms, and their corresponding levels in cocoa samples could not be directly established, as a particular sample may comprise beans from multiple farms and multiple farmers in the various towns and villages.

Over the past few decades, organochlorines have been the major insecticides used in cocoa farms in Ghana (Frimpong et al., 2012; Owusu-Ansah et al., 2010). In recent years however, the use of newer pesticides, particularly neonicotinoids with perceived less detrimental effects, has been highly endorsed and widely used without knowledge of their fate in food and the environment. Currently, imidacloprid, thiamethoxam and thiacloprid are approved for use in cocoa plantations and are recommended to be applied four times each year during the months of August, September, October and December when the mirid population is believed to be particularly high.

Table 4
Concentrations (μg/kg) of neonicotinoids present in deshelled beans(cocoa nib) and cocoa shell samples. Only samples with concentrations above the limit of quantification have been presented.

Sampling town	Imidacloprid		Thiamethoxam		Acetamiprid	
	Beans	Shells	Beans	Shells	Beans	Shells
Abochia	16.1	29.4	–	–	–	–
Achimfo	–	15.9	–		12.4	31.1
Agona Amenfi	–	23.7	–	–	–	–
Anwiaso	–	13.4	–	–	–	–
Anyinam	–	11.8	–	–	–	–
Anyinasuso	15.9	30.6	–	–	–	–
Asankragua	–	14.5	–	–	–	–
Asawinso	16.7	28.9	–	–	–	–
Dadieso	–	21.1	–	–	–	–
Dunkwa	–	22.7	–	–	–	–
Enchi A	35.6	214	–	–	–	12.9
Enchi B	–	22.7	–	–	–	–
Hohoe	24.7	55.4	–	–	–	–
Insu-Bogosu	–	12.1	–	–	–	–
Juaso	11.5	26.6	–	24.7	–	–
Manso Amenfi	17.2	89.6	–	–	–	–
Nkawie	–	16.5	–	–	–	–
Nkawkaw	–	12.8	–	–	–	–
Oda	–	13.4	–	–	–	–
Samreboi A	–	15.2	–	–	–	–
Samreboi B	–	22.6	–	39.6	–	–
Sehwi Bekwai	–	15.0	–	–	–	–
Simpa	–	20.8	–	–	–	–
Tarkwa	–	24.3	–	–	–	–
Wassa Akropong A	17.9	30.5	–	–	–	–
Wassa Asikuma	17.1	29.4	–	–	–	–

Until now, available literature on pesticide residues levels in cocoa beans has remained low. In a recent study by Zainudin et al. (2015) on pesticide levels in whole beans from different countries including Ghana, concentrations of 3 pesticides (chlorpyrifos, amethryn and metalaxyl) out of 12 studied ranged from 10 to 200 μg/kg (Zainudin et al., 2015). In a similar study of synthetic pyrethroids and organophosporus pesticide residue levels in whole beans from Ghana, concentrations ranging from 5.0–105 and 5.0–133 μg/kg respectively have been reported (Frimpong et al., 2012a, 2012b). These relatively high concentrations of insecticides suggest an increased tendency of buildup in cocoa beans.

In the EU, current maximum residue levels for all neonicotinoids studied in fermented cocoa beans (deshelled or edible part) is 50 μg/kg ("EU Pesticides database," n.d.). The limits for thiamethoxam and clothianidin (a metabolite of thiamethoxam) are expressed together as thiamethoxam with same residue limit of 50 μg/kg. Despite the lower concentrations of residues in deshelled beans found in this study, the need for greater efficiency in the application and management of neonicotinoid insecticides in cocoa farming is evident.

