Dermal Exposure and Transport of Selected Organophosphate Pesticides In Neotropical Migratory Songbirds

by

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ABSTRACT

The majority of avian pesticide field studies have focused on the ingestion of treated food items (oral exposure). Dermal exposure remains an important route of exposure, when birds come into contact with pesticides applied to foliage. The objectives of this thesis were to 1) develop, design and apply a chemical analysis methodology to detect selected organophosphate pesticides in samples of feet of migratory songbirds, and 2) to assess the potential dermal exposure of songbirds by looking at the frequency of exposure in a range of neotropical species. A total of 18 species of migratory songbirds were opportunistically sampled during the spring in 2007 and 2011 in downtown Toronto, Canada, during the migration to their breeding sites in Canada. Only the feet of dead birds colliding with lighted buildings were used for chemical analysis. The working hypothesis was that birds had been exposed via their feet to organophosphate pesticides while foraging on pesticide-treated crops in their wintering grounds. Among the four selected organophosphate pesticides in this study (i.e chlorpyrifos, fenthion, fenamiphos, and diazinon), only chlorpyrifos was detected in feet samples of six birds species, these being Black-throated blue warbler, Tennessee warbler, Northern parula, Northern waterthrush, Common yellowthroat and Blue-winged warbler.

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Dedication

This thesis is dedicated to my mother who has been a source of encouragement and inspiration to me not only throughout the times of study, but throughout my life.

I also dedicated this thesis to my wife, and sisters who have supported me all the way.

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LIST OF ABBREVIATIONS

AChE	- acetycholinesterase
APCI	- Atmospheric Pressure chemical Ionization methods
API	- Atmospheric Pressure Ionization methods
CE	- collision energy
ChE	- cholinesterase
d_{27}-TPP	- deuterated triphenyl phosphate
DCM	-dichloromethane
DSPE	-dispersive soild-phase extraction
ESI	- electrospray ionization
FLAP	- Fatal Light Awareness Program
GC-MS	-gas chromatography-mass spectrometry
H_3PO_4	-phosphoric acid
IPs	-identification points
LC/TOF-MS	- liquid chromatography-Time-of-Flight- mass spectrometry
LC-QqQ/MS/MS	- liquid chromatography- Triple Quadrupole - mass spectrometry
LC-QTOF-MS/MS	- liquid chromatography- Quadrupole-Time-of-Flight- mass spectrometry
LOD	- limit of detection
LOQ	- limit of quantitation
m/z	-mass to charge
MFOs	- mixed-function oxidases
MRM	- multiple reaction monitoring
NH_2	-aminopropyl
OPPs	- organophosphate pesticides

PSA	-primary secondary amine
QqQ	-triple Quadupole Mass Spectrometry
QuEChERS	- quick, easy, cheap, effective, rugged and safe
S/N	-signal to noise
SPE	-solid phase extraction

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CHAPTER 1

General introduction

1.1 Birds and pesticides

1.1.1 Abundance status of migratory songbirds

The abundance of migratory songbirds in both North America (Robbins *et al.*, 1989) and Europe (Peach *et al.*, 1998; Gregory *et al.*, 2002: Newton, 2004: Sanderson *et al.*, 2006) is believed to be in decline (Holmes, 2007). This has raised anew the critical question of what regulates these migratory songbird populations and when during the annual cycle these processes operate (Holmes, 2007). Several reasons might be behind these declines; one hypothesis is that migratory passerines are limited principally by events affecting survivorship in the non-breeding season (Baillie and Peach, 1992; Morton, 1992: Rappole and McDonald, 1994).

Agrochemicals have been confirmed as a major factor in reducing the entire spectrum of avian biodiversity in farmed landscapes through both direct and indirect effects (Newton, 1998). In the USA, the earliest and first overview of agrochemical use concluded that much of the significant evidence concerning the worldwide effects of insecticides has been provided by birds (Mrak, 1969: Mineau, 2005). Some of the reported effects were the deaths of seed eaters and their predators due to exposure to organochlorine (OC) insecticides.

1.1.2 Pesticide effects on birds

Since migratory birds can accumulate pesticides over vast geographic areas, (OC) pesticides contamination has been suggested as a possible cause for the decline in neotropical migrant passerines (Gard *et al.*, 1993, 1995: Block *et al*, 1995). Evidence for the harmful effects of (OC) insecticides, such as DDT, aldrin and dieldrin, on wild populations of birds contributed to the decision to impose wide ranging bans on the use of these compounds worldwide (Walker,

2003). Moreover, the use of some currently applied organophosphate, carbamate, and pyrethroid insecticides and organomercury fungicides has been placed under restrictions because of either established or perceived harmful effects upon animals in the field (Walker, 2003).

Pesticides use may be one of the important factors behind the wide ranging decline of some farmland bird species, but the exact reasons for these declines are complex and species specific (Mineau and Whiteside, 2006). They could be related to features of industrialized modern agriculture. For example, in the United Kingdom, based on widespread monitoring of bird populations, most of the avian species found on farmlands have declined (Newton, 2004), and a similar observation was found in North America (McLaughlin and Mineau, 1995).

1.2 Organophosphate pesticides

1.2.1 Chemical structures

Organophosphate pesticides (OPPs) are an important class of chemicals used to control insect pests. The general structure of OPPs can be represented as shown in Figure 1.1.

OPPs considered to be derivatives of phosphoric acid (H_3PO_4) or phosphonic acid (H_3PO_3) in which all H atoms are replaced by organic moieties. With reference to Figure 1.1, R_1 and R_2 are less reactive and can be alkoxy groups, alkyl, aryl, alkylthio or alkylamino. X is either oxygen or sulfur. The most reactive and most variable substituent is the L group (the "leaving group"). The L group is the substituent that is displaced by the OPPs phospyrylate acetycholinesterase (Chambers *et al.*, 2010a). Figure 1.2; show Nomenclature of major subclasses of OPPs.

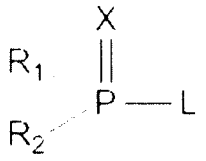


Figure 1.1 General structure of OPPs (Chambers *et al.*, 2010a)

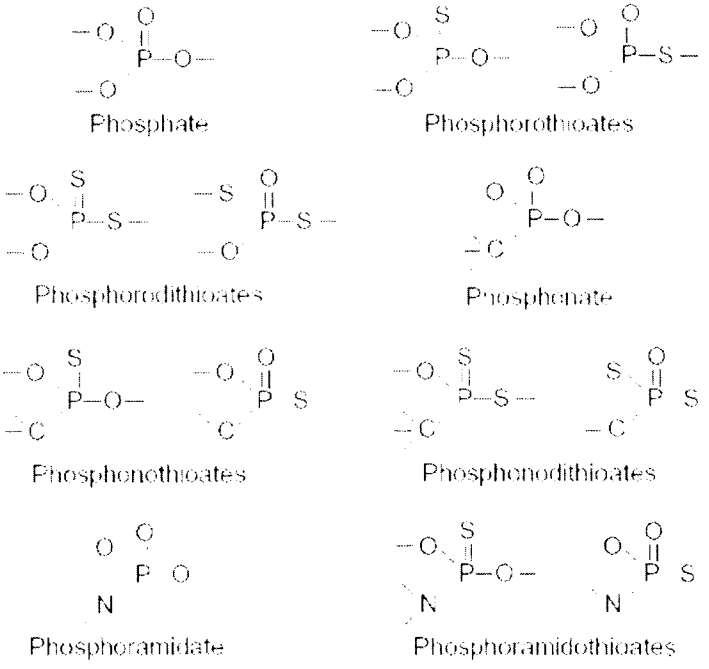


Figure 1.2 Nomenclature of major subclasses of OPPs (Chambers *et al.*, 2010a)

1.2.2 Mode of action

The principle of toxicity or mode of action of OPPs is based on disruption of the nervous system by inhibition of cholinesterase activity (ChE)–mainly acetycholinesterase (AChE)–in the central nervous system and at neuromuscular junctions (O'Brien, 1967).

Some OPPs, such as acephate and monocrotophos, can directly inhibit ChE, but the majority of OPPs, such as diazinon, malathion and parathion must first undergo an oxidative desulfuration step, which is mediated by mixed-function oxidases (MFOs) in the liver, for instance, of vertebrates, for high anticholinesterase potency (Eto, 1974).

In avian species, the lack of detoxifying acetylcholinesterase (A-esterase) activity and low microsomal monooxygenase detoxifying activity in the liver makes avian species more susceptible and sensitive to OPPs poisoning than mammals (Walker, 1983).

1.2.3 Metabolism and environmental fate

OPPs are metabolized by Phase I oxidation mediated by cytochromes P450 enzymes (the CYP450-superfamily, includes CYP 3A4, 1A2, 2B6, 2C19, and 2D6) or flavin monooxygenases, reductases and hydrolyses OPPs metabolism via Phase 2 reactions (conjugations). The metabolic and chemical lability of OPPs mean they have shorter half-lives in the environment or in an organism (Chambers *et al.*, 2010b).

There are several chemical processes (reactions) that influence the persistence of OPPs in the environment and that lead to degradation and detoxification (Caceres *et al.*, 2010). Hydrolysis is one of the most important degradation pathways for OPPs due to the susceptibility of organophosphate esters to hydrolysis reactions (Racke, 1992).

1.2.4 Bird mortality due to exposure to anticholinesterase compounds

Songbirds are routinely killed in large numbers after ingesting treated seeds or insects (Mineau, 1993), but their small body size means that these incidents may easily be overlooked and/or may only rarely involve rehabilitation (Mineau, 2002).

The majority of field studies have primarily focused on identifying mortality associated with pesticide use; on the other hand, laboratory studies have focused on the lethal potential of pesticides as well as their long-term toxicity (Hooper and La Point, 1994). Mortality of birds in the field is unlikely to be detected unless the birds in question are large and highly visible (Mineau, 2005), and happen to die in large groups in areas of high public visibility. For example, the mass mortality incident in Argentina, involving an estimated 6000 dead Swainson's hawks (*Buteo swainsoni*) between 1995 and 1996, was due to poisoning by monocrotophos used to control grasshopper outbreaks (Goldstein *et al.*, 1999). In addition, monocrotophos was the cause of death of an estimated 10,000 American Robins (*Trudus migratorius*) in two small Florida potato fields (Lee, 1972). Magpie populations declined in the western states of the USA between 1968 and 1979; this was linked to the deaths of Black-billed magpies (*Pica hudsonia*) attributed to exposure to famphur up to three months after cattle had been treated for cattle warbles (Henny *et al.*, 1985). Also, magpies (*Pica pica*) were found dead in the U.K. after cattle were treated with famphur (Felton *et al.*, 1981). Similarly, there were approximately 520 recorded incidents of raptor mortality resulting from ChE-inhibiting pesticides in the USA, U.K., and Canada in the period of 1985–1995 (Mineau *et al.*, 1999). Moreover, secondary poisoning of raptors from insecticide (a granular formulation of carbofuran) was reported in 1990 in the Fraser Delta, Canada (Elliott *et al.*, 1996). During the period from 1980 to 2000, more than 900 birds were found killed by diazinon; the majority of these species was waterfowl (Fleischli *et al.*, 2004). In

Canada, more than 50 Canada geese (*Branta canadensis*) were found dead from exposure to diazinon after it was applied to grass at golf courses (Frank *et al.*, 1991). In Bellingham, Washington, in the USA, 85 American wigeon (*Anas americana*) were found dead after application of diazinon to nine fairways at a golf course (Kendall *et al.*, 1992).

Mineau (2002) described how recent analyses and modeling of avian pesticides in field studies have led to the conclusion that bird deaths are regular and frequent in insecticide-treated fields; however, to arrive at a scientifically defensible estimate of the actual number of bodies is more difficult (Mineau, 2002). Quantifying bird presence (possible impact) in a field at the time of pesticide application is difficult. Thus, the extent of bird mortality is defined not so much by the pesticides (at least within the OPPs and carbamate families) as it is by the number of birds that are present and therefore at risk of being killed (Mineau, 2005). For example, on the basis of field studies, rough calculations for bird deaths from a granular formulation of carbofuran were conservatively estimated to be 17 to 91 million birds annually from the USA Midwest corn belt alone (Mineau, 2005). Another estimate was that 67 million birds per year were killed by direct pesticide poisoning in the USA, based on the conservative assumption that 10 percent of exposed birds were killed (Pimentel *et al.*, 1992).

1.3 Routes of pesticide exposure

Birds can be directly exposed to pesticide application by several routes or pathways: ingestion through consumption of free chemicals such as granules (primary exposure), or more commonly through consumption of contaminated food items such as insects, water, foliage, seeds, treated crops (secondary exposure), inhalation, dermal contact (Driver *et al.*, 1991), and maternal transfer (Mineau, 2005).

The majority of the studies concern bird mortality caused by either direct consumption of pesticides or secondary transfer of pesticide liquid or granular based formulations (Sotherton and Holland, 2003). There is a lack of knowledge about dermal exposure in birds due to the difficulty in performing both laboratory and field studies.

Percutaneous (dermal) hazard depends on the rate (dose and time) at which chemicals penetrate the skin. This rate varies widely among chemical formulations, site of application, and species (McCreesh, 1965). For example, some anticholinesterase pesticides have been tested by application to the wrapped feet (trasometatarsis, phalanges and webbing) of adult mallards for 24 h, and to 1 cm^2 of a featherless skin under the wing joint of a small passerine (Hudson *et al*, 1979). The same species were dosed orally (Schafer, 1973), and the LD_{50s} of both studies were positively correlated (Hill, 2003).

The residue of pesticide application on foliage further to treatment to control insects in forests, orchards and on cultivated crops such as small grains and turf grasses, has resulted in excessive mortality over the years. Foliar treatments may be especially percutaneous (dermal) hazardous when birds are in the spray zone during treatment and subject to multiple routes of exposure that includes dermal exposure (Hill, 2003). For example, turf grass treatment with diazinon has proven particularly hazardous to waterfowl (American wigeon and Canada goose) (Stone, 1985). As a result of this hazard to grazing waterfowl, the US. Environmental Protection Agency (USEPA) issued a cancellation notice for the use of diazinon products on golf courses and sod (turf) farms (US EPA, 1986).

1.4 Avian dermatology

Avian skin attached to the underlying muscles consists of outer epidermis and underlying dermis, and has the same structure as mammal's skin (Doneley, 2010). The epidermis is generally very thin and pliable. Whereas dermis is thicker contains blood vessels, fat deposits, nerves and free nerve endings, several types of neuroreceptors, and smooth muscles that move the feathers (Lucas and Stettenheim, 1972).

The basic structure of the epidermis is similar in all areas, but the thickness varies considerably. Over most of the body it is extremely thin, usually three to five cells thick, but over the naked parts of the legs and face it is much thicker. The epidermis is comprised of the stratum germinativum, or germinal layer (dividing cells), and the stratum corneum, or cornified layer (keratinized cells) (Pass, 1995). The cornified layer is composed of sheets of keratinized, anuclear, flat cells that form lamellae separated by lipids. It is thickest in the skin of scales on the legs and plantar surface of the feet. In avian skin, as in mammalian and the soft parts of reptilian skin, the keratinized cells become filled with alpha-keratin as they differentiate. The proteins differ in amino acid composition, molecular size, and organization among the various integumentary derivatives (Brush, 1980a, b; Brush and Wyld, 1982: Homberger and Brush, 1986: Stettenheim, 2000).

Avian skin lacks sweat and sebaceous glands, except for the uropygeal gland which anoints the plumage, yet the epidermis itself produces neutral fats and phospholipids in a variety of species (Lucas, 1980; Lavker, 1975). Avian keratinocytes produce large amounts of lipids. Therefore, even though avian skin has a very small number of glands, the whole integument can be considered to be an oil-producing holocrine gland. A highly lipogenic epidermis is instead thought to compensate for the paucity of sebaceous glands (Spearman and Hardy, 1985). The

lipogenic nature of avian epidermis distinguishes it from the epidermis of terrestrial mammals (Lucas and Stettenheim, 1972; Menon *et al.*, 1981). Since avian epidermal cells include both lipogenesis and keratinization in their differentiation, they have been termed "sebokeratinocytes" (Wrench *et al.*, 1980). The entire skin acts as a sebaceous secretory organ, with the preen gland and the ear glands as specialized parts (Menon *et al.*, 1981).

Sebokeratinocytes elaborate and secrete sebum-like lipids (such as sterol/wax esters, triglycerides) as they cornify (keratinization of the cell). In addition to the lipid droplets, avian epidermis elaborates, but rarely secretes, lipid-enriched organelles, the multigranular bodies. The multigranular bodies are analogous to the lamellar bodies of mammals (Menon *et al.*, 1981; Menon and Menon, 2000), the secretion of which results in formation of occlusive lipid bilayers characteristic of the mammalian stratum corneum and providing a permeability barrier. However, in contrast to mammals, the avian multigranular bodies form a reserve barrier mechanism (Menon and Menon, 2000).

1.5 Feet as a route of exposure and as a matrix for chemical analysis

Birds (mostly seabirds and top predators such as raptors) have been used to monitor the environment as qualitative and quantitative indicators of OC pesticides in food webs, taking into consideration that OC pesticides are lipophilic and highly persistent, and are accumulated in food chains through bioconcentration and biomagnifications (Furness, 1993). OC pesticides concentrations have been detected in both resident and migratory avian species, and the majority of the chemical analysis methods reported in the literature are based on the use of tissue such as muscle, liver, kidney and eggs in carcasses or captive living birds (Mora *et al*,. 1993: Auman *et al*,. 1997; Elliot and Norstrom, 1998; Gómara *et al*,. 2004; Rivera-Rodrı´guez *et al.*, 2007). with tissue analysis for pesticide residue purposes based on the assumption that birds were exposed to

pollutants through consumption of contaminated food (dietary exposure) such as fruit or prey items (insect).

Dermal exposure occurs when a bird's skin comes into contact with free pesticide spray or spills, atmospherically deposited pesticide, and pesticide in the environmental media (e.g contaminated soil, sediment, water, plants) (Driver *et al.*, 1991). Although feathers provide a barrier to much of a bird's skin (Welty and Baptista, 1988), dermal exposure may still occur through the eyes as well as the skin on the legs and feet (Burkepile *et al.*, 2002; Vyas *et al.*, 2003b).

Avian skin is in general much thinner than that of mammals (Hodges, 1974); thus, birds may be particularly vulnerable to dermal exposure to chemicals in comparison with mammals. The thickness of skin can vary considerably over different areas of the body. For example, skin is thinner in featherhead areas and thicker in unprotected areas such as the feet, tarsi, beak, and face (Hodges, 1974). For this reason, some commercial OPPs were applied to perch sites to act as a source of dermal toxicity via contact (Otis, 1987); for example, starlings and other pests were controlled using sticky formulations of OPPs on roost sites that adhere to the birds' feet. Aerial application may also be a route of dermal exposure. For example, acute toxicity of fenthion was demonstrated in weaver-birds (*Quelea quelea*) after application to the back feathers (Pope and Ward, 1972); however, it was suggested that feathers provide some protection, and sufficient amounts of solvent were needed to permeate the feathers to reach the skin. Captive forest songbirds were treated with technical phosphamidon via the dermis (feet, under the wing) using a micro-syringe and mortality was observed in birds within 15 min of exposure (Fowle, 1972). In the same study Fowle (1972) provided several songbirds (Order Passeriformes) species with either perches or a floor treated with phosphamidon, and signs of toxicity and mortality were

observed after 30 mins of exposure. In another study, House sparrows (*Passer domesticus*) were exposed to perches treated with fenthion for 30 s to 16.5 min, and signs of toxicity were observed as early as 16.5 min (Hunt *et al.*, 1991). Using cholinesterase activity measurements, dermal absorption and ingestion via preening were found to be major contributing routes of exposure for Northern bobwhite (*Colinus virginianus*) (Driver *et al.*, 1991).

Analysis of the mechanism of death and chemical residue is performed to identify the insecticide responsible for the death by analyzing the cholinesterase activity of the brain in cases when poisoning of wild birds by OPPs is suspected (Vyas *et al.*, 2003b). The conventional matrix used for the forensic investigation (chemical residue analysis) is the gastrointestinal system and its contents (Hill and Fleming, 1982; Vyas *et al.*, 2003b). Delays in the discovery, reporting, and investigation of a mortality and/or poisoning incident increase the interval between the time of mortality and carcass collection which in turn increases the chances of compromising the quality of the evidence through scavenging and decomposition (Vyas, 1999; Vyas *et al.*, 2003a,b, 2004). Consequently, when a carcass is recovered during a field investigation, the biochemical and chemical matrices that are used to investigate the cause of death from insecticide poisoning may be degraded and not suitable for analysis. The loss of these matrices introduces uncertainty in determining the cause of death and reduces the recovered carcasses to circumstantial evidence of poisoning (Vyas *et al.*, 2003b, 2004).

