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Quantification of low levels of organochlorine pesticides using small volumes (≤100 μl) of plasma of wild birds through gas chromatography negative chemical ionization mass spectrometry

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This technique allows small birds to be used as indicators of chemical contamination in habitats because pesticides can be quantified in very small volumes of plasma.

Abstract

A solid phase extraction and gas chromatography with negative chemical ionization mass spectrometry in scan mode (GC-NCI-MS) method was developed to identify and quantify for the first time low levels of organochlorine pesticides (OCs) in plasma samples of less than 100 μl from wild birds. The method detection limits ranged from 0.012 to 0.102 pg/μl and the method reporting limit from 0.036 to 0.307 pg/μl for α, γ, β and δ-hexachlorocyclohexane (HCH), heptachlor, aldrin, heptachlor epoxide, endosulfan I, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE), dieldrin, endrin, endosulfan-II, endrin-aldehyde and endosulfan-sulfate. Pesticide levels in small serum samples from individual *Falco sparverius*, *Sturnella neglecta*, *Mimus polyglottos* and *Columbina passerina* were quantified. Concentrations ranged from not detected (n/d) to 204.9 pg/μl for some OC pesticides. All levels in the food web in and around cultivated areas showed the presence of pesticides notwithstanding the small areas for agriculture existing in the desert of Baja California peninsula.

Keywords: Avian plasma; SPE; HLB-urea extraction; GC-NCI-MS; Organochlorine; Baja California Sur

1. Introduction

The use of synthetic pesticides for human health and food production occurs throughout the world. Over 800 active pesticide ingredients were formulated in approximately 21,000 commercial products in the USA during 1996–1997 (Aspelin and Grube, 1999). The tremendous usage of pesticides has also promoted toxicological studies that have demonstrated the negative effects of pesticides in both wildlife and humans, particularly organochlorine compounds (Blus, 1996; Fyfe et al., 1988; Grasman et al., 1996, 1998). Pesticides were linked to decreasing reproduction success of wild birds (eggshell thinning and endocrine disruption), behavioral changes, a reduced food supply (insects) (Fry, 1995; Grasman et al., 1996) and immunosuppression (Grasman et al., 1996, 1998). Declining bird population was one of the reported major effects of pesticide usage. In response, methods were developed to detect increasingly lower levels of pesticides and to monitor environmental health status. Sophisticated analytical techniques such as gas chromatography with electron capture or

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mass spectrometry detection have been used for the determination of organochlorine compounds. However, extraction and clean-up methods used for sample treatment play an important role and can influence the results provided by the instrumental techniques. Soxhlet extraction, sonication with solvent, solid phase microextraction (SPME) and solid phase extraction (SPE) using various single sorbents or sorbent combinations are used to extract pesticides. Clean up of the extract often requires columns filled with silica gel, alumina or Florisil or treatment with sulfuric acid prior to quantification of OCs pesticides (Aprea et al., 2002; Atuma and Aune, 1999; Brock et al., 1996; Conka et al., 2005).

Many wildlife populations such as fishes and birds have been studied to monitor known environmental problems to determine the environmental stressors (Dauwe et al., 2003; Guruge et al., 2000; Kendall, 1993). When selecting the best avian species for monitoring, consideration is given to the type and size of samples, which are often based on the proposed extraction and quantification methods. The majority of methods reported in the literature are based on the use of tissues such as muscle, liver, and lipid, which in the absence of viable carcasses, require sacrifice of the subject or blood volumes of 1 ml or larger (Auman et al., 1997; Elliot and Norstrom, 1998; Gómara et al., 2004; Mora et al., 1993). Blood represents the non-destructive sample of choice (Capen and Leiker, 1979), but sampling of smaller species is often restrictive of the metabolic test that can be run or the suite of analytes that can be monitored, e.g. in some passerines the maximum amount of blood which can be obtained without damaging the individual is 100 μl.

In the present work, we describe a method involving quantitative solid-phase urea extraction coupled with gas chromatography with negative chemical ionization mass spectrometry for the determination of OC pesticides (α-HCH, γ-HCH, β-HCH, δ-HCH, heptachlor, aldrin, heptachlor epoxide, endosulfan I, *p,p'*-DDE, dieldrin, *p,p'*-DDT, endrin, endosulfan II, endrin aldehyde and endosulfan sulfate), at low pg/μl concentrations in small plasma volumes of wild birds. To our knowledge, this is the first time a simple, non-lethal method has been reported to sample and determine OC pesticides in small volumes (≤100 μl) of wild bird plasma. We also present for the first time information on organochlorine pesticide levels in wild birds of American Kestrel, Western Meadowlark, Hooded Oriole, Northern Mockingbird, and Common Ground Dove, mainly captured in cultivated areas of Baja California Sur, México.

2. Experimental

2.1. Study area and sample collection

The avian plasma samples were collected during field trips in 2000–2005, from central Baja California Sur, México (24°50′–25°30′N; 111°30′–112°10′W). Agriculture began in the study area in the 1950s. The agricultural area covers approximately 560 km^2 and supports alfalfa, corn, sorghum, garbanzo, beans, and orange trees among others, and is bordered by sarcocaulescent scrub vegetation (Wiggins, 1980). Pesticides have been intensively applied to crop fields and although crop application of some OCs is prohibited in Mexico, their use was allowed only to control malaria and dengue (CICOPLAFEST, 2004). After 2002, the Pan American Health Organization (OPS) and Secretaría de Salud (México) agreed on the prohibition of the use of 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT) for sanitation campaigns using piretroids and organophosphorus pesticides instead of DDT. The Santo Domingo (1 and 2) sampling areas are crop fields located in the Valle de Santo Domingo. The area was intensively used to grow cotton, and DDT was extensively used. The Chametla (6 km from La Paz) sampling area has cattle pastures, which are a constant supply of water for the wildlife. The pastures are 3–5 km away from the crop fields.