4. Conclusion

The QuEChERS procedure has been successfully optimized and applied in the extraction and clean-up of neonicotinoid insecticides from cocoa beans, a complex matrix with high fat and high pigments. Sample extraction in acetonitrile using a solvent sample ratio of 5:1 and clean-up using a sorbent mixture of PSA/C18/GCB was found to be the optimum method for the matrix. The availability of different salting out/buffering procedures ensures the procedure can be adapted to other analytes with pH sensitivity. While clean-up with PSA/C18/GCB ensured good matrix cleanliness and low interference, the use of Z-Sep+ appears promising for high fat matrices. The chosen procedure enables low detection (2–5 μg/kg) and quantitation (5–10 μg/kg) limits for neonicotinoids in the cocoa matrix using LC–MS/MS. The current study has demonstrated the likelihood of accumulation of neonicotinoid insecticide residues to relatively high levels particularly in cocoa shells, most likely due to intense usage. Based on this study, greater efficiency in neonicotinoid application is recommended, in order to avoid the build-up of these insecticides to unsafe levels in cocoa beans.

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A.3 Papers Accepted for conferences:

1. Neonicotinoid insecticide residues in soils and cocoa beans following multiple applications in cocoa farming

2. Neonicotinoid application in Ghana's mass cocoa spraying exercise- Implications for research and education on policy and environmental sustainability.

Neonicotinoid insecticide residues in soils and cocoa beans following multiple applications in cocoa farming

Enock Dankyi[1], Derick Carboo[1], Chris Gordon[1], Inge Sindbjerg Fomsgaard[2*]

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[2]Aarhus University, Denmark

According to the international cocoa organization (ICCO), up to 40% of global annual cocoa production is lost to insect pests and diseases. In Ghana, the second largest producer of cocoa beans, pests and diseases account for significant losses in yields. To curtail this decline, the government of Ghana through the Ghana Cocoa Board introduced the Cocoa Diseases and Pests Control (CODAPEC) program geared towards the mass application of insecticides on cocoa farms across the country at no financial cost to farmers. Neonicotinoids (particularly imidacloprid) are the most widely used class of insecticides in cocoa production and are extensively applied under the program. Notwithstanding the increasing yields in cocoa production as a result of the free insecticide application program, the tendency for build-up of insecticides in the environment and in crops has been heightened. In our study, we examined the extent of environmental and food contamination by assessing the concentration and distribution of neonicotinoid insecticides in soils and in cocoa beans. The QuEChERS procedure was employed in both analysis in soils and in cocoa beans due to its flexibility and adaptability. Analytes were quantified using LC-MS/MS. Our findings suggest that, neonicotinoid insecticides may persist in soils for several months after application. In cocoa beans, neonicotinoids were found to selectively accumulate in shells to relatively high concentrations.

1. Dankyi E, Gordon C, Carboo D, Fomsgaard I S (2014). Quantification of neonicotinoid insecticide residues in soils from cocoa plantations using a QuEChERS extraction procedure and LC-MS/MS. Sci Total Environ. 499: 276-283
2. ICCO (2013). Regional workshop on integrated management of cocoa pests and pathogens in Africa: Controlling indigenous pests and diseases and preventing the introduction of exogenous ones; Accra, Ghana.

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Ghana Chemical Society Annual National Conference and AGM 27 – 28TH August 2015, University of Cape Coast, Ghana

Neonicotinoid application in Ghana's mass cocoa spraying exercise – Implication for research and education on policy and environmental sustainability

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Based on projections by the international cocoa organization (ICCO), pests and diseases account for about 40% of yields loses in cocoa production worldwide. Yield loses are similarly high in Ghana, the second largest cocoa producer. Due in parts to the importance of the cocoa sector to the economy of the country as well as the commitment to ensure the use of only approved pesticides, the government of Ghana introduced the cocoa mass spraying exercise over a decade ago to address the decline from pests. Under the program, pesticides are applied in cocoa farms, about four times each year, at virtually no financial cost to farmers. Neonicotinoids, currently banned in Europe due to reported detrimental effects on bee health, are perhaps the most widely used class of insecticides under the mass spraying program and has contributed to significant increases in yields among other interventions. However the scale, volume and intensity of application have invariably increased the possibility of exposure of large quantities of these chemicals in the environment. In our study, we assessed the extent of exposure of neonicotinoid insecticides in soils from cocoa farms across Ghana. Analytes were quantified by means of LC-tandem MS after extraction using the QuEChERS procedure. Our results suggest the occurrence of neonicotinoids in soils several months after application, with concentrations above 4 µg/Kg in more than 50% of samples studied. Based on our findings with its possible implications for persistence and potential mobility, as well as systemic activity and application rates of neonicotinoids in the Ghanaian environment, we suggest a critical role for research and education in ensuring environmental sustainability in pesticide application in Ghana.