It has been suggested that feet may be used in wildlife forensic laboratories for pesticide residue (Frank *et al.*, 1991; Stroud and Adrian, 1996; Vyas *et al.*, 2003b, 2004). Vyas *et al.* (2004) describe the reasons for focusing on the feet as an indicator of pesticide exposure, which are: 1) feet are likely to come into contact with insecticide (e.g walking on treated lawn and soil, perching on contaminated branches, grasping contaminated prey, and wading in contaminated

water; 2) dermal absorption of pesticide through feet can be a significant route of exposure for birds; and 3) the possible persistence of insecticide in the feet of live birds indicates a potential for detecting residue in feet, which can be explained by a study that was conducted by Henderson *et al.* (1994), in which they exposed Rock doves (*Columba livia*) to diazinon, ethyl acetate and ethyl parathion either dermally via the feet or orally by gavage. Birds dermally exposed to diazinon and ethyl parathion exhibited a slower recovery (six weeks) of plasma cholinesterase activity than birds exposed orally (three to five days), with the suggested cause being that pesticide could be stored under the scales of the feet and slowly released into the blood stream (Henderson *et al.*, 1994).

Bird feet have been used for pesticide residue detection to assess acute and chronic exposure to some currently used pesticides. For example, pesticide residue analysis of House sparrows (*Passer domesticus*) exposed to fenthion-treated perches showed that their feet and feathers had higher residue concentrations than the internal carcass (Hunt *et al.*, 1991). Foot wash from a Great-tailed grackle (*Quiscalus mexicanus*) that was exposed to a treated banana plantation field had a detectable level of ametryn (Mortensen *et al.*, 1998). Pesticide residue on the skin, and on feathers, the feet and the gastrointestinal contents, were greater in Canada goose (*Branta Canadensis*) goslings from the field than in goslings from laboratory dietary toxicity studies (Vyas *et al.*, 2006). Azinophos-methyl residues on the skin and the feathers and feet of Brown-headed cowbirds (*Molothrus ater*) were measured in order to quantify dermal exposure to songbirds that entered and inhabited an apple (*Malus x domestica*) orchard following insecticide application; the residues were detected on the skin, feathers and feet from 36 h and seven-day post application exposure periods (Vyas *et al.*, 2007).

Examination of decomposed and scavenged carcasses has revealed that the feet of many carcasses remain intact even when the remainder of the carcass may not be suitable for laboratory analysis (Vyas *et al.*, 2003b). Insecticide residue analysis of decomposed (or weathered by placing the feet outdoors) and undecomposed feet were conducted to determine whether insecticide residue could be detected from bird feet that had been weathered (Vyas *et al.*, 2003b, 2004); the results revealed that the concentration of insecticide residue on weathered feet did not exhibit a significant decline for approximately two to three weeks when compared to unweathered or undecomposed feet, and insecticides were detected from weathered or decomposed feet for up to 28 days. In another study, the liquid carbofuran formulation, Furadan (carbamate group), was poured on some pieces of dead deer, and Eastern screech owls *(Otus asio)* were placed on these pieces to simulate a raptor perched on a large insecticide-laced carcass while feeding. Carbofuran residues were detected on feet of the owls after being weathered for 28 days (Vyas *et al.*, 2005).

In general, the method of insecticide application, the chemical concentration on the matrix with which the feet were in contact, the amount of contact with uncontaminated surfaces which may remove the insecticide from the feet, the chemical absorption rate into the blood and insecticide properties such as pH and half-life and, finally, the lag time between mortality and when the feet were collected are all factors that might affect the success of detecting an insecticide on feet (Vyas *et al*, 2004).

1.6 Pesticide residue analysis

The analysis of pesticide residues in fatty matrices remains challenging, and it is difficult to avoid the co-extraction of fatty materials (Gilbert -Lopez *et al.*, 2009). The majority of the methods for analyzing pesticide residue in fatty foods are designed predominantly for OC, and

several solvents are employed for extraction, such as hexane, acetone, ethyl acetate and dichloromethane, to dissolve the lipids (Lehotay *et al.*, 2005).

One of the oldest pesticide residue methodologies is the Luke method (Luke, 1975) which was developed in the 1970s to meet the demand for a wide analytical polarity range. In this method, the sample is first extracted with acetone, and then both salt (NaCl or MgSO4) and non-polar solvent (dichloromethane, dichloromethane–petroleum etherorcyclohexane–ethylacetate) are added to induce phase separation of the acetone–water mixture.

Anastassiades *et al.* (2003) then introduced the so-called quick, easy, cheap, effective, rugged and safe (QuEChERS) method that combines pesticide isolation, extraction and clean-up. This method involves initial single-phase extraction with acetonitrile followed by salting-out partitioning by addition of $MgSO_4$ and/or NaCl (to induce the partitioning/separation), and a final step of clean-up by employing dispersive solid-phase extraction (DSPE) using several sorbents such as NH_2 and primary-secondary amine (PSA), based on the matrix (Anastassiades *et al.*, 2003; Lehotay *et al.*, 2005).

In terms of detection instruments, most pesticides can be quantified reliably by liquid chromatography- triple quadrupole mass spectrometry (LC-QqQ/MS/MS) at concentrations between 0.1 and 1 ng ml^{-1}. In contrast, the median of the limits of quantification observed by gas chromatography-mass spectrometry (GC-MS) is distinctly higher, at 100 ng ml^{-1} (Alder *et al.*, 2006). Although most OPPs are amenable to being analyzed by GC-MS, a much higher sensitivity is clearly demonstrated by LC-QqQ/MS/MS (Alder *et al.*, 2006).

1.7 Thesis objective

It has been suggested that the collection of baseline data for neotropical migrant passerines exposed to pesticide is an appropriate first step to evaluate the exposure and effects of pesticides on population dynamics (Gard *et al.*, 1993).

The main objective of my thesis was to design and develop a chemical analysis method to determine the occurrence of selected OPPs on neotropical migratory songbirds that use farmlands as wintering habitats in Mexico, Central America and Caribbean commonwealth countries, using songbirds' feet as a matrix for detection.

One hypothesis to be tested is that OPPs applied on farmlands during the period songbirds are foraging in those same farmlands, and on the same crops treated with pesticide, results in the occurrence of dermal exposure through the feet or feathers of songbirds. Although exposure to acute doses could lead to mortality of birds at their wintering sites, sub-lethal exposure may also possibly to occur, and such exposure may be correlated with pesticide use in the wintering sites.

CHAPTER 2

The isolation, identification, and determination of selected organophosphate pesticides in the feet of migratory songbirds

2.1 Introduction

To date, the chemical analysis of pesticides in biological matrices of birds has been conducted on several body compartments such as the gastrointestinal tract, liver, eggs and muscle to monitor pesticide exposure with no reference to the route of exposure. More recently, feet have been used for chemical analysis of dead birds and considered as an indicator for short and probably long term external (dermal) exposure. The feet of dead birds have been successfully used to assess exposure to pesticides; the classes of pesticides for which foot analysis has given the most valuable information about dermal exposure are OPPs and carbamate pesticides. Although feet as a route of exposure and as a matrix for analysis have received scarce attention in wildlife exposure studies, there is sufficient evidence and reports to show that feet can be a suitable matrix and indicator of dermal exposure to pesticide spillage or residue on foliage and the surface of other vegetation (Vyas *et al.*, 2003b, 2004, 2007).

The available method of pesticide residue analysis in feet that was demonstrated to be effective for OPPs analysis uses the weathered feet of birds that were experimentally exposed to pesticide-treated turf or trees (Vyas *et al.*, 2003b, 2004, 2007). The goal of these studies was to determine if insecticide residues could be detected from weathered feet (for ~28 day), thereby providing a matrix for determining insecticide exposure.

The aim of the present thesis was to develope a simple, and efficient method of extraction and clean-up, as well as evaluate the potential of sensitive LC-QqQ/MS/MS-based analytical

method for the quantitative analyses of selected OPPs at trace levels in the feet of dead migratory songbirds that are wintering in farmlands outside their breeding habitats. LC/TOF-MS and LC-QTOF-MS/MS were used for confirmation of the positive findings of selected OPPs. It was hypothesized that migratory birds that have been exposed to OPPs via their feet, retain OPP residues on their feet, which can be quantitatively determined analytically with the developed method. With a simple, and efficient chemical analysis methodology, monitoring of such OPP residues on the feet could prove to be an effective approach to monitor OPP exposure in migratory songbirds.

2.2 Avian study design

2.2.1 Selection of migratory birds

A total of 18 migratory songbirds species were opportunistically collected in 2007 and 2011 (Table 2.1) in collaboration with the Fatal Light Awareness Program (FLAP) in Toronto, Canada, http://www.flap.org/. Sampling of the birds was carried out by volunteers from FLAP, and birds were obtained with permission of Environment Canada. The birds collected for this study were found dead or had died shortly after collection. They were collected from downtown Toronto and sent to FLAP's office for identification and initial storage. The cause of death was colliding with towers and high buildings at night, because they are attracted to lights shining from skyscrapers, broadcast towers, lighthouses, monuments and other tall structures. Specimens were received and kept at the specimen bank at -40 C in the National Wildlife Research Centre, Environment Canada, Ottawa, Ontario, until the time of sample preparation. Feet of each individual birds were excised and stored in glass vials at -10 C for sample preparation.

Songbirds were classified as birds that winter in agricultural areas, while birds that winter in non-agricultural areas (one species used being the Brown creeper, *Certhia americana*) were used as the control (reference species) and to test elements of the analytical methodology's performance, such as recovery and LC-MS matrix effects. The identification of use of agricultural areas was based on information that was extracted from peer-reviewed articles (more details are in Chapter 3).

Table 2.1 Overview of feeding and migratory habits of the songbird species under study in this thesis (Poole, 2005)

English name	Scientific name	N		Breeding habitat	Wintering habitat
		2007	2011		
Indigo bunting	*Passerina cyanea*	3	1	Widespread throughout North America, north of Mexico and south of coniferous forest region, from northern Great Plains eastward to Atlantic seaboard. The main food sources are small spiders and insects, and occasionally seeds.	Largely Mexico to northern Panama, also southern Florida and Greater Antilles (Cuba and Jamaica). Small seeds,and buds are the main food taken.
Magnolia warbler	*Dendroica magnolia*	29	10	Distribution follows that of the boreal forest in Canada, but range may extend beyond the boreal forest into south-west Newfoundland, the Great Lakes-St. Laurence forest of southern Ontario and the northern Great Lakes states. Lepidopteran caterpillars constitute majority of food volume.	Primarily Mexico to Panama; in lower abundance through Greater Antilles and Bahama.
American redstart	*Setophaga ruticilla*	10	1	Breeds across much of eastern, northern U.S. and south Canada. Insects (leafhoppers, planthoppers, flies, wasps, beetles, caterpillar larvae) are the main diet.	Habitats are diverse, found in montane forests in south America and Jamaica; also Venezuela and Panama. On Caribbean islands, generally abundant in shaded coffee plantations. Frequently occurs in any habitats with woody vegetation.
Black-and-white warbler	*Mniotilta varia*	30	7	Breeds from north-eastern British Columbia to Newfoundland. In the U.S., breeds from south Maine southern-central Texas. Feeding includes trunk and larger limbs of trees, from the canopy to the ground.	Extending from south-eastern U.S. through to Central America and West Indies to northern South America.
Gray catbird	*Dumetella carolinensis*	2	-	Found in dense shrub or vine tangles, roadsides, home sites. Main food is insects, small spiders.	Uses forested habitats in tropical areas (Caribbean, Yucatan peninsula, and Central America).
Black-throated blue warbler	*Dendroica caerulescens*	29	9	Breeds at higher elevations in the southern Appalachians in the U.S. and Canada. Feeds on insects.	Winters mostly in the Greater Antilles, and parts of Central America. Insects, fruits, vegetable matter are the main food items.
Black-throated green warbler	*Dendroica virens*	8	3	Centers on north-eastern North America, primarily southern boreal coniferous forest, and south in the Appalachian Mountains. Insects are the major food items.	Winters primarily in Mexico and Central America. Small numbers winter in West Indies and South America.
Brown creeper	*Certhia americana*	30	-	Range is complex. Breeds primarily in coniferous forests throughout North America. Foods items are the same as in winter.	Winters at lower latitudes and elevations within breeding range and into lowlands, migrating as far as Gulf Coast. Food items are various insects, larvae, spiders.

Table 2.1 Continued

English name	Scientific name	N		Breeding habitat	Wintering habitat
		2007	2011		
Tennessee warbler	*Oreothlypis peregrina*	-	8	Breeds in Boreal zone in deciduous, mixed, and coniferous forests from near sea level to 450 m. Invertebrates are the main food items.	From southern Mexico through Central America to western Colombia, northern and western Venezuela, and northern Ecuador. Foods items are invertebrates, nectar; also attracted to feeders with bananas and plantains.
Common yellowthroat	*Geothlypis trichas*	-	10	Breeds throughout the continental U.S. (including part of Alaska) and in parts of all Canadian provinces. Mainly feeds on insects and spiders.	Winters in Mexico, Central America, and Caribbean. Occupies various agricultural habitats, such as coffee, citrus crops, cacao, rice, and mango.
Nashville warbler	*Oreothlypis ruficapilla*	-	6	Breeds in north-central and eastern North America, with 50% of the breeding in the U.S, and 50% in Canada. Main foods are insects, both adult and larval forms.	Winter range is from South Texas to Mexico, Belize, Guatemala and El Salvador. Casual visitor in Costa Rica, Caribbean, Haiti. Main foods are insects.
Northern parula	*Setophaga americana*	-	3	From Maritime Provinces, and south-east Manitoba in Canada, the Middle Atlantic states, to the Gulf Coast, and Florida. Main foods items are insects, spiders.	Winters in Caribbean, Bermuda, and Central America. Food items are insects and spiders; less often seeds,and nectar.
Northern waterthrush	*Parkesia noveboracensis*	-	2	Parts of Alaska and intervening parts of most of Canadian provinces to Newfoundland. Food items are mainly larval and adult insects, spiders and snails.	Mexico, Caribbean, Central America, the northern parts of Latin America. Main food items taken are beetles, ants, flies, insect larvae, snails.
Blue-winged warbler	*Vermivora cyanoptera*	-	1	North-east of the U.S., and parts of Ontario and Quebec.	Mexico, Central America, and parts of Columbia. Food items are Arthropods, especially Lepidoptera larvae and small orthopterans.
Chestnut-sided warbler	*Setophaga pensylvanica*	-	7	Primarily in northern hardwood and mixed forests of eastern North America from southern Canada southward through Appalachian Mountains to north Georgia. It is classified as insectivorous but eats some fruit.	Winters in Central America, mainly in Costa Rica, parts of Belize and Mexico. Also found in parts of Columbia and Venezuela. Main food items are foliage insects.
Yellow warbler	*Setophaga petechia*	-	2	Breeds in a very large area in North America. Also breeds in parts of Mexico.	Mexico, and South America (Amazonian Brazil).
Mourning warbler	*Geothlypis philadelphia*	-	4	Breeds from Newfoundland, Labrador, and NovaScotia west to south-eastern Yukon. Food items are mainly insects.	Costa Rica, Panama, Nicaragua, and parts of Latin America. Tree leaves and insects are its food items.
Bay-breasted warbler	*Setophaga castanea*	-	1	Inhabits boreal coniferous forests in a broad band across central and eastern Canada. Insects and spiders are the food items	Winters in southern Middle America, primarily in Panama, and the southern regions of Latin America. Insects and some nectar are the food items.

Table 2.1 Continued

English name	Scientific name	N		Breeding habitat	Wintering habitat
		2007	2011		
Black-burnian warbler	*Setophaga fusca*	-	4	Primarily West to central Alberta, north to (central Saskatchewan), east to s. Quebec and the Maritime Provinces, south along eastern seaboard to Massachusetts and New York and along Appalachian Mountains to Georgia. Insects (immature) are the food items.	Primarily s. Central America and n. South America, south in the Andes to Peru and occasionally to Amazonia.

N= number of the birds

2.3 Method development

2.3.1 Organophosphate pesticides selection and preparation of standards

Six OPPs were selected to be investigated in this thesis. These OPPs have quite distinct physico-chemical characteristics, such as a water–octanol partition coefficient ($K_{o/w}$) ranging from 2.86–4.96 (lipophilic compounds), and are hypothesized to have moderate persistency in the environment and biological matrices. Although $K_{o/w}$ is used to predict the pollutants' accumulation behavior in fatty tissue, as there is a heightened possibility of the compounds with a high partition coefficient accumulating in lipids (Gard *et al.*,1993;1995), this parameter was considered given that the analyzed matrix was relatively fatty, and there might be dermal loading (and/or storage) in the feet.

In addition, the available information in some published scientific papers, books, scientific reports, pesticide companies' websites and reviews was used to reach conclusions on the status of pesticide registration, residues, and the most likely OPPs to be used on crops in agricultural areas that are used by migratory songbirds in Central America, Mexico and the Caribbean. Methyl parathion, terbufos, fenthion, fenamiphos, chlorpyrifos and diazinon were selected for this study as they are lipophilic compounds (high $K_{o/w}$). Table 2.2 contains information about the selected OPPs.

Individual stock solutions of methyl parathion, chlorpyrifos (dursban), diazinon, fenamiphos, fenthion and terbufos were purchased from AccuStandard (New Haven, CT, USA), obtained as 1 mL solutions at a concentration of 100 μg ml^{-1} in methanol. Intermediate stock solutions for each pesticide were prepared at 10 μg ml^{-1} in methanol. A mixed working standard solution, containing 500 ng ml^{-1} of each pesticide, was prepared by dilution of intermediate stock solutions of each pesticide in methanol. The mixed working standard solution of 500 ng ml^{-1} was

used for estimates for recovery and to prepare the standard solutions to obtain the calibration curve. The individual, intermediate, and working solutions were kept in a freezer at -10 °C. The deuterated triphenyl phosphate (d_{27}-TPP), purchased from Cambridge Isotope Laboratories (Andover, MA, USA), was used as the internal standard (IS) for quantification of OPPs.

Serial dilutions of the mixed working standard solution of 500 ng ml^{-1}were performed to give six calibration solutions (2, 10, 20, 50, 100 and 200 ng ml^{-1}) in methanol, which were injected twice for each concentration. In addition to the aforementioned calibration solutions in methanol, matrix-matched solutions were prepared using sample extracts from bird species (i.e Brown creeper) that has not used agricultural areas as their wintering habitat. These matrix-matched solutions (extracts) were used only for the matrix effect study. The solutions were kept at -10 C for a period of four months.

Table 2.2 Organophosphate pesticides (OPPs) included in this thesis, and their physico-chemical properties[1]

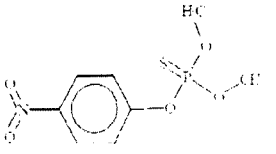
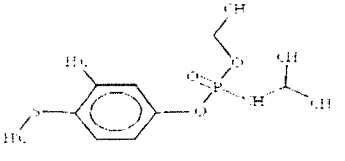
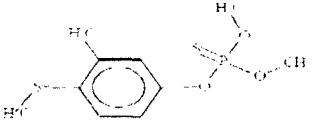
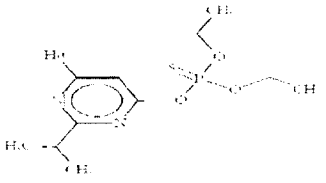
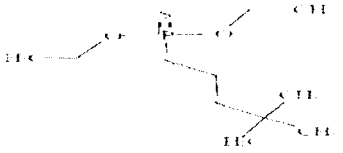
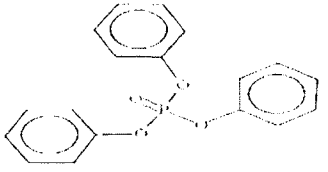
OPP	CAS number	Chemical formulae	Chemical structure	[a]Log K_{ow}	[b]Log K_{oa}
Methyl parathion	298-00-0	$C_8H_{10}NO_5PS$		2.86	8.2[(2)]
Chlorpyrifos (Dursban)	2921-88-2	$C_9H_{11}CL_3NO_3PS$		4.96	8.8[(2)]
Fenamiphos	22224-92-6	$C_{13}H_{22}NO_3PS$		3.23	10.5[(2)]
Fenthion	55-38-9	$C_{10}H_{15}O_3PS_2$		4.08	8.3[(2)]

Table 2.2 (continued)

OPP	CAS number	Chemical formulae	Chemical structure	[a]Log K_{ow}	[b]Log K_{oa}
Diazinon	333-41-50	$C_{12}H_{21}N_2O_3PS$		3.81	9.1[(2)]
Terbufos	13071-79-9	$C_9H_{21}O_2PS_3$		4.48	7.4[(2)]
triphenyl phosphate (d_{27}-TPP) *Internal standard*	115-86-6	$C_{18}H_{15}O_4P$		4.59	8.4[(2)]

[1]Information about the physico-chemical properties was obtained using EPISuite version 4.1. [a]Log K_{ow} = octanol-water partition coefficient; [b]Log K_{oa} = octanol-air partition coefficient;
[2] estimated

2.3.2 Pesticide extraction/clean up from feet

The sample preparation procedure for feet was adopted with modifications from the method described by Vyas *et al.* (2003b) for the determination of OPPs in feet. Feet of each individual bird were excised and cut into small pieces (less than 0.6 cm) using scissors, which were washed before and after use with acetone several times to avoid possible cross-contamination, and treated as a single sample. Pieces of feet of approximately 100–400 mg (depending on the species) were weighed and placed in 20 ml glass vials with screw caps, and the extraction was performed using ultrasonic solvent (assisted) extraction (USE) equipment. A 3 ml mixture of acetone:dichloromethane:hexane 1:1:1 was added to each vial and feet were extracted by immersing the vials in an ultrasonic bath for 15 min (twice). The water level was above the solvent level inside the vials. After the extraction period, the combined extracts were transferred to an empty test tube and concentrated under a flow of gentle nitrogen at 30 °C in a fume hood to 2 or 0.5 mL.