Small passerines and medium sized wild birds were mist-netted or balchatris trapped. Small blood samples (0.1–0.8 ml) were taken from the brachial or jugular vein of each bird from the American Kestrel (average weight 91 g; preys on insects, small reptiles, birds and mammals), Western Meadowlark (average weight 59 g; preys on insects), Hooded Oriole (average weight 26 g; preys on insects), Northern Mockingbird (average weight 48 g; preys on insects) and Common Ground Dove (*Columbina passerina*; average weight 39 g; seed-eater). Heparinized syringes or microcapillary tubes were used for collecting the blood. Blood was then transferred into tubes previously washed with lithium heparin to prevent coagulation. All birds were marked to prevent recapture and freed after blood collection. The blood samples were kept at 0–4 °C in cool containers and transported to the laboratory of Centro de Investigaciones Biológicas del Noroeste (CIBNOR); they were then centrifuged at 3820 rpm for 15 min at 4 °C. The plasma was stored in glass vials and frozen at −80 °C. All OC chromatography analyses were performed at the Environmental Protection Agency in Athens, Georgia.

2.2. Sample extraction

2.2.1. Hexane extraction

Each wild bird plasma sample was brought to room temperature and a 100 μl Hamilton® syringe was used to transfer an aliquot (≤100 μl) to a 1.8 ml vial and mixed with 100 μl of hexane. Approximately 0.1 g of anhydrous sodium sulfate was added to the tube to aid in breaking the emulsion. The vial was vortexed gently for 1 min, and then centrifuged until the phases were well defined. The upper part (~80 μl) of the hexane layer was transferred to a 250 μl glass insert in a 2 ml auto sampler vial.

2.2.2. HLB-urea extraction

The wild bird plasma samples were extracted improving the HLB-urea serum extraction used by Sundberg et al. (2006) for chicken serum with modifications that simplified the process and saved time. The wild bird plasma samples were brought to room temperature and a 100 μl Hamilton® syringe was used to transfer an aliquot of each sample to a 5 ml vial that contained a stirring bar. Deionized water was added to bring the final volume to 1 ml. Before extraction the plasma proteins were denatured with a 500 mg portion of urea (~8 M), and the mixture was stirred gently for 30 min. The plasma–water samples were transferred to a 1 cc/30 mg precleaned Oasis® HLB extraction cartridge (Part No. WAT094225), mounted in a SPE-12G glass vacuum manifold (J.T. Baker No. 7018-00). The cartridges were conditioned by eluting three times with 1 ml of methanol, and rinsed three times with 1 ml of deionized water and a vacuum applied to remove residual water. A low vacuum (~13 mmHg), was applied to pull the diluted plasma sample through the cartridge at low flow. The plasma sample vial was rinsed once with 1 ml of deionized water which was also passed through the cartridge. The cartridge was washed three times with 1 ml of deionized water and a vacuum was applied for 10 min to remove residual water. OCs were then eluted with 1 ml of dichloromethane (Burdick and Jackson, GC^2, USA), and extracts were collected in 2 ml vials. The extract was adjusted to 50 μl using a 50 μl Hamilton® syringe and transferred to an insert (HP 5181-1270) and this into a 2 ml autosampler vial (HP 5182-0714).

2.2.3. Method validation and recovery efficiency in small volumes (25, 50, 100 and 1000 μl)

A commercial chicken serum (Sigma C5405) was used to evaluate the recovery efficiency. A stock solution of fortified commercial chicken serum (2 pg/μl of each OC) was prepared by transferring a 50 μl aliquot of a commercial mixture of 16 OC pesticides (200 μg/ml, EPA Method

608; cat. no. 32022; Restek, USA) to a 12 ml sample using a Hamilton® syringe, allowing the solvent to evaporate by natural convection, and adding 5 ml of chicken serum. The serum was stirred gently for 1 h to ensure dissolution of the pesticides from the glass and left in the refrigerator overnight. The final serum concentration of 608 pesticide mix standard was 2 pg/μl. Prior to extraction, the serum was equilibrated to room temperature and stirred an additional 0.5 h before transferring quadruplicate aliquots (25, 50, 100 and 1000 μl) to 4 ml vials that contained small stirring bars. A spacer was placed under each vial to prevent heat transfer. After 30 min, water was added to each aliquot to make the final volume 1 ml in each vial. The samples extracted with urea were obtained as described in Section 2.2.2; with the exception that two aliquots at each volume were extracted without addition of urea, all final dichloromethane volumes of each extract were made to be equal to the volume of the extracted serum (i.e. if 25 μl of serum was extracted the final extract volume was 25 μl). This ensured the same OC concentration in all volumes and allowed a direct comparison of *m/z* abundance between the extracts and the 2 pg/μl standard. The 25, 50, 100 and 1000 μl urea and non-urea extracts (1 μl) were injected in duplicate interspersed with 23 injections of a 2 pg/ μl 608 pesticide mix standard in dichloromethane.