Appendix B.1

Table B.1 Soil properties (soil organic carbon, pH and texture classification) of cocoa growing soils

Sample	SOC (%)	Clay (%)	Sand (%)	Silt (%)	pH ($CaCl_2$)	Texture class
ADU	2.33	17.88	69.34	12.77	6.27	sandy-loam
ADW	2.52	17.91	61.62	20.47	6.03	sandy-loam
AGA	1.89	27.81	27.69	44.49	5.12	silt-loam
AGO	1.69	15.73	63.3	20.97	6.34	sandy-loam
AKY	1.89	12.6	69.76	17.64	5.1	sandy-loam
ANT	1.43	31.14	29.92	38.93	6.63	clay-loam
ANY	3.62	13.6	64.44	21.88	6.05	sandy-loam
ASA	3.57	18.99	32.16	48.85	5.69	loam
ASB	1.89	12.89	74.22	12.89	6.18	loam
ASF	3.34	16.79	66.41	16.79	5.56	sandy-loam
ASI	4.21	12.69	36.65	50.76	6.37	silt-loam
ASK	2.32	12.6	72.28	15.12	6.15	sandy-loam
ASM	4.24	20.26	44.29	50.76	7.54	loam
ASR	2.54	21.38	54.18	24.43	6.78	sandy-clay-loam
BEK	5.68	16.96	43.47	39.57	7.37	loam
BRE	2.32	37.41	26.06	34.53	6.76	clay-loam
DOR	3.56	49.51	29.71	33.99	7.53	clay
DZA	3.57	15.59	41.41	40.11	5.68	loam
ASS	1.63	23.68	57.91	28.94	4.97	sandy-clay-loam
GOA	2.7	16.76	59.30	23.94	6.67	sandy-loam
HWI	4.43	18.00	60.00	22.00	6.00	sandy-loam
JAS	1.89	22.76	44.36	32.88	5.37	loam
KAD	2.67	25.92	55.94	18.14	6.61	sandy-clay-loam
KAJ	2.88	26.69	46.62	26.69	5.67	sandy-clay-loam
KAS	2.48	16.77	52.46	30.76	5.77	loam
KON	2.68	15.89	54.98	52.99	5.77	sandy-loam
KPA	3.62	20.21	48.01	31.77	5.49	loam
LEA	4.72	12.60	69.76	17.64	5.87	sandy-loam
LEK	1.55	20.00	64.00	12.00	5.97	sandy-loam
MAN	3.92	17.3	45.62	37.07	5.58	loam
MEE	2.72	12.6	69.76	17.64	6.37	sandy-loam
MEH	5.56	16.07	46.42	37.5	7.46	Loam
MIF	2.56	20.04	31.29	48.67	7.47	Loam
MIM	3.87	33.24	31.72	42.02	7.44	clay-loam
ASU	2.79	12.35	47.51	40.14	6.2	Loam
NKA	4.46	29.93	50.12	19.95	6.94	sandy-clay-loam

Table B.1 Soil properties (soil organic carbon, pH and texture classification) of cocoa growing soils continued.