Two clean-up methods were applied:

Method 1: The sample extracts were concentrated to approximately 2 ml, and 200 mg PSA (primary secondary amine) + 700 mg of Na_2SO_4 were added directly to the test tube, and shaken using a vortex for 1 min. After that, the liquid phase was allowed to separate for 5 min, and was then transferred to another empty test tube and the solvent was evaporated to dryness and reconstituted in methanol (the initial mobile phase) up to 0.2 mL. Prior to analysis, the extract was filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter and then transferred to the LC-QqQ-MS/MS vial.

Method 2: the samples were concentrated to approximately 0.5 ml; the samples were cleaned on column ISOLUTE SPE-PSA (100 mg), (Biotage, Charlotte, NC, USA). The column was conditioned with 1 ml acetone, followed by 1 ml hexane. The extract was loaded and eluted with 3 ml acetone. The acetone extract was evaporated to dryness and reconstituted in methanol (the initial mobile phase) up to 0.2 mL. Prior to analysis, the extract was filtered through a 0.45 μm PTFE filter and then transferred to the LC-QqQ-MS/MS vial.

2.3.3 Liquid chromatography-triple quadrupole mass spectrometry

LC-QqQ-MS/MS determination first consisted of separation of all OPPs in solvent or from matrix which was carried out using a chromatographic system that consisted of a Waters Alliance 2695 separation module (Milford, MA, USA), equipped with a quaternary solvent delivery system, degasser, autosampler and column heater. Separation was carried out using a reversed phase symmetry® C_{18} analytical column (100mm × 2.1 mm internal diameter (i.d), and 3.5 μM particles size) obtained from Waters, and a guard cartridge from the same supplier . The gradient elution (or the mobile phase) was based on positive ionization mode only, in which an organic solvent containing 3 mM ammonium formate (Fluka) in methanol and an aqueous solvent containing 3 mM ammonium formate in high-performance liquid chromatography (HPLC) grade water were used. The gradient program started in 75% aqueous phase, decreased to 50% in 6 min, then to 15% in 14 min, then to 5% in 29 min, was maintained at 5% until 35 min, and returned to the initial condition (75% aqueous phase). The total run-time was 46 min. The injection volume was 10 μl, the flow rate was 0.2 ml min^{-1}, and the column was kept at 40 C.

The detection and quantification of OPPs was achieved using a Waters Quattro Ultima triple quadupole (QqQ) tandem mass spectrometry (Manchester, U.K.), and the ionization mode

used was positive electrospray ionization interference. The source of ionization parameters were: capillary voltages 2.0 Kv; sample cone voltage 35 V (for all compounds); source temperature 100 C; and desolvation gas and cone gas (N_2) were set at 100 and 600 L h^{-1}, respectively. Ions were collisionally fragmented with argon at 3.5×10^{-3} mbar. Data acquisition and processing MassLynx and QuanLynx software 4.0 was used for instrument control.

2.3.3.1 Quantitative determination of OPPs by LC-QqQ-MS/MS

The evaluation of the linearity of the analytical curves using LC-QqQ-MS/MS was undertaken based on injections of the standard OPPs solutions in methanol at concentrations of 2, 10, 20, 50, 100, and 200 ng ml^{-1}. The average peak areas, the relative standard deviations (RSD %), calibration curve equations, and coefficients (r^2) were calculated. The instrumental limit of detection (LOD) was estimated by considering to three times the background noise for the quantification ions (S/N 3:1) as a response equivalent. The instrumental limit of quantitation (LOQ) was estimated as the concentration of compound at which the signal to noise ratio was ten times the background noise (S/N 10:1).

2.3.3.2 Quality control and assurance

(i) LC-QqQ-MS/MS Matrix effect study

A matrix effect is a suppression or enhancement (decrease or increase, respectivelly) in the LC-MS response of an analyte due to co-eluting matrix constituents. It can be easily detected by comparing the detector response obtained from a standard solution in organic solvent and that from a spiked pre-treated sample (Niessen *et al.*, 2006).

To evaluate the matrix effect, matrix-matched multi-level calibration standards approach are usually used by comparing the slopes of the calibration curves of standards in pure solvent

and in matrix. In this work, the evaluation of the matrix effect was undertaken by using a sample standard addition approach (Chen *et al.*, 2012); but the weight of the replicates was not consistent, and thus the evaluation was approximate.

For matrix effect evaluation, three replicates of foot pieces were extracted—with no standard spiked before extraction—by ultrasonic assisted extraction with acetone:DCM:hexane 1:1:1, followed by addition of anhydrous sodium sulfate and purification by PSA (clean up; Method 1). The final extract of each replicate sample extraction was reconstituted to 100 µL in methanol and then divided into two sub-samples, A and B (50µL each). Sub-sample A was spiked with 50 µL of a standard solution containing a mixture of OPPs and d_{27}-TPP (IS) at a concentration of 20 ng ml^{-1} per compound. Sub-sample B was a duplicate control and was spiked with 50 µL of methanol. An external standard solution (S) was prepared by combining 50 µL of a standard solution containing a mixture of OPPs and d_{27}-TPP (IS) at a concentration of 20 ng ml^{-1} per compound with 50 µL methanol. The comparison was based on the differences in responses of the analytes in sub-samples (A) and (B) to the response of the analytes in the external standard (S). The calculation of matrix effect (*ME*) was as follows:

$$ME(\%) = A_i\text{-}B_i/S_i * 100$$

where A_i, B_i, and S_i, are the chromatographic peak areas of the analytes (i) in sub-samples A and B and external standard solution (S), respectively. The analyte detector response (signal) may be suppressed or enhanced by the co-eluted interferences in the samples if *ME*(%) is lower or higher than 100%, respectively.

(ii) Recovery experiments

The main goal of recovery experiments is to determine the method accuracy, via comparison of the real concentration of each pesticide measured by performing the complete procedure with the known pesticide concentration initially added to the matrix (Pizzutti *et al.*, 2007).

The recovery evaluation of the extraction method was performed on spiked samples with a standard solution on the control sample (reference species) using the species Brown creeper. Each of three replicates of bird feet was spiked with 20 ng each of targeted compounds and was subjected to the analytical method described above (clean-up: Method 2). Recoveries were determined using an internal standard triphenylphosphate-labelled d_{27}-TPP (IS) as a quantification procedure. The spiked matrix was allowed to stand for one hour before extraction to achieve good penetration of the pesticides into feet. Procedural blank (only solvent) assays were conducted (n = 3) to evaluate possible contamination from the proposed methodology.

2.3.4 Liquid chromatography-time-of-flight (TOF), and quadrupole time-of-flight (QTOF) mass spectrometry (MS)

An Agilent 1200 liquid chromatographic system consisting of a degasser and binary high-pressure gradient pump autosampler, coupled to an Agilent 6520A quadrupole-time-of-flight-MS (QTOF) system (Agilent Technologies, Mississauga, ON, Canada), was used to confirm the presence of positive results obtained from QqQ. LC separation parameters were the same as those used for the LC-QqQ-MS/MS analysis, except for the mobile phase flow rate which was 0.3 mL/min. The QTOF instrument was tuned and calibrated with tuning calibration solution (G1969-85000) provided by Agilent Technologies. The TOF-MS was high resolution at *m/z* 622.028413 and with an R > 20,000 and within 3 ppm mass error in a mass range from *m/z* 118

to 1700. The electrospray ionization (ESI) interface was operated in the positive mode and the capillary voltage was 4.0 kV. Nitrogen was used as a drying and nebulizing gas and helium was used as collision gas. The fragmentor and skimmer voltages were 150 V and 250 V, respectively. The gas temperature was 320 °C, dry gas 10 L/min and nebulizer 15 psi. When the QTOF was operated in MS/MS mode, the collision voltage was set at 15 V.

2.4 Results

2.4.1 Liquid chromatography-triple quadrupole mass spectrometry

2.4.1.1 Liquid chromatography (LC) optimization

Despite the type of detector used, the most common organic solvents (mobile phase) in LC/MS/MS applications are methanol and acetonitrile, with methanol generally offering slightly better chromatographic resolution of OPPs than acetonitrile. In this work, methanol/deionized water-3 mM ammonium formate (modifier) was used as a mobile phase; however, no other modifiers, such as formic acid, and acetic acid were tested. The gradient elution of methanol: water employed allowed OPPs to be separated with different retention times with respect to the separation efficiency (peak shape) and sensitivity (Figure 2.1). Jansson *et al.* (2004) evaluated six different mobile phase compositions for optimum responses of the pesticide by LC-MS/MS, and it was concluded that a methanol-ammonium formate gradient profile gave the overall best results. Moreover, ammonium formate gave the overall best results with respect to sensitivity, particularly for OPPs of intermediate polarity (García-Valcárcel and Tadeo, 2009).

2.4.1.2 Triple quadrupole mass spectrometry (QqQ-MS) optimization

The ionization techniques most widely used in pesticide residue analysis are Atmospheric Pressure Ionization methods (API); these are ESI and APCI in both positive and negative modes.

The choice of ESI over APCI in this study was because of the suitability of ESI for polar and ionic molecules (Soler *et al.*, 2008).

The identification procedure for OPPs was based on the following factors: the use of retention times and the most abundant transition ion (first transition) used as a qualifier, and secondary daughter ion, if available, used as a qualifier. Obtaining the protonated molecule (precursor ion), and selecting those transitions with a higher molecular mass in order to avoid the disruptive effects of the matrix, are two objectives for optimizing MS/MS parameters. The optimized settings for cone voltage (or fragmentor) and collision energy (CE) were tested for each compound by the injection of 10 μL of the individual pesticides standard solution at a concentration of 1 μL mL^{-1} in methanol directly into the LC effluent with a constant flow of the mobile phase at a flow rate of 0.2 mL/min (flow injection analysis).

In the initial experiments on modifications of desolvation gas flow rate, cone gas flow rate and source temperature did not improve the sensitivity of the OPPs being studied. Protonated molecular ions were chosen for each compound as precursors of product ions, and the most intense transition (abundant product ion) was used as a quantifier in multiple reaction monitoring (quantitation MRM1) and the second transition was used as a qualifier peak for confirmatory analysis in multiple reaction monitoring (confirmation MRM2). Table 2.3 lists the optimization parameters and *m/z* values for the most abundant product ions of targeted OPPs. All OPPs predominantly formed the most abundant cation, and there was no formations of sodium or ammonium adduct ions in any of the OPPs. In most cases, more than two transitions (product ions) were obtained, but the two most intense transitions were chosen; however, methyl parathion and terbufos had only one transition for each (they were already excluded from the study because of low sensitivity). Since only four compounds were monitored, the dwell time

was set at 200 ms, and no time windows were created. MRM channel (Figure 2.1) responses were acquired at one window.

Table 2.3 Operational parameters for LC-ESI (+)-QqQ-MS/MS determination of selected OPPs

Compound	Molecular weight (MW)	t*R* (min)	Type	Precursor ion $[M+H]^+$ (*m/z*)	Cone voltage (v)	Product ion (*m/z*)	Collision energy (CE)
Diazinon	304	18.43	Quantitation Confirmation	305.4 305.4	35	169.1 153.1	20 20
Cholrpyrifos (Dursban)	350.6	21.07	Quantitation Confirmation	349.6 349.6	35	198 293.9	15 15
Fenthion	278	18.30	Quantitation Confirmation	279.2 279.2	35	105 169.1	20 20
Fenamiphos	303	17.59	Quantitation Confirmation	304.1 304.1	35	234 262	20 20
Terbufos	288	20.27	Quantitation Confirmation	289	35	103.3	20
Methyl-parathion	263		Quantitation Confirmation	264	35	125.5	25
d_{27}-TPP	241	18.26	Quantitation Confirmation	342.2	100	82	40

2.4.2 Linearity study, LOD and LOQ determination

The standard curves of targeted OPPs were linear between the concentrations 2–200 ng $mL^{-1}$with coefficients of linearity (r^2) ranging from 0.995 to 0.999. Each time a new sample set was analyzed, a new calibration curve was constructed. The LOD for each compound was evaluated from the lowest standard solution (2 ng mL^{-1}) of the calibration curve. A compound that had a lower instrumental response (instrumental LOD) was considered as non-detectable (n.d). The limit of detection ranged from 0.04 to 0.1 ng $mL^{-1}$and the limit of quantification ranged from 0.1 to 0.5 ng mL^{-1} (Table 2.4). Terbufos showed no signal lower than 20 ng mL^{-1}, and methyl parathion showed no signal at 200 ng mL^{-1}. Thus, terbufos and methyl parathion were excluded from the study. The quantification in LC-ESI(+)QqQ-MS/MS was based on the instrumental LOQ.

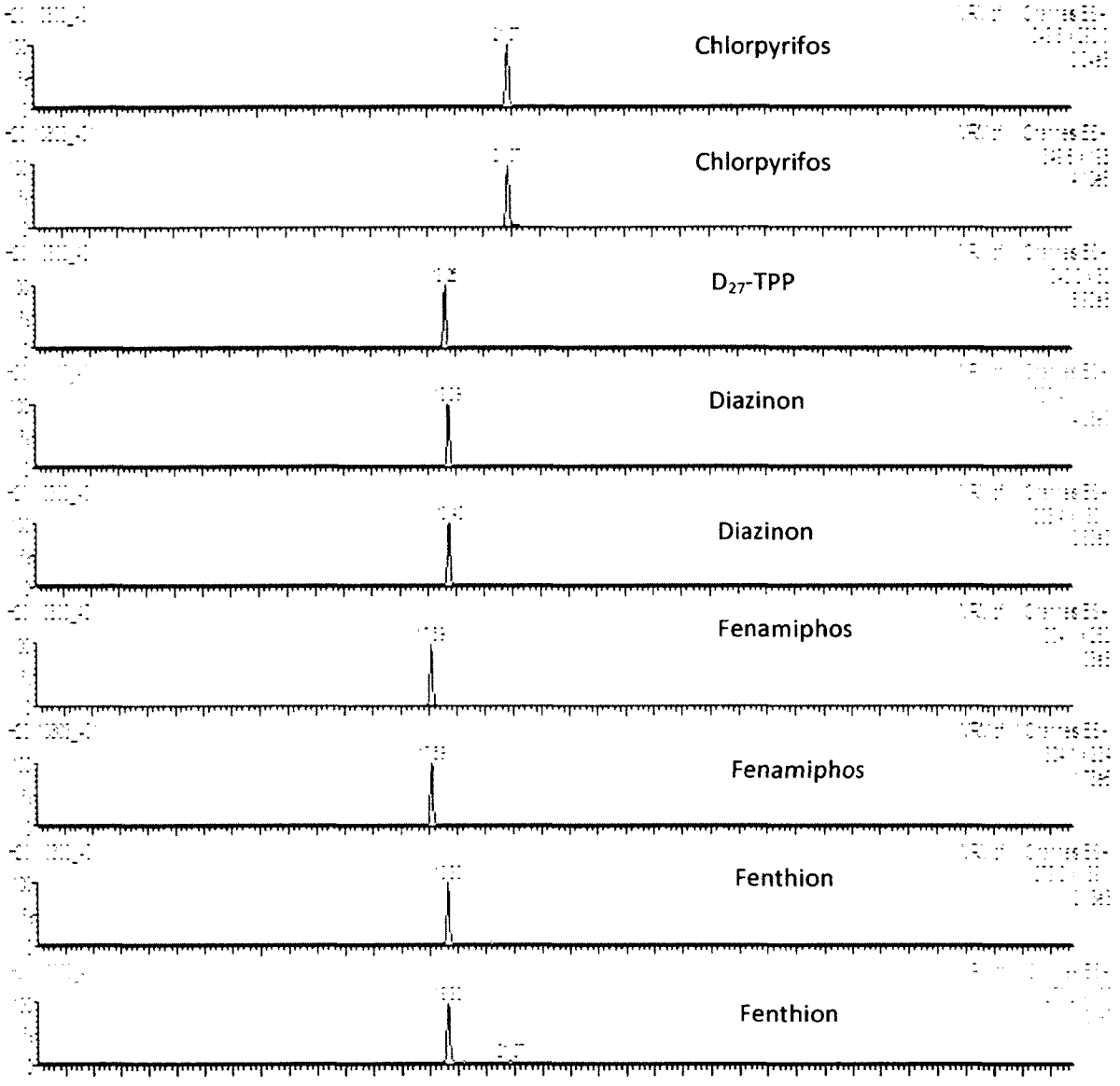


Figure 2.1 Multiple reaction monitoring (MRM) chromatograms for a standard mixture solution containing four organophosphate pesticides (chlorpyrifos, diazinon, fenamiphos, fenthion) at a concentration of 20 ng mL^{-1}, and an internal standard d_{27}-TPP, under optimized LC–ESI (+)-QqQ-MS/MS conditions.

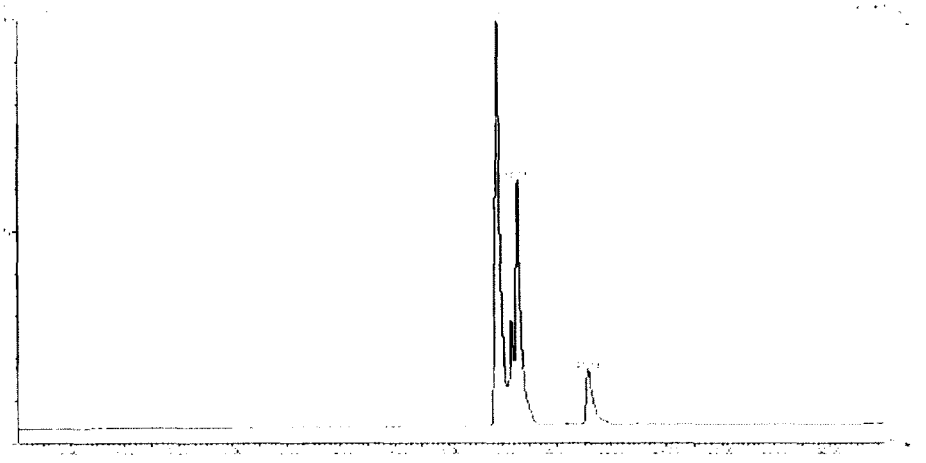


Figure 2.2 Total ion chromatogram of mixed standard solution of OPPs at concentration 20 ng mL^{-1} using LC–ESI (+)-QqQ-MS/MS.

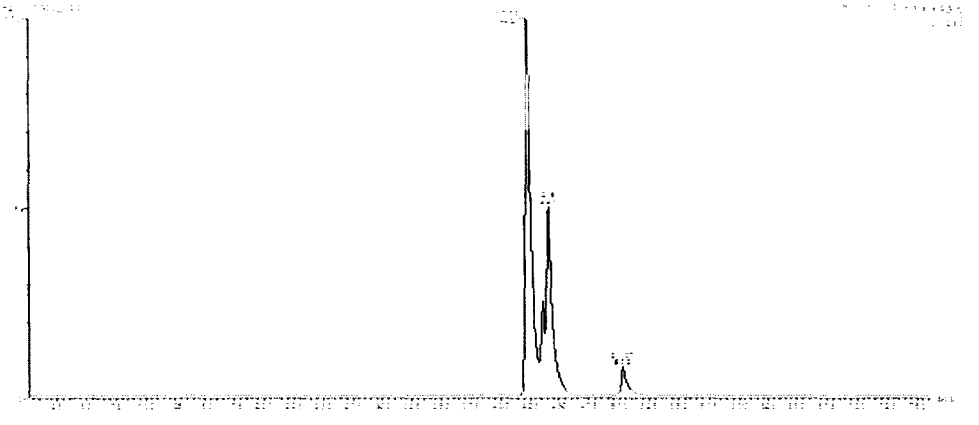


Figure 2.3 Total ion chromatogram of feet matrix (Bown creeper) spiked with mixed standard solution of OPPs at concentration 20 ng for each target compound using LC–ESI (+)-QqQ-MS/MS

As the internal standard is used generally to improve the results of the method by correcting for the matrix effect, the labelled Triphenylphosphate (d_{27}-TPP) was used as an internal standard for the targeted OPPs. However, using the labeled standard for each compound is preferable, but such standards are unavailable commercially and/or are very expensive. d_{27}-TPP is similar to OPPs and has been used mainly as an internal standard in organophosphate flame retardants (Chen *et al.*, 2012).