2.3. GC-ECD and GC-NCI-MS

2.3.1. GC-ECD

The hexane extracts of plasma samples were analyzed on a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a ^{63}Ni electron capture detector (ECD) and 7673 autosampler (Palo Alto, CA, USA). A DB-5 column (J&W Scientific, Folsom, CA), 30 m × 0.25 mm ID; 0.25 μm film thickness was used. The column temperature was programmed as follows: 100 °C, held for 1 min and programmed at 4 °C/min to 250 °C and held for 2 min. A 1 μl aliquot of the hexane extract was injected in the splitless mode for 1 min. A calibration standard (DDX) that contained *o,p*-DDT, *p,p'*-DDT, *o,p*-DDE, *p,p'*-DDE, *o,p*-DDD, and *p,p'*-DDD was also injected on the GC-ECD.

2.3.2. GC-NCI-MS

The HLB-urea extracts were analyzed on an Agilent Technologies (Palo Alto, CA) 6890 GC equipped with a 5973N mass selective detector (MSD) that was operated in the negative chemical ionization mode (NCI) with methane reagent gas. Compound identification was performed on a J&W Scientific (Agilent Technologies) DB-5 capillary column, 30 m × 0.25 mm ID; 0.25 μm film thickness. Quantification of OCs was on a Restek (Bellefonte, PA, USA) Rtx-CLPesticides2 capillary column, 30 m × 0.32 mm ID; 0.25 μm film thickness. Injector and interface temperatures were set at 250 °C and 290 °C, respectively. The column temperature was programmed as follows: 100 °C, held for 1 min and programmed at 4 °C/min to 250 °C and held for 2 min. The carrier gas was helium at a constant flow of 1 ml/min. The MSD operating parameters were set by the tune file. EM volts were set at +400 tune volts. Source temperature was set at 130 °C and the quadrupoles at 106 °C. The GC-MSD was programmed to scan ions from *m/z* 30 to 500. A selected ion monitoring (SIM) program was constructed in which quantifying ions and qualifying ions were separated into groups based on elution times of identified OC pesticides, fragment ions are listed in Table 1. The higher masses in Table 1 are the most abundant *m/z* ions in chlorine isotope clusters of the pesticides. The SIM method detection limit (MDL) was determined for each pesticide from seven replicate injections of a 0.2 pg/μl 608 and computed as the product of the standard deviation and the critical *t*-value (3.14). Based on the 0.2 pg/μl signal to noise (S/N) ratio, e.g. γ-HCH (47), aldrin (32), endosulfan I (156), and *p,p'*-DDE (49), the method reporting limit (MRL) was set at three times the method detection limit (MDL). Three calibration tables were used: 0–2 pg/μl, 2–20 pg/μl, and 20–200 pg/μl and were used to calculate the concentrations of pesticides in the plasma extracts. Injections of the 2 pg/μl standard were interspersed with injections of the plasma extracts.

The presence of overlapping *m/z* 35 and *m/z* 37 peaks in the natural abundance ratio 3:1 was good evidence of a chlorinated substance presence. Individual scans and the software average of 8–12 scans of each chlorine containing peak in the TIC were interpreted by comparison with a NCI-MS spectral peak index for 361 environmental contaminants and related compounds (Stemmler and Hites, 1988) and with a pesticide spectral library that was created on the GC-NCI-MS by injecting reference standards of OC pesticides.

The pesticide identification in TICs was based on: a retention time match with a pesticide standard, the EIC plots for *m/z* 35 and *m/z* 37 and matching chlorine ion clusters in the TIC plasma extract compared to the pesticide standard. While endosulfan I and II and endosulfan sulfate generate a negligible abundance of *m/z* 35 and 37, endosulfan I and II exhibit abundant ion clusters at *m/z* 406, 372, 270 and 242 and endosulfan sulfate has significant abundance of *m/z* 97 (HSO_4^-) and abundant chlorine clusters *m/z* 422, 386 and 352. Likewise, the abundance of *m/z* 35 and 37 is low for the HCH isomers that are characterized by intense *m/z* ions at 253 and 255 $(M - Cl)^-$ and 71 and 73 (HCl_2).

2.4. Analysis of wild bird plasma samples

Eight of the 15 wild bird samples analyzed by the hexane extraction method (Section 2.2.1), with enough plasma samples, were also processed as indicated in HLB-urea extraction (Section 2.2.2) and quantified as in Section 2.3.2. The results were then compared between them.