Sample	SOC (%)	Clay (%)	Sand (%)	Silt (%)	pH ($CaCl_2$)	Texture class
NKE	3.52	21.42	67.89	10.7	6.9	sandy-clay-loam
NKI	2.33	13.97	58.10	27.93	6.73	sandy-loam
NKR	1.98	20.39	38.84	40.77	7.17	Loam
NKW	2.59	26.84	38.27	34.89	6.28	loam
NSO	7.43	15.6	81.8	2.6	4.88	sandy-loam
OBU	2.22	15.34	43.72	40.93	6.83	loam
ODA	1.89	21.8	70.02	8.17	4.62	sandy-clay-loam
NED	2.11	20.23	50.87	28.9	5.45	sandy-clay-loam
OFF	2.56	21.1	49.35	29.54	6.84	loam
OFO	1.51	24.83	67.72	7.45	5.79	sandy-clay-loam
SAE	3.79	26.11	55.61	18.28	7.21	sandy-clay-loam
SAN	2.83	27.19	33.25	39.56	6.56	clay-loam
SUN	2.79	19.42	27.86	52.72	6.48	silt-loam
TAF	3.13	24.12	68.64	7.23	4.46	sandy-clay-loam
TEP	1.89	20.16	72.26	7.58	4.62	sandy-clay-loam
TWH	2.48	15.14	69.72	15.14	6.16	sandy-loam
TWP	1.28	15.43	69.14	15.43	6.62	sandy-loam
AVERAGE	2.94	20.41	52.94	27.80	6.20	

Appendix B.2 Analysis of Variance

EFFECT OF EXTRACTION SALTS

```
. bysort pesticide: oneway recovery salts, bonferroni tabulate
```

```
-> pesticide = imida
```

Salts	Summary of Recovery Mean	Std. Dev.	Freq.
acetate	83.791667	2.8697416	6
unbuffere	91.25	4.7355042	6
citrate	85.166667	2.148643	6
Total	86.736111	4.6363908	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	189.048611	2	94.5243056	8.04	0.0042
Within groups	176.385417	15	11.7590278		
Total	365.434028	17	21.4961193		

Bartlett's test for equal variances: chi2(2) = 2.9644 Prob>chi2 = 0.227

Comparison of Recovery by Salts
(Bonferroni)

Row Mean- Col Mean	acetate	unbuffer
unbuffer	7.45833 0.006	
citrate	1.375 1.000	-6.08333 0.023

```
-> pesticide = thiac
```

Salts	Summary of Recovery Mean	Std. Dev.	Freq.
acetate	80.791667	2.4208297	6
unbuffere	89.291667	3.171816	6
citrate	77.5	3.9749214	6
Total	82.527778	5.955033	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	444.256944	2	222.128472	21.01	0.0000
Within groups	158.604167	15	10.5736111		
Total	602.861111	17	35.4624183		

Bartlett's test for equal variances: chi2(2) = 1.0940 Prob>chi2 = 0.579

Comparison of Recovery by Salts
(Bonferroni)

Row Mean-Col Mean	acetate	unbuffer
unbuffer	8.5 0.001	
citrate	-3.29167 0.300	-11.7917 0.000

-> pesticide = thiam

Summary of Recovery

Salts	Mean	Std. Dev.	Freq.
acetate	84.916667	17.14181	6
unbuffere	86.083333	5.9595861	6
citrate	90.083333	7.3223402	6
Total	87.027778	10.854617	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	88.1111111	2	44.0555556	0.35	0.7136
Within groups	1914.875	15	127.658333		
Total	2002.98611	17	117.822712		

Bartlett's test for equal variances: chi2(2) = 6.0299 Prob>chi2 = 0.049

Comparison of Recovery by Salts
(Bonferroni)

Row Mean- Col Mean	acetate	unbuffer
unbuffer	1.16667 1.000	
citrate	5.16667 1.000	4 1.000

-> pesticide = aceta

Salts	Summary of Recovery Mean	Std. Dev.	Freq.
acetate	92.375	2.2513885	6
unbuffere	93.875	3.1809983	6
citrate	86.666667	2.8838631	6
Total	90.972222	4.1381376	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	173.590278	2	86.7951389	11.08	0.0011
Within groups	117.520833	15	7.83472222		
Total	291.111111	17	17.124183		

Bartlett's test for equal variances: chi2(2) = 0.5508 Prob>chi2 = 0.759

Comparison of Recovery by Salts
(Bonferroni)

Row Mean- Col Mean	acetate	unbuffer
unbuffer	1.5 1.000	
citrate	-5.70833 0.009	-7.20833 0.001