2.4.3 Optimization of solvent extraction, and clean up technique

Due to the low sample size, it was not possible to conduct several experiments to test the effect of sonication time, number of sonication cycles, solvent polarity using different solvents, or solvent volume. However, it has been shown that the recovery of extracted pesticides from soil did not improve clearly when the extraction time increased from 30 to 40 min (Tor *et al.*, 2006), and the performance of two 15 min sonication cycles allowed satisfactory pesticide recoveries to be obtained (Boti *et al.*, 2007). Thus, the extraction of the samples with solvent was undertaken for two 15 min sonication cycles, using 3 mL solvent for each cycle.

Although acetonitrile has been used as the best solvent for pesticide residues (Anastassiades *et al.*, 2003), it is mainly used for low-fat matrices such as fruit and vegetables (Lehotay *et al.*, 2005). Lipids are not very soluble in acetonitrile and/or water, and fats typically form an oily film on the surface of these solvents or an emulsion during extraction; consequently the lipophilic pesticides remain or partition into the undissolved fats, which results in their lower recovery in the acetonitrile extract (Lehotay *et al.*, 2005). Based on these reasons, the initial stage of analyzing the method efficiency was compared by using one extraction method (USE) with six solvent systems, these being acetone, acetone:DCM:hexane 1:1:1, acetone:DCM 1:1,

acetone:hexane 1:1, acetonitrile-1% acetic acid, and methanol. The preliminary results indicated that, except for acetone, methanol and acetonitrile, the differences in the recovery between the other solvent systems were not significant. Although the polarities of the targeted compounds are intermediate, acetone, methanol and acetonitrile yielded very low recoveries which may have been affected by the lipid content of feet; however, lipid weight measurements were not performed. Typically, all extracts yielded a yellowish trace after solvent evaporation (concentrated extracts), but the extent of this color was observed more in the solvent combinations. As acetone:DCM:hexane yielded the highest recovery, it was chosen over the others as the extraction solvent.

In the initial trials for optimizing the extraction solvent, the matrix effect study was evaluated by testing four solvents; acetone, acetone:DCM:hexane 1:1;1, aceton:DCM 1:1, and acetonitrile-1% acetic acid to evaluate the impact of the extraction solvent on the ion response (in the sample) compared to pure solvent (methanol). All the tested solvents (except acetone) showed ion suppression (up to approximately 20%). Only for acetone was a response enhancement observed (up to approximately 30%) (Data not shown). The extraction solvent that induced the lowest matrix effect (suppression) was acetone:DCM:hexane 1:1:1, and the use of PSA for clean-up—discussed below—decreased the matrix effect further. This solvent system showed the lowest matrix effect and the highest recovery, and was considered as an extraction solvent.

Pizzuti *et al.*, 2007 used the solvent system of acetone:petroleum ether:DCM 1:1:1with anhydrous sodium sulfate using a polytron high-speed blender to extract 169 pesticides from mixed soya grain, and the samples were extracted in one step without clean-up and were directly analyzed by LC-MS/MS. Using this method, the matrix effects were negligible for around 90%

of the pesticides, and more than 70% of pesticides analyzed met the acceptability criteria of recovery (70–120%).

After the extraction solvent was established (acetone:DCM:hexane), the next step was to evaluate the clean-up procedure. Only one sorbent-based technique was tested (PSA) as a sorbent for clean-up (either in column or as dispersive). The reason behind focusing on one sorbent was that PSA is the sorbent universally adopted in pesticide residue analysis, and was found to remove many matrix coextractives without affecting pesticide recoveries (Schenck *et al.*, 2002; Anastassiades *et al.*, 2003). Additionally, the weight of the matrix was very small (average ~ 200 mg), thus using a more intensive clean-up/lipid removal technique such as gel permeation chromatography (GPC) is not needed. In the initial work of the study, 50 mg of PSA was used, but the chromatographic interferences were very high when using this amount. The amount of PSA added to the extracts in the dispersive format (clean-up: Method 1) was then increased to 200 mg PSA per sample; however, this amount is relatively larger than the original method, in which 50 mg was used. No other sorbents were tested or added to the PSA in this work. In general, it has been shown that NH_2 or PSA alone can achieve acceptable clean-up of extracts and the use of common additional solid phase extraction (SPE) columns provides little benefit and essentially serves to increase the cost of analysis and solvent consumption (Schenck *et al.*, 2002).

2.4.4 Matrix effect and recovery experiments

After optimization of the extraction solvent and the clean-up technique, the matrix effect evaluation and recovery study were performed on one representative bird species (Brown creeper). This species was chosen because there are no records that indicate use of agricultural area by this species, thus there is no likely exposure to any of the target compounds.

The accuracy of the method was approximately estimated by means of recovery experiments at one concentration level. Figures 2.2, and 2.3 show the total ion chromatogram of mixed standard solution, and feet matrix spiked with mixed standard solution, respectively. During the initial trials, no difference in the recoveries between clean-up formats (either column or dispersive) was found using PSA as a sorbent. Using column SPE for clean-up (clean-up: Method 2), the results indicated that all the targeted compounds gave satisfactory mean recoveries ranging from 80% to 100%, except for fenthion, with a mean recovery of 64%, and the RSD was below 20%. Matrix effect results, however, were obtained using dispersive solid phase clean-up (clean-up: Method 1). The mean matrix effect ranged from 84.5 to 105 % and the RSD was below 20%. The ion suppression/enhancement effect might be related to the co-extractive interferences that eluted at the same or close to retention time. The results of recovery and matrix effects (Table 2.4) were based only on the use of LC-ESI (+)-QqQ-MS/MS.

Vyas *et al.* (2003b) reported acceptable recovery of diazinon ranging from 80–120% from birds spiked at a concentration of 5.36 μg. In another study, the average spiking recovery was 102% for birds' feet spiked with azinphos-methyl (Vyas *et al.*, 2007). The same author reports that diazinon and chlorpyrifos recovery was 112% and 90%, respectively, in bird feet. All these studies were consistent in the chemical analysis procedures used, which were gas chromatography-mass spectrometry as the instrument of detection, and acetone:dichloromethane as a solvent of extraction. Although no clean-up step was performed in these studies, the extraction was done without using sonication. The use of ultrasonic-assisted extraction probably extracts more interference and consequently affects the recovery of spiked compounds.

The limit of quantification or the quantitative sensitivity was based primarily on the estimation of the instrumental signal-to-noise ratios (S/N = 10) of the compound peak at the

lower concentration in respective standards (Table 2.4). No method of limit of quantification was performed based on the spiked matrix.

Table 2.4 Calibration data, limit of detection and quantitation (pg mg^{-1}), mean percentage matrix effect, mean overall recoveries for the target OPPs (±SD)

OPP	r^2 (2-200) ng ml^{-1}	LOD[1]	LOQ[2]	Mean matrix effect %[3]	Mean overall recoveries %[4]
Chlorpyrifos	0.997	0.1	0.3	87 (3)	80 (14.3)
Diazinon	0.995	0.05	0.2	105 (11.7)	100 (12.5)
Fenamiphos	0.999	0.04	0.14	84.5 (4.7)	91(11)
Fenthion	0.998	0.1	0.5	90 (14)	64 (15.7)

1 based on S/N=3:1
2 based on S/N=10:1
3 clean up based on Method 1
4 clean up based on Method 2

2.5 Analysis of real samples

Birds' feet of 18 species were analyzed individually using the LC-ESI (+)-QqQ-MS/MS based on method 1 and no pooling of samples was performed. Among all the targeted OPPs being studied, only chlorpyrifos (Dursban) was detected (>LOD, Table 2.4) in some species under investigation. Figure 2.4 shows the total ion chromatogram of chlorpyrifos in a positive sample compared to control. Concentrations of chlorpyrifos were low in all these species, and ranged from 0.5–1.2 pg mg^{-1} feet (wet) .weight. One sample of Black-throated blue warbler had an exceptional concentration with 52 pg mg^{-1} feet.weight, and the mean of this species was 17.9 pg mg^{-1} feet (wet) .weight (±29) for 2007 year (Table 2.5).

Table 2.5 Concentration pg mg^{-1} feet.weight (±SD) of chlorpyrifos in migratory songbirds using quantification based on using LC–ESI (+)-QqQ-MS/MS (nd not detected).

species	2007		2011	
	Concentration	Positive samples	Concentration	Positive samples
Gray catbird	nd	0/2	-	-
Indigo bunting	nd	0/3	nd	0/1
American redstart	nd	0/10	nd	0/1
Magnolia warbler	nd	0/29	nd	0/10
Black and white warbler	nd	0/30	nd	0/7
Blue winged warbler	-	-	0.9	1/1
Blackburnian warbler	-	-	nd	0/4
Mourning warbler	-	-	nd	0/4
Chestnut-sided warbler	-	-	nd	0/7
Northern parula	-	-	1.2	1/3
Tennessee warbler	-	-	1 (±0.8)	3/8
Common yellowthroat	-	-	1 (±0.3)	5/10
Yellow warbler	-	-	nd	0/2
Northern waterthrush	-	-	0.6	1/2
Bay-breasted warbler	-	-	nd	0/1
Nashville warbler	-	-	nd	0/6
Black throated green warbler	-	0/8	nd	0/3
Black throated blue warbler	17.9 (±29)[1]	3/29	0.5 (±0.1)	3/9

[1] One sample had a high concentration with 52 pg mg^{-1}

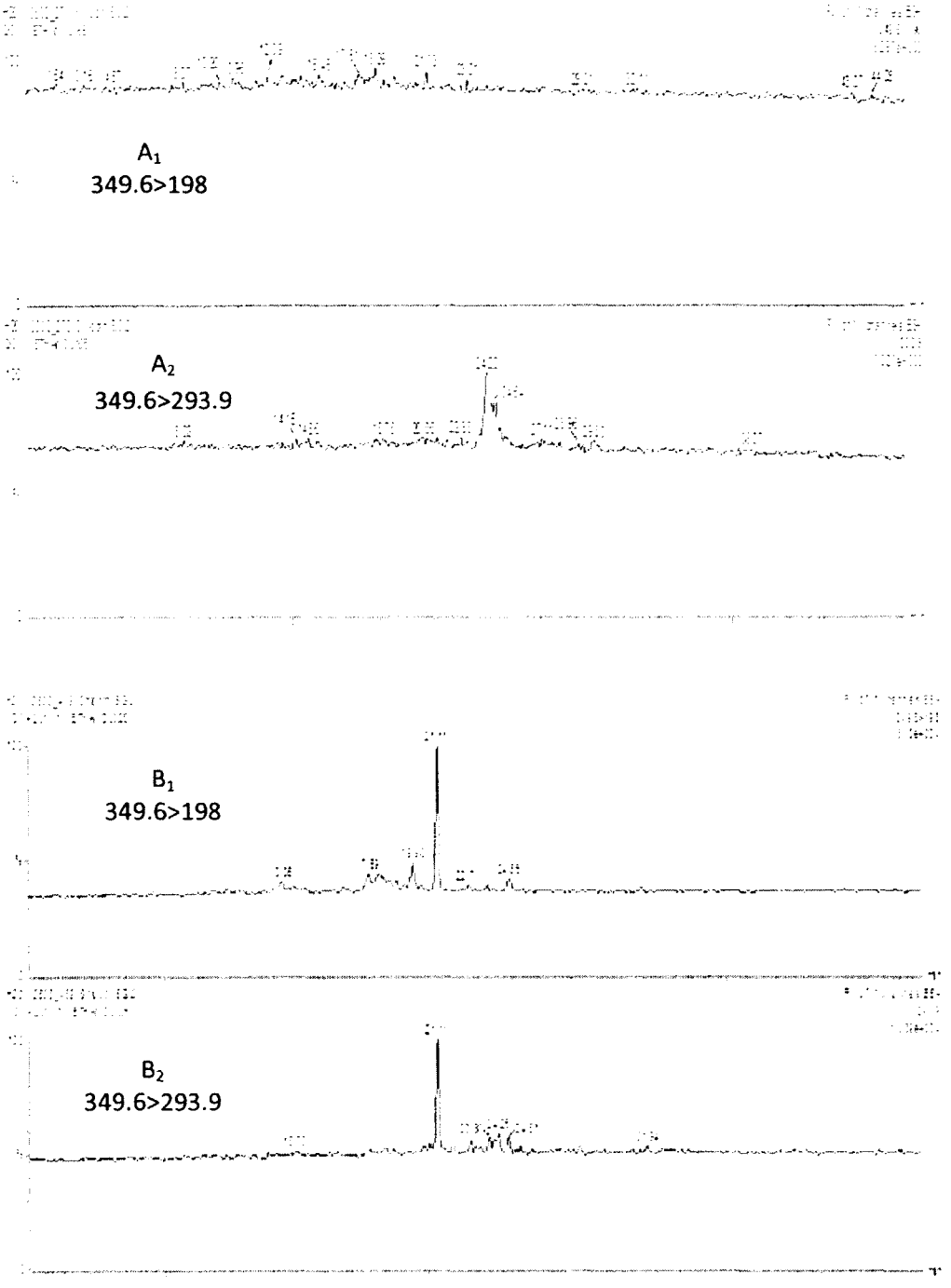


Figure 2.4 LC-ESI (+)-QqQ-MS/MS analysis and MRM chromatograms (two transitions) for chlorpyrifos in feet extract from (A) control and (B) a sample (Northern waterthrush) positive to chlorpyrifos.

2.5.1 Strategy of identification and quantification using LC-QqQ-MS/MS

The MS-based method could be considered as highly selective, but the occurrence of false positives is possible mainly in the analysis of relatively dirty samples, as some interference can share the same MS properties (MRM or SRM transitions) as the analyte (Hernandez *et al.*, 2004). One of the guidelines in chemical residue identification criteria is the European Commission Guidelines for identification and quantification of organic residues and contaminants (EU Commission Decision-SANCO document 10684/2009/EC; European Commission, DG-SANCO, 2009), which are based on the use of the criterion of identification points (IPs) (Table 2.6). It represents a new approach for setting up quality criteria for the spectrometric identification and confirmation of organic contaminants. The number of IPs "earned" by the detection of a precursor/product ion depends on the technique used (Hernandez *et al.*, 2004; European Commission, DG-SANCO, 2009). In general, these guidelines have several requirements for overall analytical methodology, but for chromatography and mass spectrometry there are some specific requirements that have to be fulfilled in order to report the identification of analyte in the sample. It should be noted that although these guidelines are intended for laboratory control in the monitoring of pesticide residues in food and feed, we followed these criteria as we were expecting low concentrations of the targeted compounds in the birds' feet. These concentrations might be as low as those that are used for enforcement actions, and for checking compliance with maximum residue limit (MRL) for food safety purposes.

Table 2.6 The number of identification points (IP) earned for the range of LC-MS techniques (Petrovic and Barcelo, 2006; European Commission, DG-SANCO, 2009).

Technique	Number of IP earned per ion	Example of ions	IP earned
LC-MS (Q)	1	1 ion (SIM)	1
LC-MS2 (QqQ)	1 for precursor ion 1.5 for product ion	1 precursor, 1 product (SIM) 1 precursor, 2 products (2 SRM) 2 precursors, each with 1 product (2 SRM)	2.5 4 5
LC-MS (IT)	1 for precursor ion 1.5 for product ion	1 precursor, one MS2 product and two MS3 products	5.5
LC-TOF-MS	2	1 ion	2
LC-QqTOF-MS	2 for precursor ion 2.5 for product ion	1 precursor, 1 product (MS/MS) 1 precursor, 2 products (MS/MS)	4.5 7.5

Despite the high specificity of QqQ, there is still a risk of reporting false positives when analyzing real samples owing to the acquisition of only one MS/MS transition (Hernández *et al.*, 2006). In the present study, the two most abundant transitions for the targeted compounds were acquired by LC–ESI (+)-QqQ-MS/MS with optimal mass spectrometer operation parameters, and led to mass chromatograms with sufficient chromatographic- and mass-resolved separation. Chlorpyrifos had m/z 349.6>198 amu as a quantification transition, and *m/z* 349.6>293.9 amu as a confirmation transition. Based on the criterion of identification points in the SANCO guidelines 1068/2009, the number of IPs earned was four, which was enough for identification (Table 2.6). In this case, the detection of two transition product ions (198, and 293.9 for chlorpyrifos) yielded three IPs (1.5 IP for each) and one IP for the parent ion (349.6). In most of the targeted compounds, more than two transitions were available, but it has been suggested that recording of more transitions usually requires a decrease in the dwell time in order to maintain satisfactory chromatography, affecting the sensitivity and making confirmation difficult at low concentration levels (Hernández *et al.*, 2004). In the case of chlorpyrifos, the transition *m/z* 349.6>97 amu was observed but was not as abundant as 293.9 as a confirmation transition.

Terbufos, however, obtained only one transition (m/z 289>103.3amu) which can be used for quantification and confirmation.

Chlorpyrifos is a chlorinated molecule that has three chlorine atoms, and there is a possibility of choosing the most abundant transitions by selecting different precursor ions, leading to two transitions with the same order of sensitivity. This could present an advantageous isotopic pattern in some compounds (Hernández *et al.*, 2004). Hernández et al. (2004) explains that the presence of a chlorine atom in the compound terbuthylazine allowed the inclusion of a second abundant transition with a different precursor ion *m/z* 232.1>176.1 (corresponding to ^{37}Cl) to (or beside) the first abundant transition with the precursor ion 230.1 (*m/z* 230.1>174.4); this yields an ion ratio of 3 and increase the IPs to five. This is the case for a noticeable difference in the sensitivity between the first and the second fragment at the same precursor ion. In this work, however, the sensitivity of chlorpyrifos using one precursor ion with two product ions was high, and no significant difference in the sensitivity was found between the two transitions.

The ion ratio MRM (area of quantification)/MRM (area of confirmation) in the reference standard was nearly 1.84. Following SANCO/10684/2009 (European Commission, DG-SANCO, 2009), this relative intensity is more than 50%, and the tolerance was 1.84 ± 20% (1.47–2.2). Experimental ion ratios in samples were compared to the ion ratios of the reference standard, and the deviations (relative error) were calculated (Table 2.7). Although there were noticeable deviations in the ion ratios of chlorpyrifos in the samples of different species compared to the ion ratio of chlorpyrifos in the standard, the confirmation of the compound based on the ion ratio criterion was successful since all the sample deviations were within the range ±20%.

Samples that had ion ratios outside the acceptable range (different to that of the reference) were treated as non-detected. It should be noted that the confirmation transition (*m/z* 293.9) in these samples suffered extreme interference from matrix bioorganic components, and as a result the calculated ion ratios in the samples were affected. It would sometimes be possible to include more than two product ions to compensate any change in the ion ratio. Although a third transition was observed in chlorpyrifos fragmentation, at 349.6>97, this transition was slightly lower than the 293.9 in the abundance (in the reference standard). The positive samples were re-analyzed and added the transition 349.6>97 (as a third transition), and as expected the intensity of the ion was very low and/or the ion was not detected. In this case, the clean-up technique had to be improved in order to remove as much of the co-extractives as possible, enabling inclusion of the low abundance transitions. This will eventually help to detect and confirm the compound at very low concentrations.

The use of a signal to noise ratio is another criterion for identification. As stated in SANCO/10684/2009, the signal to noise ratio must be greater than 3 for the precursor ion and the two transitions. The signal to noise ratios of the quantification ion *m/z* 349.6>198 were greater than 3 in all positive samples, but the signal to noise ratios of the confirmation ion 349.6>293.9 in some samples were not. The confirmation ion was greatly affected by the matrix effect of the high intensity peaks at close retention time (most likely bioorganic materials). In general, the signal to noise criterion for identification was not fully successful in some samples for identification, except for the first ion transition (quantification).

Table 2.7 Ion ratios for chlorpyrifos found in some migratory songbirds compared to the reference standard using LC–ESI (+)-QqQ-MS/MS.