A set of 49 Common Ground Dove 2001–2005 plasma samples of different volumes (6–100 μl), were analyzed by HLB-urea extraction (Section

Table 1
Ions used for single ion monitoring (SIM) pesticide identification and quantification by GC-NCI-MS analysis

Pesticide	Ions *m/z*[a]	SD	MDL (pg/μl)[b]	MRL (pg/μl)[c]
α-HCH	35, 37, 71*, 73, 253, 255	0.008	0.026	0.078
γ-HCH	35, 37, 71*, 73, 253, 255	0.007	0.023	0.069
β-HCH	35, 37, 71*, 73, 253, 255	0.005	0.015	0.045
δ-HCH	35, 37, 71*, 73, 253, 255	0.027	0.084	0.252
Heptachlor	35, 37, 237, 266, 300*	0.033	0.102	0.307
Aldrin	35, 37, 237, 330*	0.018	0.056	0.168
Heptachlor epoxide	35, 37, 237, 318, 388*, 390	0.016	0.051	0.154
Endosulfan I	35, 37, 370, 372, 404, 406*	0.027	0.027	0.081
p,p'-DDE	35, 37, 283, 316, 318*	0.039	0.039	0.117
Dieldrin	35, 37, 237, 346, 380, 382*	0.042	0.042	0.126
Endrin	35, 37, 239, 272, 308, 346, 380	0.004	0.012	0.036
Endosulfan II	35, 37, 370, 372, 404, 406*	0.006	0.019	0.056
p,p'-DDT	35, 37, 71, 283, 316, 318	0.019	0.059	0.178
Endrin aldehyde	35, 37, 272, 306, 346, 380	0.011	0.036	0.107
Endosulfan sulfate	35, 37, 71, 97, 352, 384, 386, 422*	0.017	0.054	0.163

[a] SIM ions; ions marked with an asterisk were quantification ions.
[b] Method detection limit.
[c] Method reporting limit.

2.2.2), but in this case the final extract volume was adjusted to 500 μl using a 500 μl Hamilton® syringe and quantified by GC-NCI-MS (Section 2.3.2), in order to make a validation of the method in wild bird small plasma samples. Pesticide concentrations obtained were compared among plasma samples of different volumes.

Quantification was done based on comparison of peak areas of the sample to the response of a calibration made with different concentrations of standard injection.

2.5. Statistical analysis

The recoveries of pesticides were compared among different volumes of serum (25, 50, 100 and 1000 μl) and treatment with and without urea (one-way ANOVA). A Tukey multiple comparisons procedure was used to determine significant differences in mean values of concentration recoveries and effect of urea denaturation. Pesticide concentrations were compared among different plasma volumes of common ground doves using a one-way ANOVA-GLM. A GLM simple regression was performed to estimate the correlation between pesticide concentration and the different plasma volumes analyzed. All statistical analyses were performed using Statistica 6.2 software. The level of significance was set at 0.05.

3. Results

3.1. Hexane extraction

The hexane extracts of avian plasma were analyzed by GC-ECD and each of the chromatograms contained one to four major peaks. The retention times of *o,p'*-DDT and *p,p'*-DDT and the two corresponding DDE and DDD degradation products (DDX) were compared with peak retention times in the plasma chromatograms and possible matches for *p,p'*-DDE, *o,p*-DDT, and *p,p'*-DDT were observed in several of the extracts. Hexane extracts were co-injected with the DDX mixture, and *p,p'*-DDE was the only component of the DDX calibration mixture that co-eluted with a plasma peak in 9 of the 15 extracts. The peak shape and retention time of the tentatively identified *p,p'*-DDE peak was not changed and the unknown peak area increased in proportion to the added *p,p'*-DDE. The *p,p'*-DDE concentrations in Table 2 were calculated based on comparison of the peak areas of the sample to the response of *p,p'*-DDE standard. The concentration of *p,p'*-DDE in the plasma samples ranged from 1 to 69 ng/ml (Table 2). Nine of the 15 samples (60%) analyzed showed the presence of at least 1 pg/μl of *p,p'*-DDE. The highest concentration was found in the Western Meadowlark using 38 μl of plasma.

3.2. HLB-urea extraction and quantification of OC pesticides in wild bird avian plasma

To detect elution of compounds that contained chlorine, the HLB-urea extracts were analyzed by GC-NCI-MS in scan mode (*m/z* 30–500) to generate a total ion chromatogram (TIC). Extracted ion chromatograms (EIC) for *m/z* 35 and *m/z* 37 (two isotopes of chlorine) were plotted for each plasma extract IC (Fig. 1). One or more of the following pesticides were identified in each of the eight wild birds plasma extracts (Section 2.4, Fig. 2): *p,p'*-DDE, α-, β-,γ-, and δ-HCHs, heptachlor, aldrin, heptachlor epoxide, endosulfan I and II, endosulfan sulfate, endrin, endrín aldehyde and dieldrin, concentrations are reported in Table 3. The metabolite of *p,p'*-DDT (*p,p'*-DDE) was detected in all of the plasma samples and in a higher concentration compared to those detected by hexane extraction even using a higher volume of plasma sample (Table 2). The highest concentration was found for dieldrin in the American Kestrel sample no. 4 from the field crop area near La Paz city.

3.3. Recovery efficiency of OC pesticides from fortified small volumes (25, 50, 100 and 1000 μl) of serum

The percent recovery of each OC from 25, 50, 100 and 1000 μl of the chicken serum fortified at 2 pg/μl 608 pesticide

Table 2
Levels of *p,p'*-DDE found using hexane extraction in plasma samples of American Kestrel (*Falco sparverius*), Western Meadowlark (*Sturnella neglecta*), Hooded Oriole (*Icterus parisorum*) and Northern Mockingbird (*Mimus polyglottos*) near fields crops in Baja California Sur