-> pesticide = clothi

Salts	Summary of Recovery Mean	Std. Dev.	Freq.
acetate	88.875	3.8103478	6
unbuffere	96.791667	1.4354152	6
citrate	90.166667	6.4858821	6
Total	91.944444	5.4755845	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	216.465278	2	108.232639	5.54	0.0158
Within groups	293.229167	15	19.5486111		
Total	509.694444	17	29.9820261		

Bartlett's test for equal variances: chi2(2) = 8.1786 Prob>chi2 = 0.017

Comparison of Recovery by Salts
(Bonferroni)

Row Mean- Col Mean	acetate	unbuffer
unbuffer	7.91667 0.022	
citrate	1.29167 1.000	-6.625 0.061

EFFECT OF D-SPE CLEAN-UP SORBENTS

```
      name:  <unnamed>
       log:  C:\Users\Eric\Dropbox\Enok_Eric\e1.smcl
  log type:  smcl
 opened on:   6 Jun 2014, 22:42:39

. bysort pesticide: oneway recovery sorbent, bonferroni scheffe sidak tabulate

-------------------------------------------------------------------------------
-> pesticide = Imidac
```

Sorbent	Summary of Recovery Mean	Std. Dev.	Freq.
none	91.25	4.7355042	6
psa	98.625	3.204489	6
c-18	96.333333	1.921371	6
gcb	94.5	1.5652476	6
Total	95.177083	4.0043992	24

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	174.632812	3	58.2109375	6.00	0.0044
Within groups	194.177083	20	9.70885417		
Total	368.809896	23	16.0352129		

```
Bartlett's test for equal variances:  chi2(3) =   6.6956  Prob>chi2 = 0.082
```

Comparison of Recovery by Sorbent (Bonferroni)

Row Mean-Col Mean	none	psa	c-18
psa	7.375 0.003		
c-18	5.08333 0.063	-2.29167 1.000	
gcb	3.25 0.515	-4.125 0.197	-1.83333 1.000

Comparison of Recovery by Sorbent
(Scheffe)

Row Mean- Col Mean	none	psa	c-18
psa	7.375 0.006		
c-18	5.08333 0.076	-2.29167 0.660	
gcb	3.25 0.377	-4.125 0.189	-1.83333 0.792

Comparison of Recovery by Sorbent
(Sidak)

Row Mean- Col Mean	none	psa	c-18
psa	7.375 0.003		
c-18	5.08333 0.061	-2.29167 0.770	
gcb	3.25 0.417	-4.125 0.181	-1.83333 0.901

-> pesticide = Thiaclo

	Summary of Recovery		
Sorbent	Mean	Std. Dev.	Freq.
none	89.291667	3.171816	6
psa	97.125	1.9348773	6
c-18	95.541667	3.3668111	6
gcb	91.666667	3.0808549	6
Total	93.40625	4.1870741	24

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	230.070313	3	76.6901042	8.86	0.0006
Within groups	173.15625	20	8.6578125		
Total	403.226563	23	17.5315897		

Bartlett's test for equal variances: chi2(3) = 1.5083 Prob>chi2 = 0.680

Comparison of Recovery by Sorbent
(Bonferroni)

Row Mean-Col Mean	none	psa	c-18
psa	7.83333 0.001		
c-18	6.25 0.009	-1.58333 1.000	
gcb	2.375 1.000	-5.45833 0.026	-3.875 0.202

Comparison of Recovery by Sorbent
(Scheffe)

Row Mean-Col Mean	none	psa	c-18
psa	7.83333 0.002		
c-18	6.25 0.014	-1.58333 0.832	
gcb	2.375 0.591	-5.45833 0.036	-3.875 0.192

Comparison of Recovery by Sorbent
(Sidak)

Row Mean- Col Mean	none	psa	c-18
psa	7.83333 0.001		
c-18	6.25 0.009	-1.58333 0.933	
gcb	2.375 0.690	-5.45833 0.026	-3.875 0.186

-> pesticide = Thiame

Summary of Recovery

Sorbent	Mean	Std. Dev.	Freq.
none	86.083333	5.9595861	6
psa	93.958333	8.5738216	6
c-18	92.375	9.5691562	6
gcb	85.291667	6.0444534	6
Total	89.427083	8.1579113	24