Species	Mean ion ratio (Q/q)		
	Standard	Sample	Deviation (%)
Blue winged warbler	1.84	1.72	-6.5
Northern parula	1.84	1.93	4.5
Tennessee warbler	1.84	1.60	-13
Common yellowthroat	1.84	1.89	2.7
Northern waterthrush	1.84	1.85	0.5
Black throated blue warbler 2007	1.84	1.70	-7.6
Black throated blue warbler 2011	1.84	1.70	-7.6

Q quantification ion
q Confirmation ion

Table 2.8 The obtained retention time ratios for chlorpyrifos found in some migratory songbirds compared to the reference standard using LC–ESI (+)-QqQ-MS/MS.

Species	Mean ratio chlorpyrifos/IS in standard solution	Mean ratio chlorpyrifos/IS in the samples	Deviation/%
Blue winged warbler	1.154	1.155	0.08
Northern parula	1.154	1.151	-0.26
Tennessee warbler	1.154	1.155	0.08
Common yellowthroat	1.154	1.155	0.08
Northern waterthrush	1.154	1.151	-0.26
Black throated blue warbler 2007	1.154	1.151	-0.26
Black throated blue warbler 2011	1.154	1.154	0

Moreover, the ratio of the chromatographic retention time of the peak (in the sample) to that of the suitable internal standard, i.e. the relative retention time of the analyte, should correspond to that of the calibration solution with a tolerance of ± 2.5%, according to SANCO/10684/2009 (European Commission, DG-SANCO, 2009). Although shift in the retention times was clearly observed, both chlorpyrifos and the internal standard d_{27}-TPP were affected. The ratio of the reference standard (chlorpyrifos) to the internal standard in the calibration solutions (methanol) to that in the samples (unknown peaks) was calculated (Table 2.8), and all the positive samples met the criterion of tolerance ± 2.5%. Figures 2.5, and 2.6 show the shifts in the retention times in both chlorpyrifos and internal standard where the difference (2.81) was identical for both samples.

In terms of the fragmentation pathway, the product ion *m/z* 198 was observed in several studies in which chlorpyrifos was analyzed using LC–ESI (+)-QqQ-MS/MS (Sancho *et al.*, 2000; Hiemstra and de Kok., 2007; Salm *et al.*, 2009), and the product ion *m/z* 198 was used for quantification. The confirmation product ion *m/z* 293.9 has not been observed in many studies. However, Sinha *et al.* (2011) propose a fragmentation pathway for chlorpyrifos under a LC-ESI (+)-MS/MS-QqQ condition, and the product ion *m/z* 293.4 was proposed as a result of the loss of two ethylene groups. Other transitions were also observed in this work, such as (as described previously for m/z 97), *m/z* 125, *m/z* 213 and *m/z* 115, but they were in lower abundance than those that were selected. In general, changing the collision energy is probably the most important factor for producing product ions with different abundance.

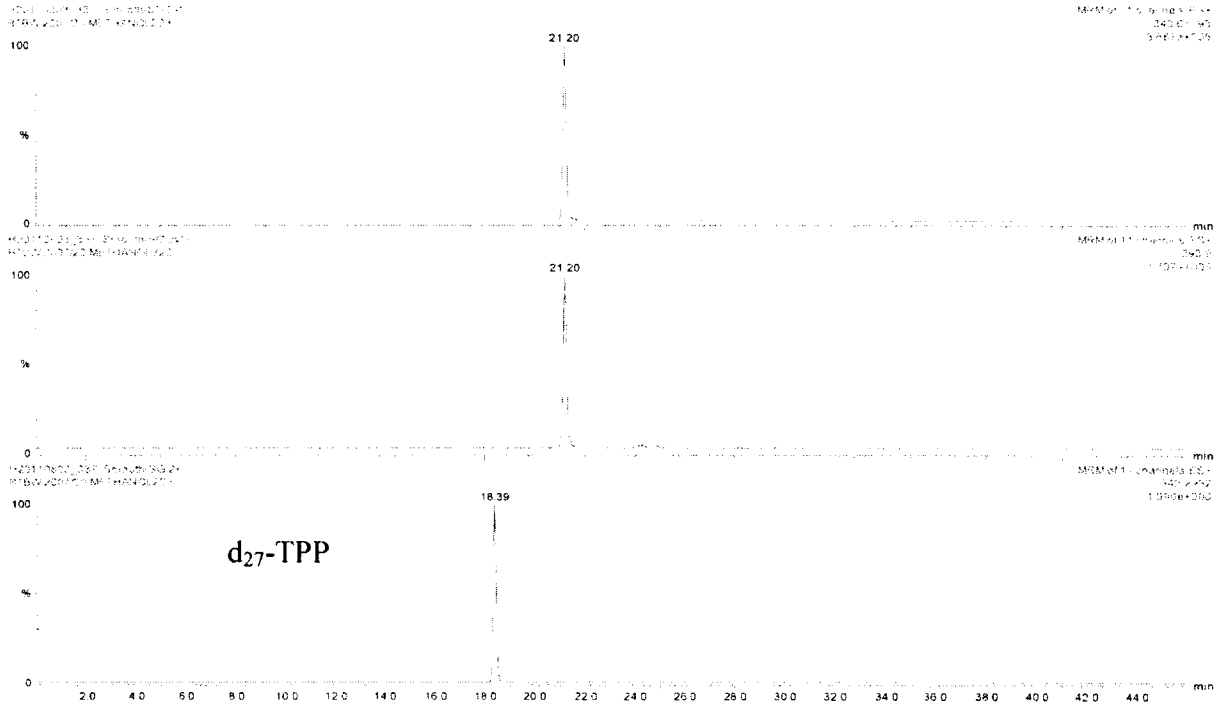


Figure 2.5 LC–ESI (+)-QqQ-MS/MS analysis and MRM chromatograms for real feet extract sample of Black throated blue warbler containing chlorpyrifos.

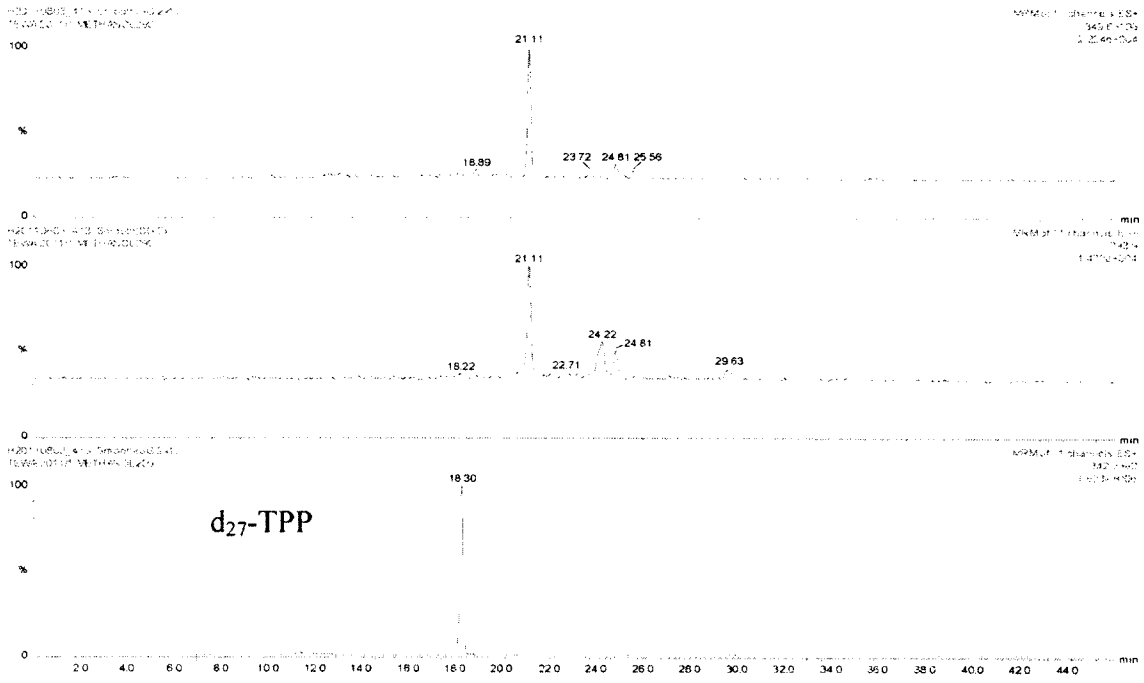


Figure 2.6 LC–ESI (+)-QqQ-MS/MS analysis and MRM chromatograms for real sample of Tennessee warbler containing chlorpyrifos.

2.5.2 Confirmation of the identification using time-of-flight (TOF), and quadrupole time-of-flight (Q-TOF) mass spectrometry

The TOF instruments accurately measure mass-to-charge ratios on the basis of mass dependent flight time differences from the entrance of the analyzer to the detector; this affords fast full-spectral acquisition rates and full-spectral sensitivity at high mass resolution with high mass accuracy to pesticide analysis (Botitsi *et al.*, 2010).

When using a TOF instrument, a single ion gives only two identification points, which is not sufficient to confirm the identity of environmental contaminants. Obtaining three to four IPs, needed for positive confirmation of target contaminants, is feasible only for compounds showing an easy in-source fragmentation or which have a characteristic isotopic pattern (Petrovic and Barcelo, 2006). As chlorpyrifos was the compound under investigation, it is easily fragmented in the source and also in collision (in the case of QTOF), depending on the concentration (Grimalt *et al.*, 2010), and since it is a chlorinated compound, it has a characteristic isotopic pattern. Thus, using a TOF instrument would be suitable for confirmation of chlorpyrifos in the real samples.

The reason for using TOF and QTOF in the prsent work was for confirmation of the positive findings obtained by QqQ. As the positive samples were observed at low concentrations, the positive samples from each species were combined in one fraction, and the volume was reduced to approximately 0.1 ml. These combined fractions were then injected in TOF. The reason for combining steps is that TOF and QTOF have lower sensitivity compared to QqQ in single reaction monitoring (SRM) mode (note: TOF works in a scan mode and no ion selection can be performed) (Hernández *et al.*, 2004). The goal was to increase the concentration to a level that can be detected by TOF instruments. Chlorpyrifos, theoretically, shows ^{35}Cl $[M+H]^+$ 349.9336, and $^{35/37}$Cl $[M+H]^+$ 351.9306. The monitoring of these isotopic characteristics allowed

us to use TOF successfully for confirmation. Although some fragment ions, such as *m/z* 199.9 and *m/z* 197.9, were monitored using TOF instrumentation (Grimalt *et al*, 2010), these ions were not observeed in this work. This is probably due to their low abundance.

As a reference standard of chlorpyrifos was used (Figure. 2.7), the identification and confirmation of chlorpyrifos in the real samples (Figure. 2.8) were accomplished by accurate mass measurement of protonated molecule using its isotopic signature, and retention time that compared to reference standard.

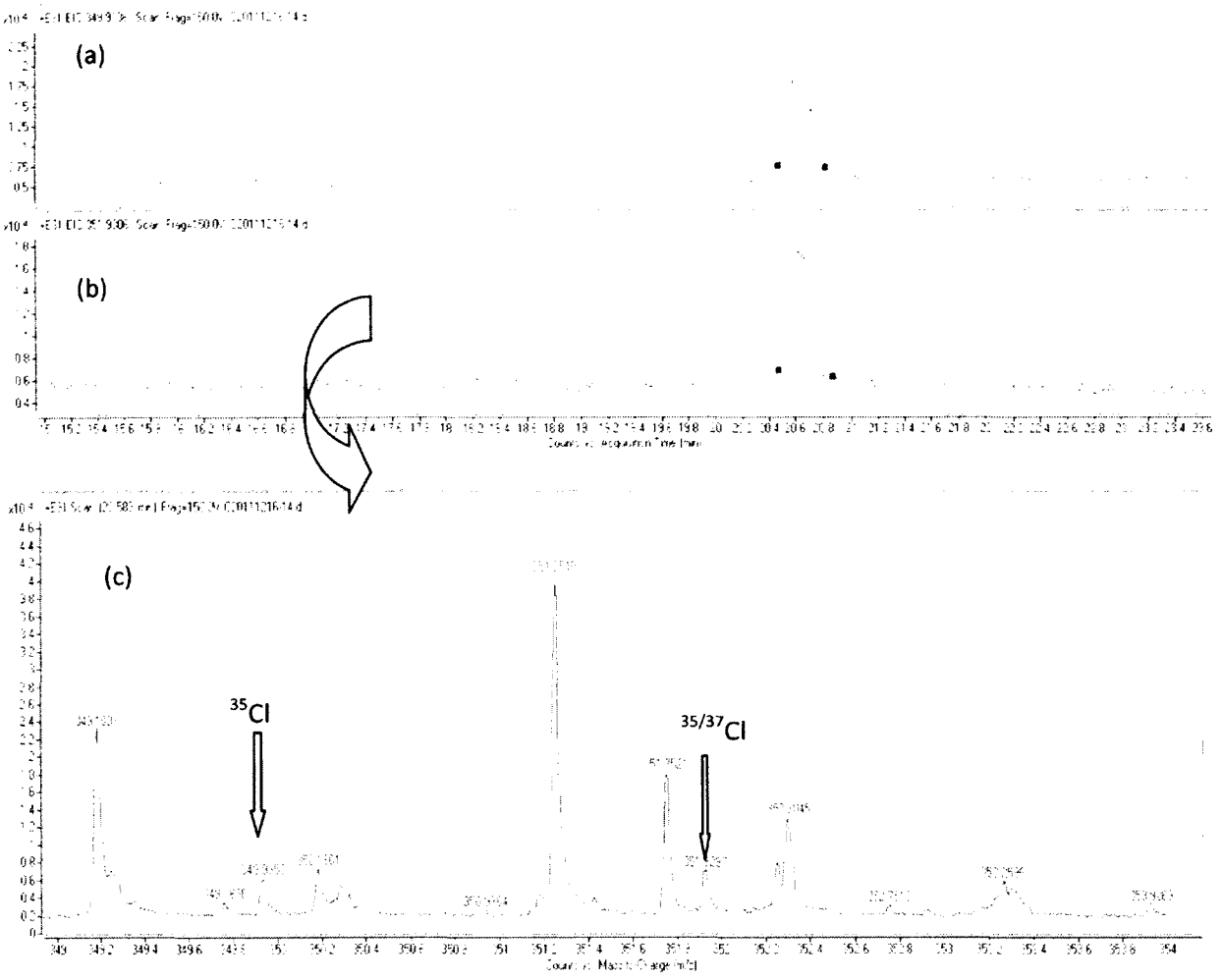


Figure 2.7 Extracted ion chromatogram corresponding to the LC/TOFMS analysis of chlorpyrifos standard solution at 2 ng ml^{-1} (a) for ^{35}Cl, (b) for $^{35/37}Cl$, and (c) accurate mass spectrum of chlorpyrifos obtained at 20.6 min for ^{35}Cl and $^{35/37}Cl$.

The quantification using TOF and QTOF was not of priority as the focusing was only on confirmation, and thus the quantification part was omitted. The optimization for confirmation was performed where a fixed cone energy ramp was applied for the chlopyrifos reference standard to obtain a single acquisition: the protonated molecular ion. Chlorpyrifos fragmented at 150v yielded clearly two precursor ions, these being (measured) ^{35}Cl $[M+H]^+$ 349.9353, and $^{35/37}$Cl $[M+H]^+$ 351.9297; these two isotopic molecular ions were used for confirmation purposes. Using the TOF instrument, two ions must be monitored to achieve the minimum IPs required (Hernández *et al.*, 2004). In the case of chlorpyrifos, however, some fragment ions might be obtained by increasing the cone voltage in the ion source (electrospray), but the two isotopic signature ions were enough to achieve at least four IPs. As the concentrations of chlorpyrifos were very low, increasing the cone voltage might result in losing the abundance of chlorine isotopic ions, which are important for monitoring in TOF. Moreover, Grimalt *et al.* (2010) concluded that the problem of low in-source fragmentation of chlorpyrifos could be solved, as the chlorpyrifos offered an abundant characteristic isotopic distribution (chlorine isotopic ions) for obtaining an increased confirmation power. In the TOF study, the evaluation was based on retention time, mass accuracy and isotopic signature as the single MS was used, but no ion ratios were evaluated. The retention times of the reference standard matched those of real samples (deviation ~0.9%).

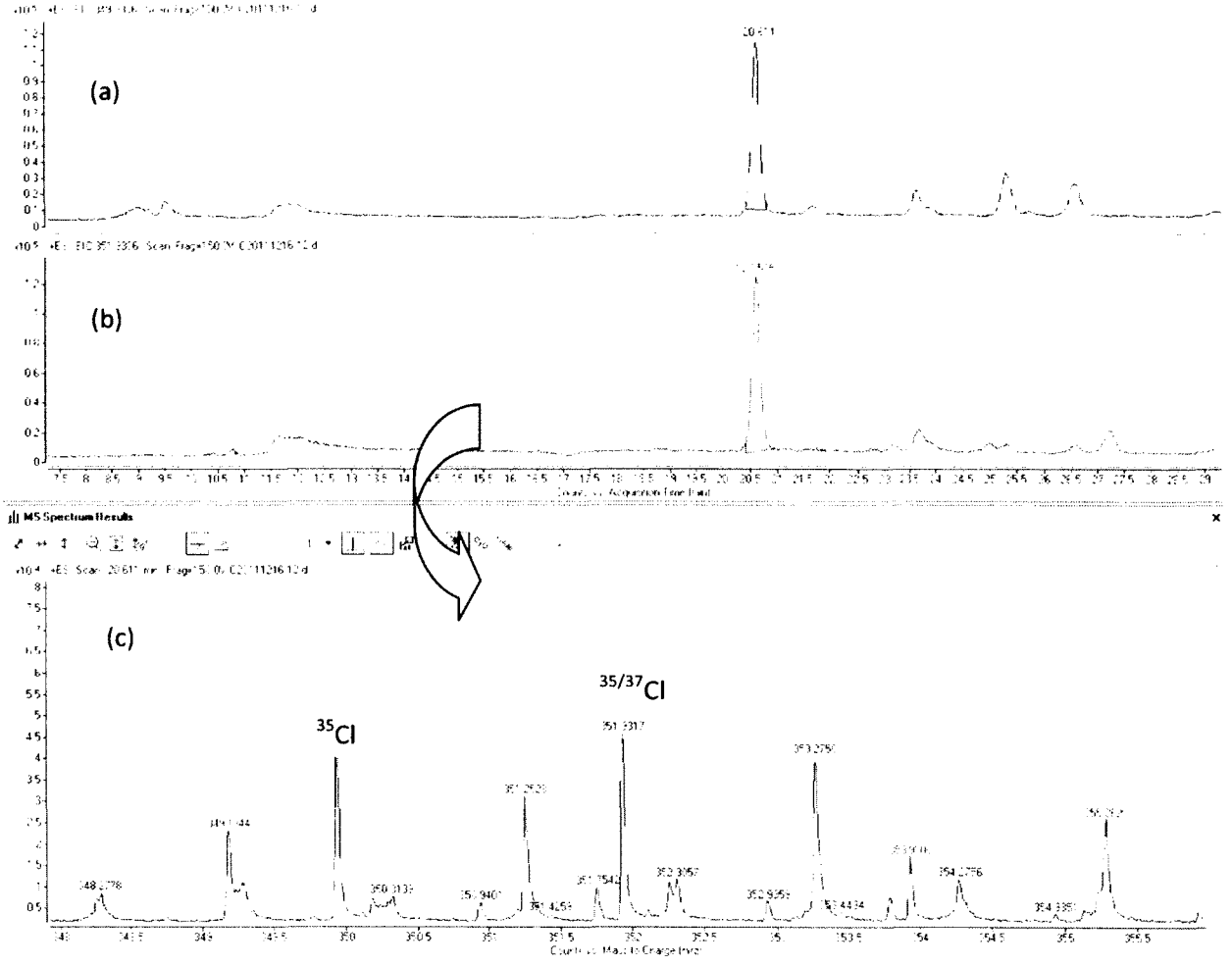


Figure 2.8 Extracted ion chromatogram corresponding to the LC/TOFMS analysis of chlorpyrifos for (a) ^{35}Cl, (b) $^{35/37}Cl$, and (c) accurate mass spectrum of chlorpyrifos obtained at 20.6 min for ^{35}Cl and $^{35/37}Cl$ of a Black throated blue warbler-2007.

Some of the positive samples obtained from QqQ were not detected in TOF; this might be due to the very low concentrations of chlorpyrifos in these samples. This is consistent with Grimalt *et al.* (2010), where the positive result of chlorpyrifos (detected as lower than 0.01 mg/kg) in a tomato sample could not be confirmed using TOF due to the very low concentration. Species that were confirmed using TOF to have chlopyrifos were: Black-throated blue warbler-2007, Common yellowthroat, Northern parula, Tennessee warbler and Blue-winged warbler. Table 2.9 shows the species positive to chlorpyrifos using TOF, the measured mass, and the deviation (mDa). The identification points earned according to the criterion of SANCO/10684/2009, in which the precursor ion obtained from high resolution gains 2.0 points, were four for all species (Table 2.9). It should be noted that this criterion for IPs is based on the resolution power of TOF. Another criterion (alternative) was proposed by Hernández *et al.*,2004, in which assignment of IPs is based on accurate mass measurements (mass errors in mDa). In this work, the original criterion was followed (i.e. SANCO/10684/2009).