Plasma number	Date sampling	Location[a]	Avian species	Hexene extraction		HLB-urea extraction	
				μl plasma	*p,p'*-DDE (pg/μl)	μl plasma	*p,p'*-DDE (pg/μl)
1	2001	1	*F. sparverius*	100	n/d	50	1.21
2	2001	1	*F. sparverius*	95	5 ± 0.02[b]	15	7.80
3	2000	A	*F. sparverius*	100	n/d	50	1.15
4	2001	3	*F. sparverius*	100	1 ± 0.03	25	3.71
5	2000	2	*F. sparverius*	40	n/d		
6	2001	2	*S. neglecta*	38	69 ± 0.33		
7	2000	2	*S. neglecta*	87	n/d		
8	2001	2	*S. neglecta*	98	6 ± 0.03		
9	2001	2	*S. neglecta*	100	24 ± 0.22	100	131.71
10	2001	2	*S. neglecta*	82	25 ± 0.58		
11	2001	2	*S. neglecta*	100	8 ± 0.06	50	28.78
12	2001	2	*I. parisorum*	48	n/d		
13	2001	2	*I. parisorum*	62	n/d		
14	2001	2	*M. polyglottos*	100	1 ± 0.01	50	141.65
15	2001	2	*M. polyglottos*	100	1 ± 0.11	40	147.59

[a] (1) Santo Domingo 1, road near sorghum and corn field crop; (2) Santo Domingo 2 near alfalfa field crop; (3) Chametla cattle pasture; (A) remote natural vegetation area. n/d, not detected.

[b] Average and deviation of duplicate injections of the hexane extract.

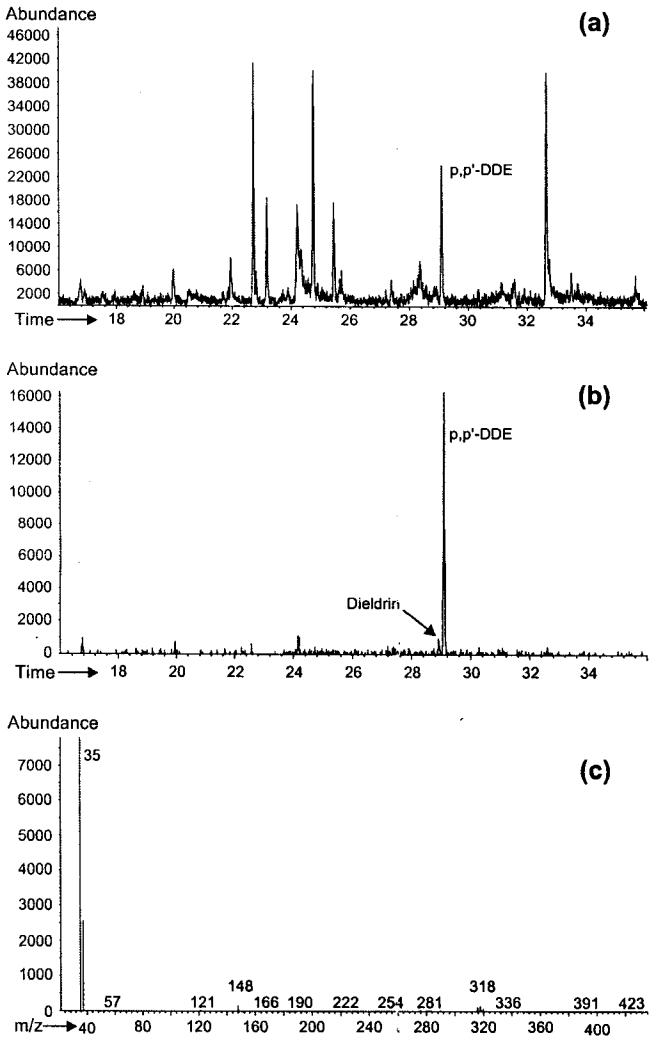

Fig. 1. Western Meadowlark no. 11 plasma sample. (a) Scan run (*m/z* 30–550), DDE eluted at 29.09 min, (b) extracted ion plot of *m/z* 35 from the same scan, (c) averaged mass spectra of the elution time for DDE. See Section 3.2.

mix standard is reported in Table 4. The recoveries on the same sample volume without the use of urea to denature the protein (Table 4) were lower and statistically different to the those prepared with urea except for γ-HCH ($F = 2.29$; df = 7, $p = 0.06$, marginally significant). Statistical differences ($p < 0.05$) in recoveries were found between the 25 μl volume and the other volumes in α-HCH ($F = 5.73$, df = 3, $p = 0.01$), β-HCH ($F = 5.77$, df = 3, $p = 0.01$), δ-HCH ($F = 5.89$, df = 3, $p = 0.01$), heptachlor epoxide ($F = 5.78$, df = 3, $p = 0.01$), endosulfan I ($F = 6.41$, df = 3, $p < 0.01$), *p,p'*-DDE ($F = 7.31$, df = 3, $p < 0.01$), endrin ($F = 8.94$, df = 3, $p = 0.01$), endosulfan II ($F = 5.20$, df = 3, $p = 0.01$), endrin aldehyde ($F = 4.05$, df = 3, $p = 0.03$), endosulfan sulfate ($F = 0.25$, df = 3, $p < 0.01$).