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	345.028646	3	115.009549	1.94	0.1557
Within groups	1185.65625	20	59.2828125		
Total	1530.6849	23	66.5515172		

Bartlett's test for equal variances: chi2(3) = 1.5991 Prob>chi2 = 0.660

Comparison of Recovery by Sorbent
(Bonferroni)

Row Mean- Col Mean	none	psa	c-18
psa	7.875 0.550		
c-18	6.29167 1.000	-1.58333 1.000	
gcb	-.791667 1.000	-8.66667 0.392	-7.08333 0.760

Comparison of Recovery by Sorbent
(Scheffe)

Row Mean- Col Mean	none	psa	c-18
psa	7.875 0.394		
c-18	6.29167 0.582	-1.58333 0.988	
gcb	-.791667 0.998	-8.66667 0.313	-7.08333 0.485

Comparison of Recovery by Sorbent
(Sidak)

Row Mean- Col Mean	none	psa	c-18
psa	7.875 0.439		
c-18	6.29167 0.679	-1.58333 1.000	
gcb	-.791667 1.000	-8.66667 0.333	-7.08333 0.557

-> pesticide = Acetam

Sorbent	Summary of Recovery Mean	Std. Dev.	Freq.
none	93.875	3.1809983	6
psa	102.875	1.8957189	6
c-18	99.583333	4.0331956	6
gcb	96.458333	1.2289901	6
Total	98.197917	4.326347	24

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	273.049479	3	91.0164931	11.56	0.0001
Within groups	157.447917	20	7.87239583		
Total	430.497396	23	18.7172781		

Bartlett's test for equal variances: chi2(3) = 6.7309 Prob>chi2 = 0.081

Comparison of Recovery by Sorbent
(Bonferroni)

Row Mean- Col Mean	none	psa	c-18
psa	9 0.000		
c-18	5.70833 0.013	-3.29167 0.334	
gcb	2.58333 0.759	-6.41667 0.005	-3.125 0.408

Comparison of Recovery by Sorbent
(Scheffe)

Row Mean- Col Mean	none	psa	c-18
psa	9 0.000		
c-18	5.70833 0.020	-3.29167 0.279	
gcb	2.58333 0.484	-6.41667 0.008	-3.125 0.321

Comparison of Recovery by Sorbent
(Sidak)

Row Mean- Col Mean	none	psa	c-18
psa	9 0.000		
c-18	5.70833 0.013	-3.29167 0.291	
gcb	2.58333 0.556	-6.41667 0.005	-3.125 0.345

-> pesticide = Clothian

Sorbent	Summary of Recovery Mean	Std. Dev.	Freq.
none	96.791667	1.4354152	6
psa	104.79167	2.2990034	6
c-18	98.625	3.8818488	6
gcb	97.333333	4.3001938	6
Total	99.385417	4.4183284	24

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	244.466146	3	81.4887153	7.97	0.0011
Within groups	204.53125	20	10.2265625		
Total	448.997396	23	19.5216259		

Bartlett's test for equal variances: chi2(3) = 5.9176 Prob>chi2 = 0.116

Comparison of Recovery by Sorbent
(Bonferroni)

Row Mean- Col Mean	none	psa	c-18
psa	8 0.002		
c-18	1.83333 1.000	-6.16667 0.020	
gcb	.541667 1.000	-7.45833 0.004	-1.29167 1.000

Comparison of Recovery by Sorbent
(Scheffe)

Row Mean- Col Mean	none	psa	c-18
psa	8 0.004		
c-18	1.83333 0.805	-6.16667 0.028	
gcb	.541667 0.993	-7.45833 0.007	-1.29167 0.920

Comparison of Recovery by Sorbent
(Sidak)

Row Mean- Col Mean	none	psa	c-18
psa	8 0.002		
c-18	1.83333 0.912	-6.16667 0.019	
gcb	.541667 1.000	-7.45833 0.004	-1.29167 0.983