Although the presence of chlorpyrifos in some migratory songbirds species using QqQ and then TOF instruments was confirmed based on comparison with the reference standard, one sample had an exceptionally high concentration, and this sample was analyzed further by QTOF (a tandem MS instrument, but using TOF as a second MS instead of Q). This sample was from Black-throated blue warbler, and the goal was for further monitoring of the product ions.

Table 2.9 Exact mass measurements and mass errors (deviations) for chlorpyrifos identified in some migratory songbirds using TOF-MS

Species	theoretical mass	measured mass	deviation(mDa)	assigned IPs[1]
Chlorpyrifos standard	Cl^{35}349.9336	349.9353	1.7	2
	$Cl^{35/37}$351.9306	351.9336	3	2
			Total IPs	4
Blue winged warbler	Cl^{35}349.9336	349.9387	5.1	2
	$Cl^{35/37}$351.9306	351.9312	0.6	2
			Total IPs	4
Northern parula	Cl^{35}349.9336	349.9360	2.4	2
	$Cl^{35/37}$351.9306	351.9251	-5.5	2
			Total IPs	4
Tennessee warbler	Cl^{35}349.9336	349.9336	0	2
	$Cl^{35/37}$351.9306	351.9329	2.3	2
			Total IPs	4
Common yellowthroat	Cl^{35}349.9336	349.9308	-2.8	2
	$Cl^{35/37}$351.9306	351.9304	-0.2	2
			Total IPs	4
Black throated blue warbler-2007	Cl^{35}349.9336	349.9344	0.8	2
	$Cl^{35/37}$351.9306	351.9317	1.1	2
			Total IPs	4

1 SANCO/10684/2009, IPs Identification points

Quadrupole time of flight (QTOF) has the highest confirmation capacity as a consequence of the high efficient fragmentation attainable in the collision energy, as well as the accurate mass of product ions measured by the second mass analyzer (Grimlat *et al.*, 2010). As a chlorpyrifos reference standard was used, the retention time of the peak in the real sample (Black-throated blue warbler-2007) was monitored and compared to the reference standard. The retention time of the real sample was successfully matched with that of the reference standard of chlorpyrifos at 20.8 min (deviation ~0 %). MS/MS acquisition was performed at one collision energy (15 eV), and five fragment ions (product ions) were monitored; those were *m/z* 321, *m/z* 293, *m/z* 197.9 *m/z* 153 and *m/z* 124.9. The fragments were observed in some other studies taking into account the different collision energies that were applied (Hernández *et al.*, 2009). Although the use of one collision energy was successful to induce the fragmentation in the present work,

the use of different collision energies for comparison purposes would be preferable. The fragment ions in the real samples were exactly observed in the reference standard (Table 2.10); however, noticeable deviations also occurred.

Table 2.10 Exact masses of protonated chlorpyrifos and its ion products obtained from QTOF MS/MS (chlorpyrifos precursor ion was *m/z* 349).

Exact mass (theoretical)	**Reference standard**		**Positive sample**	
	measured	mass deviation (mDa)	measured	mass deviation (mDa)
321.9023	321.8998	-2.5	321.9058	3.5
293.871	293.8698	-1.2	293.8739	2.9
197.9275	197.9296	2.1	197.9297	2.2
153.0134	153.0146	1.2	153.0173	3.9
124.9821	124.9823	0.2	124.9834	1.3

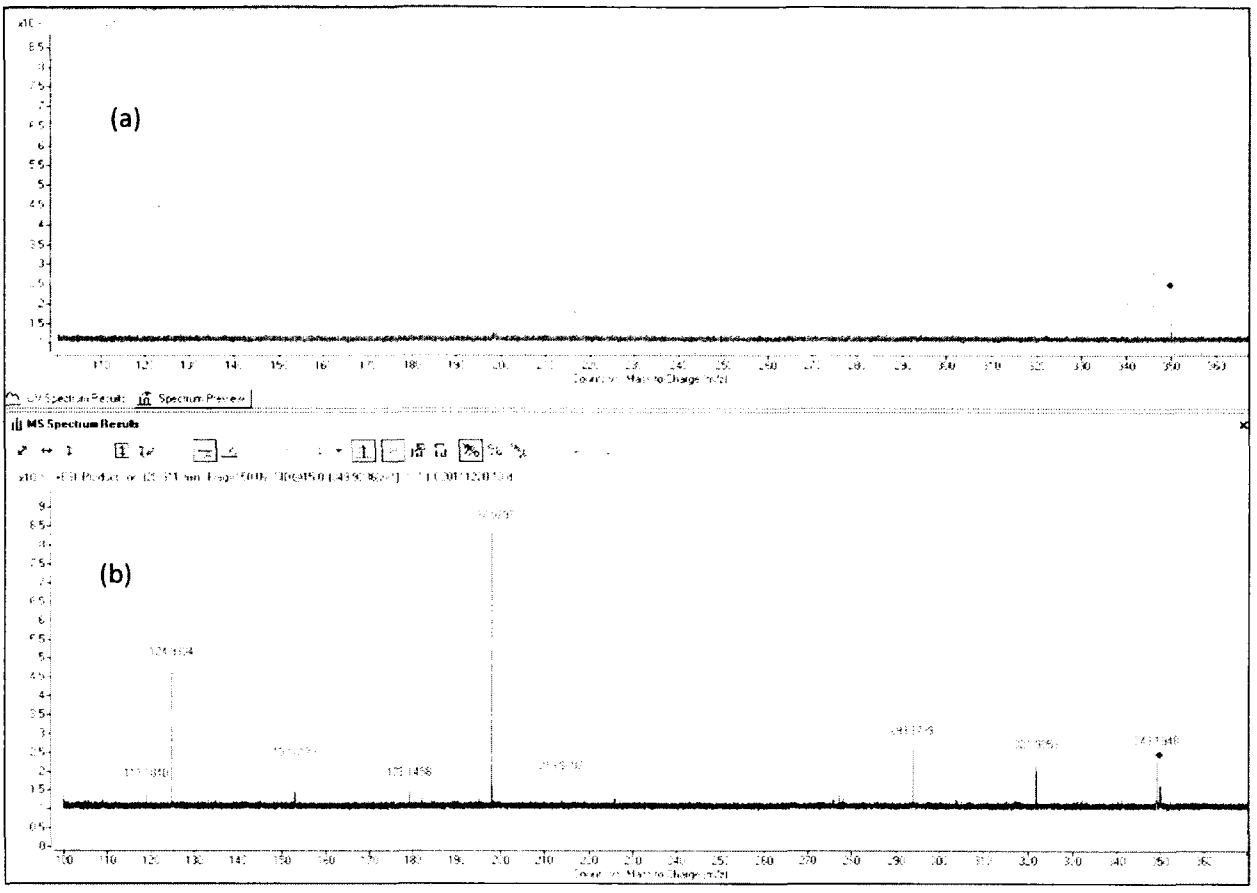


Figure 2.9 Product ion spectra obtained from QTOF for chlorpyrifos: (a) standard solution at 10 ng ml^{-1} and (b) feet extract positive for chlorpyrifos from Black-throated blue warbler-2007.

2.6 Discussion

It was hypothesized that if OPPs are applied on crops at the same time as songbirds are foraging on farmlands, and on the crops treated with OPPs, then dermal exposure occurs via the feet or feathers of the songbirds. This thesis hypothesis was supported as one of the OPPs tested, i.e. chlorpyrifos, was detected and confirmed in the feet of some migratory songbirds. Furthermore, the feet of migratory songbirds were proven useful as a matrix to monitor dermal exposure of farmland birds to OPP application to several crops.

Chlorpyrifos is a lipophilic compound relative to all other OPPs in used, and the opportunity for it to adhere to feet and then be absorbed through the skin is relatively high. Chlorpyrifos is applied on the field in different formulations, but the wettable powder formulation is that most used on foliage as a spray to control pests (DowAgroScience[2]). The residue on foliage may be a source of possible dermal exposure for birds that are perching on the treated crop. However, irrigation after pesticide application might also play a role in dermal exposure. For example, it was shown that irrigation immediately following pesticide application significantly increased the dissipation rate of chlorpyrifos residues from foliage (Goh *et al.*, 1986), which might decrease the possibility of dermal exposure. In general, dislodgeable foliar residues of chlorpyrifos dissipated to less than 0.01 mg m^{-2} by 14 days after treatment (Sears *et al.*, 1987).

The assumption in this case is that birds are exposed dermally by perching on treated crops rather than oral intake. If birds were exposed to chlopryrifos via dietary intake, chlorpyrifos would undergo activation and detoxification in the blood, liver, or brain by CYP enzymes or paroxanase, and the half-life of the parent compound would be measured in hours. The study of Henderson *et al.* (1994) is the best available evidence for AChE taking a long time

(days) to recover to its normal activity after dermal exposure to parathion and diazinon, and for oral exposure taking less time (hours). These observations indicated that the skin might store the parent compound and/or its oxon, and oral exposure was unlikely to be detected in any part of the body due to the quick recovery of the enzyme activity.

In terms of dermal studies on chlorpyrifos, to our knowledge-no study is available on dermal absorption of chlorpyrifos on birds. In humans, a study found that 1% of metabolites of chlorpyrifos were recovered in the urine (Griffin *et al.*, 1999). In another study, 1.28% of the dermally applied dose of chlorpyrifos was recovered in the urine after 24 h when the study was conducted in six human volunteers (Nolan *et al.*, 1984). Two doses of chlorpyrifos (5 and 15 mg) were applied to the foreams of human volunteers to determine chlorpyrifos absorption. The results indicated that the non-absorbed fraction amounted to 42–67% of the applied doses. The excreted amount of TCPy (a major metabolite of chlorpyrifos) was approximately 4.3% for the 5 mg dose, and the percentage for 15 mg was similar to that for 5 mg. The clearance of chlorpyrifos was not completed until five days post-exposure. This indicates that chlorpyrifos or TCPy could be retained in the skin (specifically at the application site) or by other tissues in the body (Meuling *et al.*, 2005).

Distribution studies of chlorpyrifos and chlorpyrifos oxon in different tissues were conducted on rats. It was found that both parent OPP and its metabolite had the highest concentration in fatty tissues, followed by/or equal to brain, then liver and finally kidney (Timchalk *et al.*, 2002). Although protein-binding and the influence of biotransformation are not considered in this study, it has been suggested that chlorpyrifos and its oxon accumulate in the adipose tissue (Testai *et al.*, 2010).

2.7 Conclusion

The main goal of the present study was to design and develop a simple method of pesticide residue analysis using the feet of dead migratory songbirds, and to apply the developed method to real samples to investigate whether or not the feet of dead migratory songbirds can be used as a matrix for OPP residue analysis. Chlorpyrifos residues were detected in six species (Black-throated blue warbler, Common yellowthroat, Northern parula, Northern waterthrush, Tennessee warbler and Blue-winged warbler) indicating that a portion of chlorpyrifos is in intact feet for relatively a long time without being metabolized.

In terms of analytical methodology, the method was based on the principle of the well-known pesticide residue method, i.e., the QuEChERS method; however, solvent combination was used rather than acetonitrile. The method has several advantages, such as being simple, efficient, easy and economical, and was applied for the determination of OPPs in the feet of real samples of birds. In the extraction procedure, the solvent system used yielded an acceptable recovery and minimum matrix effect. In the clean-up technique, sorbent PSA was the best in terms of removal of the co-extractive materials, but adding other sorbents such as C_{18} may enhance the clean up results by removing some fatty materials.

Although OPPs are amenable to GC-MS, the use of LC-(ESI+)-QqQ-MS/MS offers high sensitivity (low limit of detection to parts per billion levels). The use of LC-(ESI+)-QqQ-MS/MS in this work allowed us to detect chlorpyrifos at low concentrations, which may not be achievable using GC-MS. Additionally, the use of tandem MS reduced the matrix interferences, which can affect the signal in the chromatograms due to the matrix being fatty and possibly containing some lipid materials. SANCO/10684/2009 criteria were followed for identification and quantification of chlorpyrifos in the real samples using QqQ instruments. The confirmation

procedure for positive results was carried out using LC-(ESI+)-TOF-MS, and LC-(ESI+)-QTOF-MS/MS (based on an extracted ion chromatogram at the exact two isotopic chlorine signatures of chlorpyrifos). Due to the low sensitivity, LC-(ESI+)-TOF-MS and LC-(ESI+)-QTOF-MS/MS can be used only for confirmation purposes for the target analysis.

Overall, the method was preliminarily (not fully) developed, and, as described, the sample size was not sufficient to perform several experiments in order to test every aspect in the analytical methodology, such as the method limit of quantification, which is usually used to accurately measure the sensitivity of the overall analytical method and instrumentation. The sensitivity parameter that was used, however, was based on the signal to noise ratio in the standard solution. Moreover, the weight of each sample was not consistent among all replicates of control species, making the results of recovery and matrix effect approximate.

CHAPTER 3

The use of crops by neotropical migratory birds and their possible source of pesticide exposure: an investigation of the bird-crop interaction

3.1 Introduction

The term "wintering habitat" is used to refer to the tropical area of Mesoamerica, the Caribbean and South America that provide the winter habitat for a massive number of birds that retreat each year from their temperate zone breeding areas (Norris *et al.*, 2004). The neotropical migratory bird species cover the land mass of the wintering habitat, but the greatest diversity, density, and relative density (migrants/total birds) is found in the Northern Neotropics, particularly in Mexico and the greater Antilles (Greenberg, 1992).

It has been hypothesized that food influences the winter distribution of migratory birds (Leisler, 1990; Hutto, 1992: Wunderle and Waide, 1993; Jones *et al.*, 1996; Katti and Price, 1996; Johnson and Sherry, 2001). In the winter, migratory songbirds are forced to occupy agricultural and disturbed habitats, which is comprised of immature insects in the non-breeding (winter) season (Petit *et al.*, 1999: Hughes *et al.*, 2002).

Migratory birds are mobile animals, and have the ability to move through several habitats in their wintering sites searching for food. They are opportunistic foragers, entering crop lands as they are attracted by agricultural pests as well as weed seeds, which are the main food items for migratory songbirds (Kirk, 1996; Mineau, 2005). The incidence of mortality of wild birds from exposure to highly toxic chemicals in insecticide-treated fields has provoked researchers to calculate and document these incidences to estimate direct bird losses in farm fields (Mineau, 2005).

The association between the use of farmland in the wintering habitats by migratory songbirds and the detected chlorpyrifos in some of these birds was investigated using data on bird–crop interactions obtained from literature. The aim of this chapter was to investigate whether there is a relationship between birds that were positive to chlorpyrifos residue and the use of specific crops i.e. if birds that had chlorpyrifos residue use or visit a particular crop in high abundance which might be the source of pesticide exposure.

3.2 Methods

3.2.1 Migratory bird habitats

In this study, migratory songbirds were sampled in May, and the specimens were received in the laboratory in late June. These specimens were considered to have arrived from the wintering habitat, and were thus treated as representative of the non-breeding rather than breeding season. For example, Black throated-blue warblers leave their breeding grounds from late August to mid-September and they arrive in their winter grounds in early to mid-October. The migration from the winter sites starts in late March to April, and they arrive in breeding areas from late April to early–late May (Poole, 2005). The breeding habitat of the majority of songbirds is forests; for example, the breeding habitat of Black-throated blue warbler is mainly undisturbed deciduous or mixed deciduous/coniferous forests such as maples, and beech in North America (Poole, 2005).

3.2.2 Bird–crop interaction data

Information about use of crops by migratory songbirds in the overwintering sites was obtained from peer-reviewed articles. Table 3.1 summaries the most available data on the use of crops by songbirds in their wintering habitats. All these studies investigated the abundance of birds on crops for a great number of species, whether residents and migrants, but the focus in this work was on the species that were included in chemical analysis for OPPs residue (See Chapter 2 for a list of species). The usage of crops and the abundance of each species on the crops were obtained from each article.

There are many methodologies or techniques for undertaking bird census on the farms; however, the fixed-radius point counts technique is the most popular (Greenberg *et al.*, 2000). The majority of the available data was obtained using the fixed-radius point technique, but the comparison of the abundance of each species on a particular crop between the different studies (pooled data) may not be fully acceptable (from methodological point of view) due to the differences in these studies in terms of number of sites, timing, date, habitat location, duration of the study and number of replicates. As a result, a comparison was made firstly within each study i.e. taking the differences in the abundance among species on each crop within one study. Secondly, abundance data of each species on a particular crop from different studies was pooled in order to obtain a picture of the abundance of birds on each crop among different studies.

Some studies, such as Greenberg *et al.* (1997a, b), recorded observations of birds on more than one site per crop. So, the results from all these sites were combined to facilitate the comparison.

3.3 Results

Using the most available information on the neotropical migrants' use of crops as wintering habitats, the majority of songbirds under study were reported to use farmlands as wintering habitats. The geographic pattern of these species in terms of the use of crops indicated that coffee plantations, citrus, and cacao were the dominant crops used in five wintering sites: Mexico, Guatemala, the Dominican Republic, Jamaica, and Puerto Rico, and Guatemala (Table 3.1).

Table 3.1 shows the species that were included in the study, both the species positive and negative to chlorpyrifos, and the number of positives per species. Species where chlorpyrifos was detectable were Black-throated blue warbler, Northern parula, Blue winged warbler, Common yellowthroat, Tennessee warbler and Northern waterthrush (See chapter 2).

In cacao plantations, two studies were conducted in Mexico in which Magnolia warbler and American redstart had the highest abundance, followed by Black-throated green warbler, while other species had relatively equal abundance (Greenberg *et al.*, 2000) (Figure. 3.1). In another study by Estrada *et al,*(1997), the same trend was found, with Magnolia warbler and American redstart having the highest abundance compared to other species (Figure. 3.2). In the study by Robbins *et al.* (1992) in Belize the Black and white warbler and American redstart had the highest abundance, followed by Northern waterthrush and Gray catbird, whereas Northern parula had the lowest abundance in cacao plantations (Figure.3.3).

In coffee plantations, in the study of Greenberg et al. (1997b) (Figure. 3.4), the abundance of birds on coffee plantations was high for Black throated green warbler, Magnolia warbler, American redstart, and Black and white warbler, which were all negative to

chlopryrifos. However, Tennessee warbler had a relatively moderate abundance. In contrast, in the study of Greenberg *et al.* (1997a) (Figure 3.5) in coffee plantations, the trend is relatively different, with Tennessee warbler having the highest abundance followed by Black-throated green warbler, Indigo bunting and Yellow warbler. In Jamaica, Black-throated blue warbler had the highest abundance, while Black and white warbler, American redstart and magnolia warbler had less than one point in banding per site (Robbins *et al.*, 1992) (Figure.3.6). Black-throated blue warbler was also in higher abundance in shaded coffee plantations than in unshaded coffee plantations in a study in Puerto Rico (Figure. 3.7) (Robbins *et al.*, 1992).

In citrus plantations, one study was conducted using different census techniques whereby the individual number of birds of each species on crops at five citrus orchards in Belize was recorded (Figure. 3.8), and the results indicated that Black and white warbler and Magnolia warbler comprised the highest total number for all orchards (Mills and Rogers, 1992). Furthermore, in the study of Estrada *et al.* (1997), Magnolia warbler and American redstart were the most abundant species in citrus plantations (Figure. 3.2). Table 3.2 summarizes the rank-abundance for all birds on coffee, citrus, and cacao with number 1 as a highest abundance. Figure 3.9 is the histogram of the rank-abundance for all birds on coffee, citrus, and cacao with the highest number is the highest abundance.

Table 3.1 The migratory songbirds included in the study and their use of crops at wintering habitats, their abundance on crops, their status of whether positive and negative to chlorpyrifos, and concentration of chlorpyrifos in positive samples.