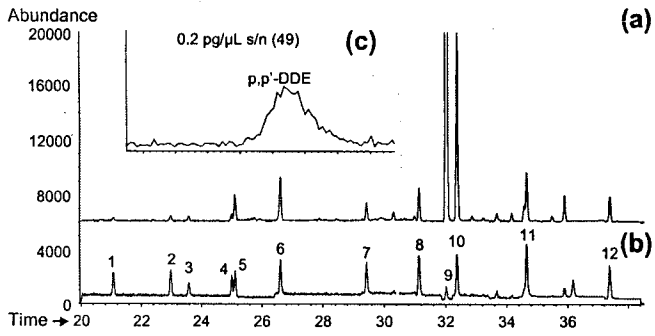

Fig. 2. SIM chromatograms of plasma no. 9 (a) and 2 pg/μl 608 standard mix (b). See Table 3 for name of numbered peaks. (c) SIM signal-to-noise check for *p,p'*-DDE in 0.2 pg/μl standard.

The levels of pesticides in the Common Ground Dove plasma samples collected in crop fields of Baja California Sur ranged from not detected (n/d) to 204.9 pg/μl. Fig. 3 shows the concentrations of *p,p'*-DDE, and dieldrin, which were detected in all plasma samples, plotted against the plasma sample volumes of the 49 samples. Table 5 summarizes the results of three plasma sample sizes (25, 50 and 100 μl). No statistical differences were found between concentrations detected related to plasma volume size (ANOVA test, $p > 0.05$). No correlation was found between the volume of plasma sample and concentrations detected for any of the pesticides (range of r for pesticides 0.118–0.390, $p > 0.05$). Some individuals showed high concentrations of a particular pesticide, like one dove with 204.9 pg/μl of aldrin. Although we found 15 of 16 pesticides in this Common Ground Dove plasma sample, aldrin was two orders of magnitude higher than the other pesticide concentrations in all other common Ground Doves.

4. Discussion

Factors that confound the identification and quantification of pesticides in wildlife samples include low extraction efficiency of the analytes, incomplete removal of matrix components in clean up steps, and degradation of unstable compounds, e.g. when sulfuric acid is used for lipid removal. Thus, we needed a fast extraction and clean up method that would enable the processing of a large number of small-volume avian plasma samples. We successfully developed such a method for wild birds. First, it was reported that protein denaturation of chicken serum with urea yielded high recoveries of OC pesticides from serum fortified at 10–400 pg/μl without clean up by GC-ECD (Sundberg et al., 2006). Second, the concentrations of OC pesticides in wildlife plasma samples are most often in the low or sub-pg/μl range. Thus, we improved and developed a new technique for wild birds using small volumes of plasma. GC-MS operated in the negative chemical ionization mode has proven to be a valuable tool for the identification and quantification of low levels of pollutant with high confidence (Aprea et al., 2002; Conka et al., 2005; Covaci and Schepens, 2001; Gómara et al., 2002; López et al., 2001). When the extraction of OCs was made by hexane extraction, the concentrations of *p,p'*-DDE determined are similar to the results from other methods using 1 ml of human fortified serum sample and extraction with hexane or hexane–acetone (Lino et al., 1998), and although other organochlorine pesticides were not initially identified using this first method,

Table 3
Organochlorine levels (pg/μl), found in American Kestrel (samples 1–4), Western Meadowlark (samples 9 and 11), and Northern Mockingbird (samples 14 and 15), near crop fields in Baja California Sur using HLB-urea extraction (n/d, not detected)

Pesticide		Sample number (μl of serum)							
		1 (50)	2 (15)	3 (50)	4 (25)	9 (100)	11 (50)	14 (50)	15 (40)
α-HCH	1	n/d	n/d	n/d	n/d	0.2	n/d	n/d	n/d
γ-HCH	2	0.4	n/d	0.2	n/d	0.5	n/d	n/d	n/d
β-HCH	3	0.7	n/d	0.3	n/d	1.6	n/d	n/d	n/d
δ-HCH	4	0.7	n/d	0.4	n/d	0.8	n/d	n/d	n/d
Heptachlor	5	0.7	1.3	0.5	2.1	1.6	0.2[a]	2.3	2.4
Aldrin	6	0.9	1.0	0.5	2.9	2.5	0.3	2.7	2.8
Heptachlor epoxide	7	0.4	n/d	0.3	0.4	1.1[a]	0.2	0.1[a]	0.2
Endosulfan I	8	0.5	0.4	0.3	0.5	1.2	0.3	0.2	0.3
p,p′-DDE	9	1.2	7.8	1.2	3.7	132	29	5.2	6.1
Dieldrin	10	24	56	26	153	32	5.8	142	148
Endosulfan II	11	0.7	0.7	0.4	1.0	1.8	0.5	0.3	0.4
Endosulfan sulfate	12	0.4	0.3	0.3	0.8	1.2	0.3	0.1[a]	0.1[a]

[a] Greater than MDL but less than MRL.

this step suggested that the extraction and quantification of pesticides in small plasma volumes was feasible. As explained before the detection of halogenated compounds by negative chemical ionization is more sensitive by at least an order of magnitude when compared to electron ionization mass spectrometry.

Our results confirm that the dilution of bird samples to 1 ml using deionized water and adding urea helps to reduce the matrix interference of plasma compounds and increases pesticide recovery using HLB cartridge. The combination of an efficient extraction method and a detector with greater selectivity and sensitivity allows the identification and quantification of low levels of OCs pesticides in small volumes of wild bird plasma.

Summarizing, organochlorine pesticides (α-HCH, γ-HCH, β-HCH, δ-HCH, heptachlor, aldrin, heptachlor epoxide, endosulfan I, *p,p′*-DDE, dieldrin, *p,p′*-DDT, endrin, endosulfan II, endrin aldehyde and endosulfan sulfate) can be both identified and quantified successfully even in volumes of plasma of wild birds as low as 15 μl by using this new technique.

The detection of the metabolite of *p,p′*-DDT (*p,p′*-DDE) in all of the plasma samples was anticipated as *p,p′*-DDE is found in the majority of human and wildlife plasma samples (Jarman et al., 1994; Glynn et al., 2000; Gómara et al., 2004). The more unstable metabolite dieldrin was also detected in all wild bird plasma samples and in six samples, the dieldrin concentration was higher than *p,p′*-DDE levels. The detection of aldrin in all of the plasma samples was also noted. Aldrin is rapidly metabolized to dieldrin in biological organisms. Thus, the detection of aldrin is an indication of recent exposure. During fieldwork we found empty bottles of endosulfan and lindane around crop fields; our results confirm their presence in wild bird plasma samples. A recent report also suggests that OCs (DDTs) are currently used in the area because they were found in the liver of Common Ground Doves and House Sparrow *Passer domesticus* (Jiménez et al., 2005). We wanted to compare our results with other studies of birds from agricultural environments but we found that a comparison of the results and concentration values with other studies was not feasible and even not recommended because we could easily fall into speculation. The methods are different and most have used tissues for pesticide

Table 4
Pesticide recovery efficiency from chicken serum samples, 2 pg/μl 608 mix standard (duplicate injections of duplicate aliquots)

Pesticide	Percent recoveries							
	1 ml (mean ± SD)		100 μl (mean ± SD)		50 μl (mean ± SD)		25 μl (mean ± SD)	
	Urea	Without urea	Urea	Without urea	Urea	Without urea	Urea	Without urea
α-HCH	71 ± 5.0	48 ± 11.3	44 ± 4.6	37 ± 2.4	63 ± 14.6	46 ± 7.4	48 ± 13.3	45 ± 13.7
γ-HCH	84 ± 8.8	64 ± 18.7	63 ± 1.3	56 ± 5.7	68 ± 2.2	66 ± 6.3	87 ± 27.7	77 ± 20.6
β-HCH	119 ± 3.4	88 ± 25.7	108 ± 15.3	108 ± 9.6	132 ± 11.1	108 ± 10.5	200 ± 66.3	144 ± 28.1
δ-HCH	126 ± 8.6	98 ± 20.2	136 ± 2.8	113 ± 12.0	138 ± 7.1	131 ± 12.6	195 ± 50.1	175 ± 26.8
Heptachlor	63 ± 12.0	25 ± 7.6	53 ± 2.6	51 ± 4.7	69 ± 2.8	57 ± 7.8	67 ± 20.5	49 ± 10.5
Aldrin	74 ± 6.9	24 ± 5.1	54 ± 4.8	50 ± 3.6	74 ± 37.3	55 ± 6.6	73 ± 15.2	57 ± 11.7
Heptachlor epoxide	93 ± 11.8	62 ± 12.9	99 ± 3.8	88 ± 14.1	119 ± 20.1	118 ± 30.9	226 ± 100.5	174 ± 31.1
Endosulfan I	98 ± 5.7	66 ± 12.7	77 ± 3.0	67 ± 2.6	82 ± 2.7	74 ± 2.4	116 ± 26.9	95 ± 15.3
p,p′-DDE	89 ± 6.0	36 ± 4.9	78 ± 5.4	77 ± 6.9	96 ± 15.9	98 ± 8.1	139 ± 35.7	151 ± 32.9
Dieldrin	81 ± 7.5	48 ± 10.3	81 ± 2.5	70 ± 5.1	111 ± 57.8	82 ± 5.4	119 ± 31.2	111 ± 11.8
Endrin	104 ± 12.8	57 ± 12.8	121 ± 0.8	100 ± 17.4	134 ± 19.2	115 ± 10.8	196 ± 48.6	165 ± 29.8
Endosulfan II	99 ± 7.4	61 ± 12.1	89 ± 1.5	74 ± 4.3	99 ± 10.5	86 ± 5.5	140 ± 37.6	115 ± 17.6
Endrin aldehyde	18 ± 2.1	8 ± 2.8	19 ± 2.2	24 ± 2.6	37 ± 11.6	25 ± 17.1	41 ± 20.9	3 ± 1.7
Endosulfan sulfate	100 ± 10.6	68 ± 14.6	94 ± 1.6	83 ± 7.8	103 ± 6.2	46 ± 7.4	153 ± 41	130 ± 16.5