Species	Positive/negative to chlorpyrifos[11]	Crops	Country	Abundance	Reference
Gray catbird	Negative (0/2)	Cacao	Mexico	0.04	Greenberg *et al.*, 2000[3]
		Cacao, citrus	Belize	1,1.4	Robbins *et al.*, 1992[4]
		Coffee	Mexico	0.46	Greenberg *et al.*, 1997b[2]
		Coffee plantations	Guatemala	0.08	Greenberg *et al.*, 1997a[9]
		Citrus	Belize	17	Mills and Rogers, 1992[7]
Indigo bunting	Negative (0/4)	Cacao	Mexico	<0.01	Greenberg *et al.*, 2000[3]
		Cacao, coffee	Mexico	0.29,0.36	Estrada *et al.*,1997[5]
		Coffee plantations	Guatemala	1.17	Greenberg et al., 1997a[9]
		Citrus	Belize	32	Mills and Rogers, 1992[7]
American redstart	Negative (0/11)	Cacao	Mexico	0.44	Greenberg *et al.*, 2000[3]
		Sun-exposed and shaded coffee Citrus, coffee Citrus, cacao	Puerto Rico, Jamaica, Belize	0.1,0.6 0.6,0.6 1.6,2.25	Robbins et al., 1992[4]
		Shaded coffee	Dominican Republic	Nr [10]	Wunderle and Latta, 1996[8]
		Cacao, coffee, citrus	Mexico	1.03,1.97,2.17	Estrada *et al.*,1997[5]
		Coffee	Mexico	0.58	Greenberg *et al.*, 1997b[2]
		Citrus	Belize	44	Mills and Rogers, 1992[7]

Table 3.1 Continued

Species	Positive/negative to chlorpyrifos[11]	Crops	Country	Abundance	Reference
Magnolia warbler	Negative (0/39)	Shaded coffee (modern vs traditional farms)	Guatemala	Traditional	Calvo and Blake, 1998[6]
		Cacao	Mexico	0.46	Greenberg *et al.*, 2000[3]
		Citrus, coffee, cacao	Mexico	4.19, 1.67,1.62	Estrada *et al.*,1997[5]
		Coffee	Mexico	1.24	Greenberg et al., 1997b[2]
		Coffee plantations	Guatemala	1.88	Greenberg *et al.*, 1997a[9]
		Citrus	Belize	80	Mills and Rogers, 1992[7]
Black and white warbler	Negative (0/37)	Cacao	Mexico	0.07	Greenberg *et al.*, 2000[3]
		Sun- and shaded coffee Citrus, coffee Citrus, cacao Citrus	Puerto Rico, Jamaica, Belize Costa Rica	0.6,1.3 1,0.6 2.4,3 0.3	Robbins *et al.*, 1992[4]
		Coffee	Mexico	0.44	Greenberg *et al.*, 1997b[2]
		Citrus	Belize	97	Mills and Rogers, 1992[7]
		Coffee	Dominican Republic	0.7	Wunderle and Latta, 1996[8]
Blue winged warbler	Positive (1/1) *0.9	Citrus	Belize	12	Mills and Rogers, 1992[7]
		Cacao	Mexico	0.02	Greenberg *et al.*, 2000[3]
		Coffee	Mexico	0.06	Greenberg *et al.*, 1997b[2]

Table 3.1 Continued

Species	Positive/negative to chlorpyrifos[11]	Crops	Country	Abundance	Reference
Black brunian warbler	Negative (0/4)				
Mourning warbler	Negative (0/4)				
Chestnut-sided warbler	Negative (0/7)	Cacao	Mexico	0.01	Greenberg *et al.*, 2000[3]
		Coffee	Mexico	0.08	Greenberg *et al.*, 1997b[2]
		Coffee	Guatemala	0.12	Greenberg *et al.*, 1997a[9]
Northern parula	Positive (1/3) *1.2	Cacao	Mexico	0.06	Greenberg *et al.*, 2000[3]
		Citrus,coffee Cacao, citrus Citrus	Jamaica, Belize Costa Rica	0.6,0.1 0.5,0.2 0	Robbins *et al.*, 1992[4]
		Coffee	Mexico	0.02	Greenberg *et al.*, 1997b[2]
		Citrus	Belize	2	Mills and Rogers, 1992[7]
Tennessee warbler	Positive (3/8) *1 (±0.8)	Citrus	Belize	6	Mills and Rogers, 1992[7]
		Coffee	Guatemala	2.75	Greenberg *et al.*, 1997a[9]
		Coffee (modern vs traditional farms)	Guatemala	Both	Calvo and Blake, 1998[6]
		Citrus, cacao Citrus	Belize, Costa Rica	1.2,2.5 1	Robbins *et al.*, 1992[4]
		Shaded coffee	Mexico	0.56	Greenberg *et al.*, 1997b[2]

Table 3.1Continued

Species	Positive/negative to chlorpyrifos[11]	Crops	Country	Abundance	Reference
Common yellowthroat	Positive (5/10)	Citrus	Belize	49	Mills and Rogers, 1992[7]
		Citrus	Mexico	0.24	Estrada *et al.*,1997[5]
		Coffee	Dominican Republic	1.9	Wunderle and Latta, 1996[8]
	*1 (±0.3)	Coffee	Mexico	0.01	Greenberg *et al.*, 1997b[2]
Yellow warbler	Negative (0/2)	Cacao	Mexico	0.25	Greenberg *et al.*, 2000[3]
		Coffee	Mexico	0.04	Greenberg *et al.*, 1997b[2]
		Coffee	Guatemala	0.56	Greenberg *et al.*, 1997a[9]
Northern waterthrush	Positive (1/2)	Citrus	Belize	27	Mills and Rogers, 1992[7]
		Citrus,	Jamaica	0.3	Robbins *et al.*, 1992[4]
		Citrus, cacao	Belize	0.4,1.5	
	*0.6	Citrus	Costa Rica	1	
		Cacao	Mexico	0.01	Greenberg *et al.*, 2000[3]
Bay-breasted warbler	Negative (0/1)				
Nashville warbler	Negative (0/6)	Cacao	Mexico	<0.01	Greenberg *et al.*, 2000[3]
		Coffee	Mexico	0.15	Greenberg *et al.*, 1997b[2]

Table 3.1 Continued

Species	Positive/negative to chlorpyrifos[11]	Crops	Country	Abundance	Reference
Black throated green warbler	Negative (0/11)	Cacao	Mexico	0.23	Greenberg *et al.*, 2000[3]
		Coffee	Mexico	1.76	Greenberg *et al.*, 1997b[2]
		Cacao, coffee, citrus	Mexico	0.34,1.07,0.1	Estrada *et al.*,1997[5]
		Coffee	Guatemala	1.95	Greenberg *et al.*, 1997a[9]
Black throated blue warbler	Positive (6/38) *For 2007 samples: 17.9 (±29)[1] *For 2011 samples: 0.5 (±0.1)	Shades/sun-exposed coffee Coffee, citrus	Puerto Rico, Jamaica	2,0.3 2.3,0.3	Robbins *et al.*, 1992[4]
		Shaded coffee	Dominican Republic	Nr	Wunderle and Latta, 1996[8]
		Shaded coffee (modern vs traditional farms)	Guatemala	Both	Calvo and Blake, 1998[6]
		Coffee	Mexico	0.01	Greenberg *et al.*, 1997b[2]
		Cacao, citrus	Mexico	0.1,0.1	Estrada *et al.*,1997[5]

*Concentration of chlorpyrifos pg mg $^{-1}$, feet weight (±SD)

[1] One sample had high concentration 52 pg mg -1, feet weight

[2] The total number of birds per point detected on point counts in planted and rustic coffee plantations

[3] The number of birds per point detected on point counts in cacao plantations

[4] Values shown are the mean three-day banding per site

[5] Values shown are the percentage of individuals contributed by each species to the total number of birds recorded per habitat

[6] Abundance in modern or traditional farms (no ranking was reported)

[7] Numbers captured in mist nets in citrus orchards

[8] Capture rate in mist nets

[9] Mean number of individuals per point of birds (minimum 0.05 ind.point) on point counts in three habitats studied over two periods (six observations)

[10] Not reported

[11] total number of samples for 2007, and 2011

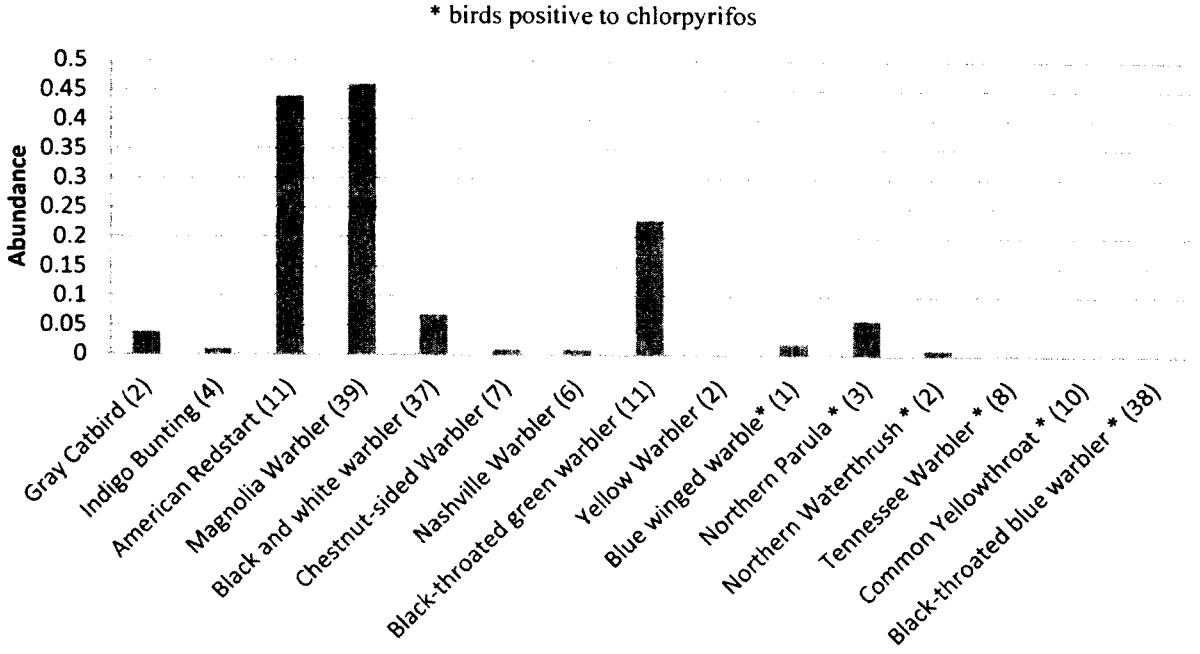


Figure 3.1 The abundance represented by the number of birds per point detected on point counts in cacao plantations in Mexico (Greenberg *et al.*, 2000). Total number of birds in parentheses

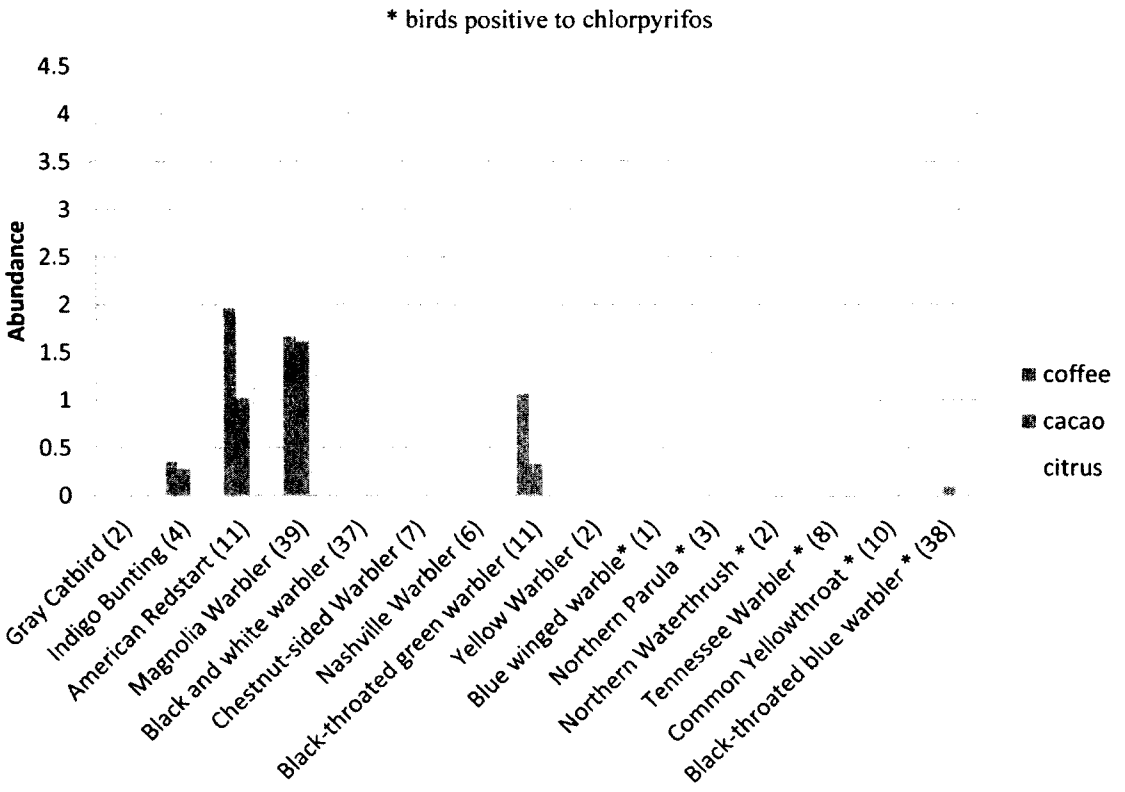


Figure 3.2 The abundance represented by the percentage of individuals contributed by each species to the total number of birds recorded for three habitats (coffee, cacao and citrus) in a study in Mexico (Estrada *et al.*, 1997). Total number of birds in parentheses

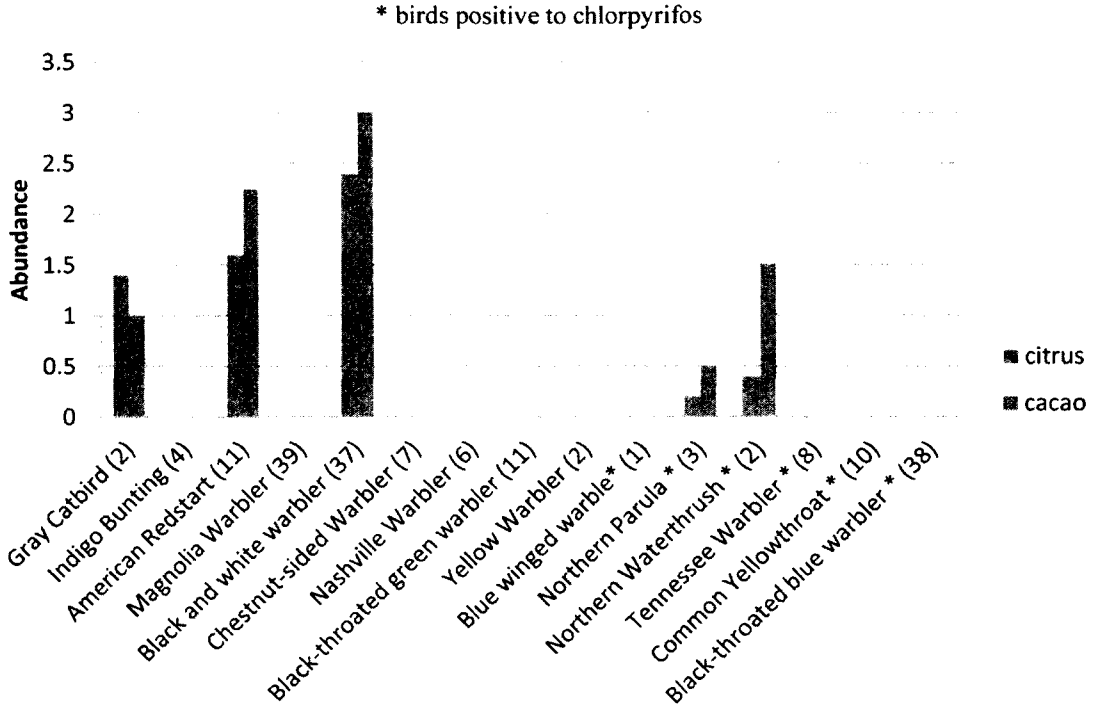


Figure 3.3 The abundance of birds represented by the mean 3-day banding per site for citrus and cacao plantations in Belize (Robbins *et al.*, 1992). Total number of birds in parentheses

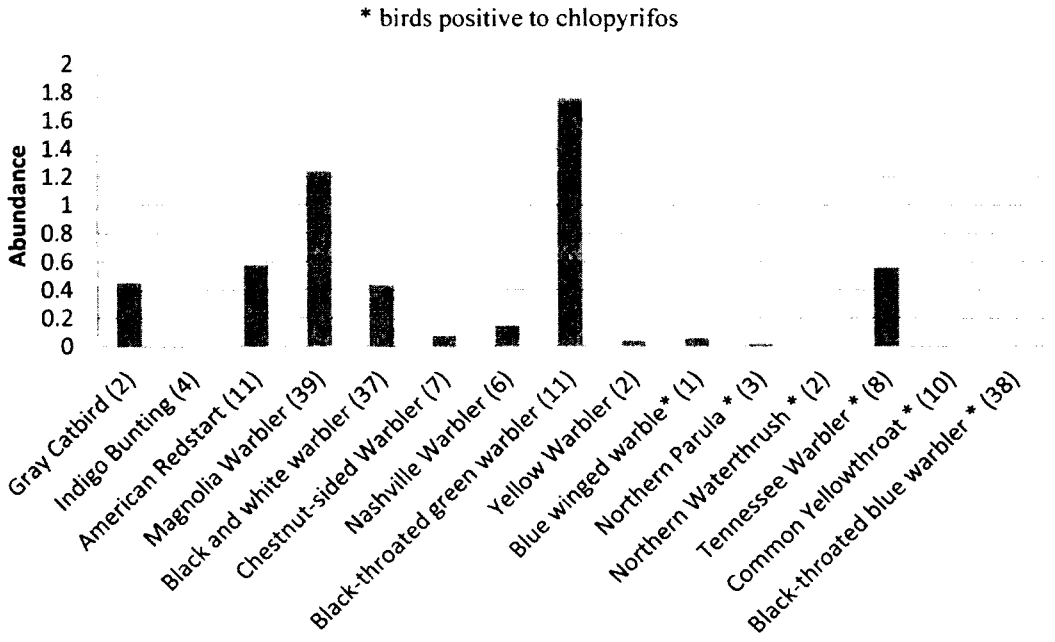


Figure 3.4 The abundance represented by the total number of birds per point detected on point counts in planted and rustic coffee plantations in Mexico (Greenberg *et al.*, 1997b). Total number of birds in parentheses

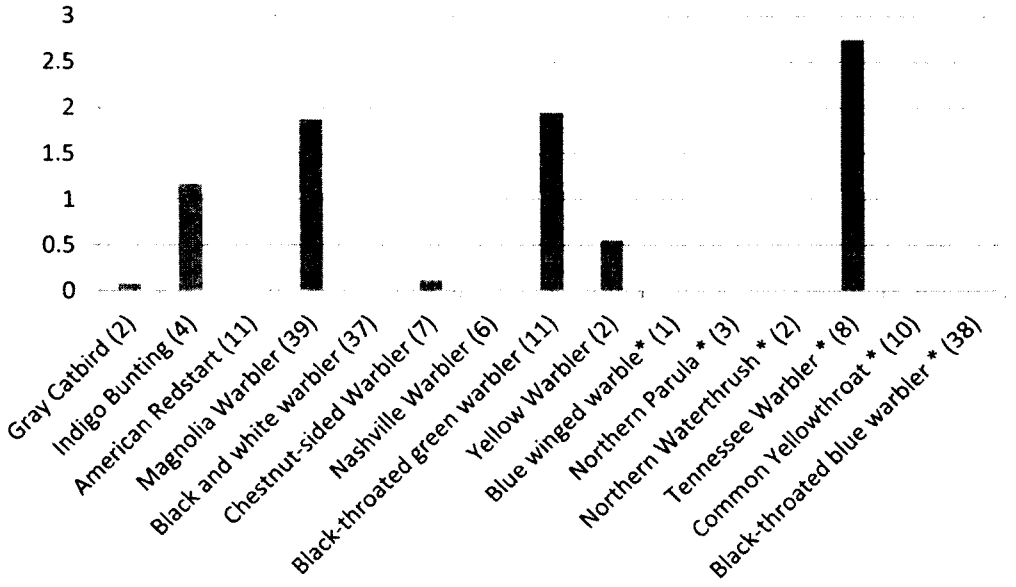


Figure 3.5 The abundance represented by mean numbers of individuals per point of birds on point counts in three coffee habitats studied over two periods in Guatemala (Greenberg *et al.*, 1997a). Total number of birds in parentheses

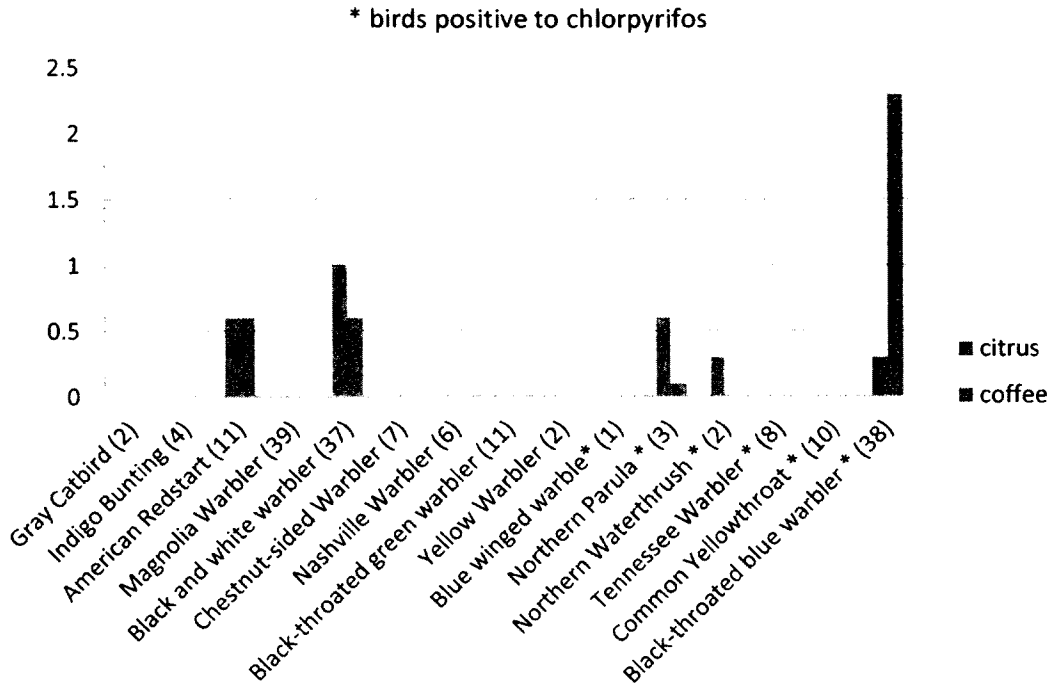


Figure 3.6 The abundance of birds represented by the mean three-day banding per site for citrus and coffee plantations in Jamaica (Robbins *et al.*, 1992). Total number of birds in parentheses

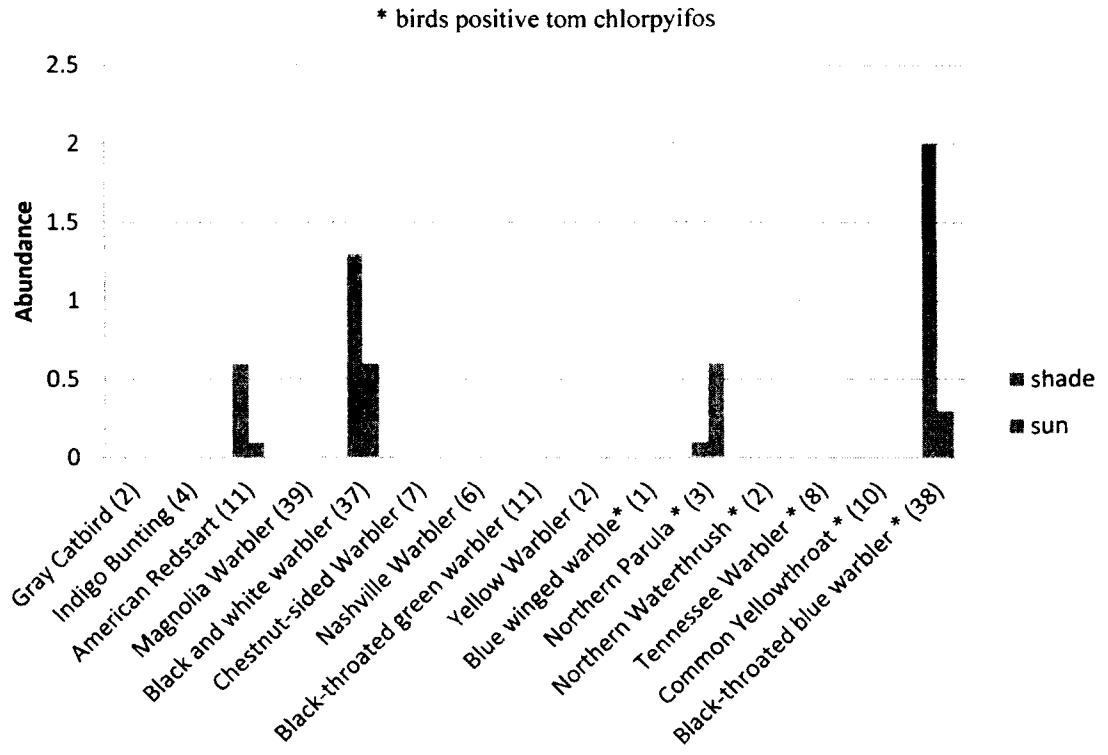


Figure 3.7 The abundance of birds represented by the mean 3-day banding per site for coffee plantations (shade and sun) in Puerto Rico (Robbins *et al.*, 1992). Total number of birds in parentheses

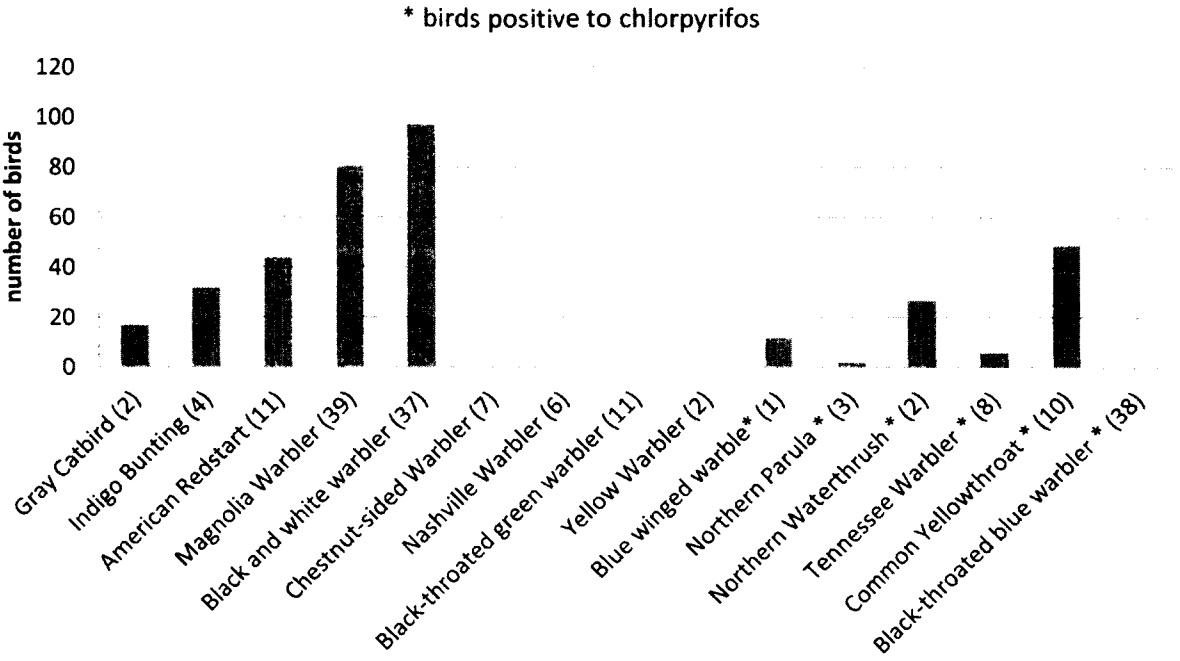


Figure 3.8 the abundance represented by numbers of birds captured in mist nets in five citrus orchards in a study in Belize (Mills and Rogers, 1992).Total number of birds in parentheses

Table 3.2. Summary of rank-abundance for all birds on coffee, citrus, and cacao

Species	Greenberg *et al.*, 2000	Greenberg *et al.*, 1997b	Greenberg *et al.*, 1997a	Mills and Rogers, 1992	Estrada *et al.*, 1997			Robbins *et al.*, 1992 Belize		Robbins *et al.*, 1992 Jamaica		Robbins *et al.*, 1992 Puerto Rico
	Cacao	Coffee	Coffee	Citrus	coffee	Cacao	Citrus	Citrus	Cacao	Citrus	coffee	Coffee (sun+shade)
Blue winged warble*	7	9		8								
Northern Parula *	5	11		10				3	4	2	3	3
Tennessee Warbler *		4	1	9								
Common Yellowthroat *		12		3			3					
Black throated blue warbler *		12				4	3			3	1	1
Gray Catbird	6	5	7	7				2	3			
Indigo Bunting	8		4	5	4	3						
American Redstart	2	3		4	1	2	2	2	2	2	2	3
Magnolia Warbler	1	2	3	2	2	1	1					
Black and white warbler	4	6		1				1	1	1	2	2
Chestnut-sided Warbler	8	8	6									
Northern Waterthrush	8			6				3	3	3		
Nashville Warbler	8	7										
Black throated green warbler	3	1	2		3	3	3					
Yellow Warbler		10	5									

*Birds positive to chlorpyrifos

Abundance ranking: *1 is highest abundance*

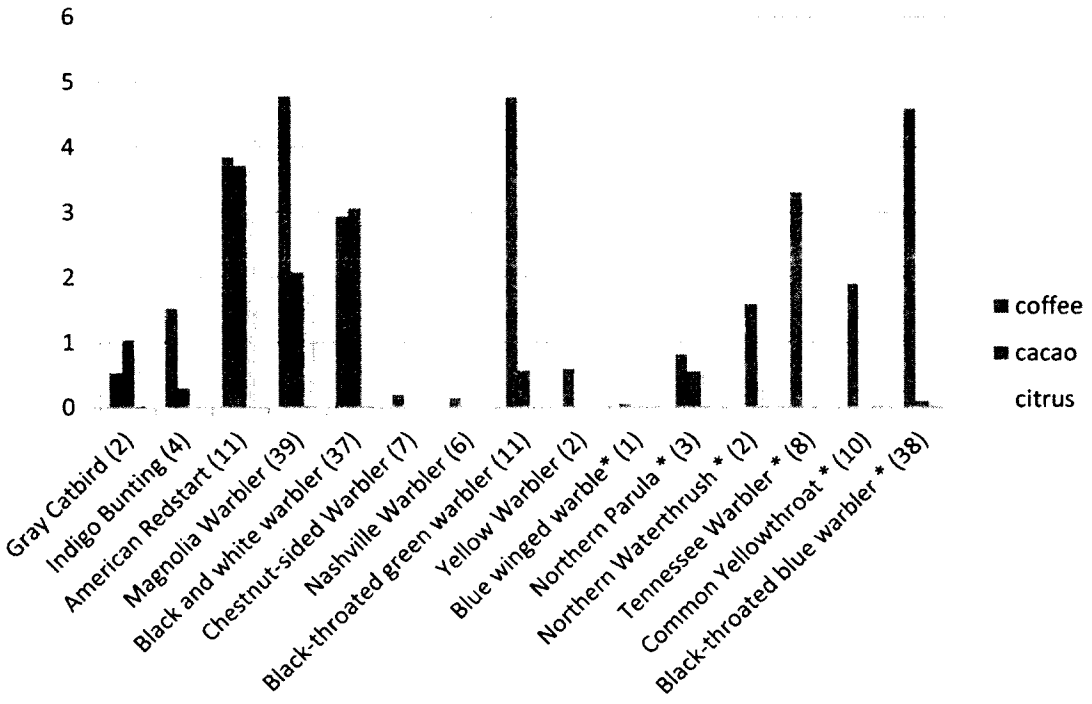


Figure 3.9 Rank-abundance for all birds on coffee, citrus, and cacao (pooled data). Total number of birds in parentheses

3.4 Discussion

The number of birds available for sampling varied greatly among species. Some species negative for the presence of chlorpyrifos such as Indigo bunting, Gray catbird, Yellow, Bay breasted, Blackburnian and Mourning warblers had very low sample sizes; the chance of detecting chemical residues would undoubtedly increase with more sampling. Nevertheless the single Blue winged warbler analysed tested positive for residues of chlorpyrifos whereas species with high sample sizes such as Magnolia warbler and American redstart had no residues of any of the OPPs under study. Clearly, there is a great deal of chance involved in detecting any given species-pesticide combination.

Based on Table 3.1, and the related figures for each study, the abundance of each bird on the corresponding crops shows that there is no clear relationship between birds that are positive to chlorpyrifos and their use of crops, as the abundance of these species was not particularly high in crops. In fact, the abundance of birds that were negative for chlorpyrifos, such as Magnolia warbler and American redstart, was higher in several crops (coffee, citrus and cacao) than the abundance of birds with chlorpyrifos residues. This suggests that the abundance of birds on each crop may not be an accurate indicator of possible pesticide exposure. Only two studies indicated that Black-throated blue warbler, a species positive for chlorpyrifos, had the highest abundance in coffee plantations compared to other species in Jamaica and Puerto Rico (Robbins *et al.*, 1992; Figures. 3.6 and 3.7).

The preliminary results obtained in this work might lead to a tentative conclusion that coffee plantations and citrus were the most likely source of pesticide exposure as most species were observed on these crops. However, the observations differ in terms of the abundance of each species on any particular crop, and the trend of crop usage by each species is inconsistent

for the available data. Although the species that tested negative for chlorpyrifos residues were also reported to use coffee and citrus plantations, and thus might have had contact with chlorpyrifos, it seems to be a matter of chance that species negative to chlorpyrifos interacted with the plantation at a time when no application was being performed.

Species that were reported to use croplands but were negative for chlorpyrifos residues might either have not been exposed to chlopryifos, or it was depleted in the tissue of birds taking into consideration the period and distance of traveling from their wintering to breeding habitat. It should be emphasized that all species under study might be exposed to pesticides other than those that were included in this study.

Chlorpyrifos is registered in Costa Rica, Dominican Rebublic, Nicaragua, Belize, Jamaica, Mexico and Guatemala (DowAgroSciences[1]). Chlorpyrifos is used on a variety of crops such as citrus (DowAgroSciences[2]). In terms of actual usage of chlorpyrifos, a study on pesticide use on plantain in Costa Rica, which engaged in interviews with plantain farmers, showed that over 97% of farmers used the insecticide chlorpyrifos on plantain (Polidoro *et al.*, 2008). Also, many export farmers still use chlorpyrifos as one of the OPPs that are used for foliar application in Northern Cartago and the Ujarrás Valley (Galt, 2008).

Reports on the effect of chlorpyrifos on birds in agro-ecosystems are not conclusive. A few incidences of bird mortality following chlorpyrifos use were documented; for example, dead geese found on golf courses that had been treated with chlorpyrifos, whether alone or in combination with diazinon (US EPA, 1981). In terms of agricultural-related uses of chlorpyrifos, two cases were documented in which chlorpyrifos was used in conjunction with carbofuran

(Smith, 1987). In experimental studies, significant mortality of young mallard ducks following treatment with chlorpyrifos was reported (Hurlbert, 1977).

In a study in Northern Senegal, two buffalo weaver bird colonies were found abandoned after one-day post-treatment with 270 g a.i/ha of chlorpyrifos. Chlorpyrifos was applied as part of locust control in a savannah plot. The investigation of brain cholinesterase levels of adult and juveniles indicated depression by 14 and 38%, respectively, compared to control birds (Mullie and Keith, 1993). The same study design but a different application rate of chlorpyrifos (387 g a.i/ha), resulted in three dead Abyssinian leaf rollers, and one dead Singing bush lark in the treatment plot compared to no deaths in the control plot. The brain cholinesterase level of adult bush larks collected after treatment on study plots was depressed by 17% of levels compared to control birds (Mullie and Keith, 1993).

Chlorpyrifos is used in large amounts in one or more of the seven Central American countries (Wesseling *et al.*, 2005). Chlorpyrifos was also one of the compounds that were suggested in 2000 to be restricted or banned in the seven Central American countries due to the acute poisoning incidences among farmers (Wesseling *et al.*, 2005). A final conclusion on what crop is deemed to be the source of exposure could not be reached, and needs further study.

CHAPTER 4

Conclusion and Future directions

4.1 Conclusion

The ultimate goal of this thesis was to test the feasibility of assessing exposure of songbirds in agricultural areas of their wintering range. The result of this study will contribute to and support avian pesticide field studies with pesticide-exposure data. Although the real toxicological interpretation of the observed identification of chlorpyrifos in the feet of some migratory songbirds species is difficult, the results indicate an important point, which is the possibility of using the feet of dead birds for wildlife forensic investigation purposes. In this work, feet were used to assess the exposure of migratory songbirds that are wintering in agricultural areas, to OPPs which are applied widely in agricultural areas as an insecticide and acaricide. The feet of dead birds can provide valuable information about the short term (acute) or even long term dermal exposure to pesticides; however, the possibility of detection depends on the persistence of compounds in animal tissues (skin).

Finally, the results cannot be used for a "weight of evidence" approach, and we cannot confirm the environmental risk of any detected compound in feet or any part of the body of migratory songbirds. But, as the exposure of chlorpyrifos residue was confirmed in this work, migratory songbirds would be at risk of mortality losses due to being exposed to highly toxic compounds such as chlorpyrifos, which might lead to acute toxicity incidences (for a review, see Mineau 2004; 2005). The results can also be used to increase our understanding of dermal exposure, and how this exposure route can be of as much importance as dietary intake.

4.2 Future directions

There is a paucity of data on currently used pesticide exposure for a great number of migratory songbirds associated with farmlands. Dermal exposure to pesticides seems to be of importance, as it was confirmed in the results from this work. We monitored a limited number of OPPs, and we recommend expanding the number of analytes by including other plant protection products such as fungicides and herbicides. This will give an overall view on the actual diversity of chemicals that may affect wild birds.

Skin permeability data of avian species is absent. There are many limitations to conducting *in vivo* dermal studies on avians, such as lack of facilities and finding a surrogate species. The determination of absorption mechanism(s) in dermal exposure is still a challenging task. We would also recommend (in the case of wild bird specimens) analyzing other exterior compartments of the bird body, such as skin, feathers and even the interior compartments such as the liver and brain. This might help to address the possible distribution behavior of the compounds that are assumed to be absorbed via feet.

We also recommend supporting studies into avian exposure to pesticides in wintering habitats by ensuring adequate, conclusive and accessible data on both pesticide usage on crops, and bird–crop interactions. These data will provide researchers with sufficient information to build up integrated ecological estimation models of the risk of chemicals to wild birds.

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Appendix 1

Organochlorine pesticides analysis-preliminary results

In order to investigate the usefulness of songbirds feet as a matrix for analyzing OC pesticides, the methanol fractions that were used for OPPs analysis in LC/MS/MS, were combined for each species. These combined fractions were then injected in GC/MS for organochlorine pesticides analysis.

In short, the methanol solvent was switched to TMP (2,2,4-Trimethylpentane), then 50 ul of Internal standard of the following compounds (1,2,4,5-Tetrachlorobenzene; 1,2,3,4-Tetrachlorobenzene; Pentachlorobenzene; alpha-Hexachlorobenzene; Hexachlorobenzene; beta-Hexachlorocychlohexane; gamma-Hexachlorocychlohexane; Octachlorostyrene; Heptachlor epoxide; Oxychlordane; t-chlordane; c-chlordane; t-Nonachlor; p,p'-DDE; Dieldrin; p,p'-DDD; c-Nonachlor; p,p'-DDT; and Mirex) was added as a mixture.

Then, all TMP-based fractions (combined fractions into one vial for each species) were injected into GC/MS using an established temperature program. The samples were run with blanks to monitor any possible cross-contamination from the original method (See chapter 2). The overall results indicated that some OC pesticides (data not shown); those were beta-Hexachlorocychlohexane; gamma-Hexachlorocychlohexane; Heptachlor epoxide; t-Nonachlor; p,p'-DDE; p,p'-DDT and c-Nonachlor were at detectable levels among the majority of birds species. However, beta-Hexachlorocychlohexane; gamma-Hexachlorocychlohexane; and Heptachlor epoxide were detected at low concentration in the blanks.

As the sample and lipid weight were not taken into account for calculations; these results are considered as preliminary qualitative results. The results show how birds feet could be used as a

matrix for not only current use pesticides such as OPPs but also for legacy OC pesticides as well as the ability of the developed method to be used for both OPPs and organochlorine pesticides analysis. Further studies are required to accurately measure OC pesticides levels in feet and to compare the OC pesticides levels in feet to some conventional biological matrices such as liver, blood, muscle, and eggs to understand whether feet would be a suitable matrix and give valuable information for short/long-term exposure to OC pesticides or not.

It should be noted that these preliminary results do not confirm that OC pesticides levels in feet are predictive of the risk of these pollutants to birds, and more studies are needed for a definitive conclusion.