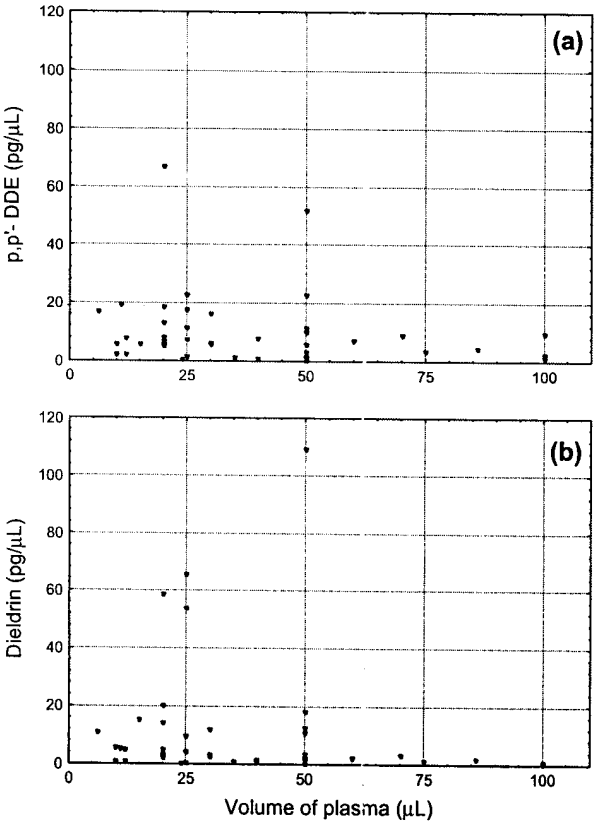

Fig. 3. Concentrations of *p,p'*-DDE (a) and dieldrin (b) in 49 Common Ground Dove plasma samples (6–100 µl).

determination and the sensitivity of the equipment for analysis was different. Also, a comparison of results from individual values with those that present a value from a pool seems to be a mistake. We propose the build of databases for different species that clearly indicate the use of each analytical method. These databases could permit the comparison of pesticide concentrations in different bird species and habitats.

The recovery efficiency of OC pesticides from fortified small volumes (25, 50 and 100 µl) of serum was high even for the 25 µl volume size. The higher recoveries found in the 25 µl volume set seem to be most likely due to loss of dichloromethane. This can be confirmed as we did not find any correlation between the concentration of pesticides and the serum volume. The loss of solvent was also indicated by the increase in response of the second injection by 20–30% for each OC.

Of the 49 Common Ground Dove individuals analyzed to determine recovery efficiency using this method, some showed different concentrations of a particular pesticide, like one dove with 204.9 pg/µl of aldrin. The high level of aldrin detected suggests recent usage of this pesticide which is rapidly metabolized to dieldrin. This is supported by the elevated dieldrin concentrations from 5.8 to 153 pg/µl. The *p,p'*-DDE concentrations ranged from 1.2 to 132 pg/µl. Although we found 15 of 16 pesticides in this Common Ground Dove plasma sample, aldrin was two orders of magnitude higher than the other pesticide concentrations. The 49 samples demonstrated that there can be a large variation in the concentrations of pesticides in individual birds even when they are trapped in the same area and share the same source of food. This variation in concentration strongly suggests the need to sample individual birds without pooling the plasma samples. It is clearly interesting that because this technique improved the sensitivity to detect low levels of persistent pollutants at the individual level, now it is feasible to design biological tests that may help to identify both bird species and individuals with non-lethal responses to different pesticides. That could help for example to better identify species useful for monitoring chemical effects in a system.

Finally, all wild bird species captured mainly in cultivated areas (but also the bird captured in a natural area) showed a diverse array of organochlorine pesticides used in agriculture in Baja California Sur. Some individuals showed pesticides with

Table 5
Organochlorine concentration levels (pg/µl), found in three serum sample sizes of Common Ground Doves using HLB-urea extraction

Pesticide	Serum sample volumes		
	25 µl ($n = 6$)	50 µl ($n = 9$)	100 µl ($n = 4$)
α-HCH	0.163 ± 0.233 (n/d–0.516)	0.119 ± 0.197 (n/d–0.610)	0.031 ± 0.036 (n/d–0.066)
β-HCH	0.238 ± 0.279 (n/d–0.647)	0.489 ± 0.856 (n/d–2.640)	0.032 ± 0.064 (n/d–0.128)
δ-HCH	1.006 ± 2.115 (n/d–4.784)	0.177 ± 0.311 (n/d–0.897)	0.016 ± 0.019 (n/d–0.038)
γ-HCH	0.434 ± 0.757 (n/d–1.752)	0.303 ± 0.618 (n/d–1.865)	n/d
Heptachlor	2.428 ± 3.317 (n/d–6.370)	1.468 ± 1.801 (n/d–4.209)	0.050 ± 0.040 (0.013–0.104)
Aldrin	6.152 ± 8.434 (n/d–16.127)	2.718 ± 4.783 (n/d–14.854)	0.024 ± 0.031 (n/d–0.070)
Heptachlor epoxide	0.714 ± 1.229 (n/d–2.840)	0.619 ± 1.076 (n/d–3.257)	0.006 ± 0.011 (n/d–0.022)
Endosulfan	0.919 ± 1.257 (n/d–2.753)	0.879 ± 1.145 (n/d–3.149)	0.027 ± 0.022 (n/d–0.050)
p,p'-DDE	12.124 ± 8.193 (1.748–22.627)	12.020 ± 16.600 (0.122–52.018)	4.046 ± 3.873 (1.459–**9.807**)
Dieldrin	26.856 ± 30.568 (0.678–**65.778**)	17.868 ± 34.821 (0.191–**109.239**)	0.748 ± 0.081 (0.679–0.857)
Endrin	0.984 ± 1.711 (n/d–3.964)	1.612 ± 2.317 (n/d–5.960)	0.062 ± 0.021 (0.040–0.085)
Endosulfan II	1.364 ± 2.865 (n/d–6.484)	0.751 ± 1.196 (n/d–3.181)	0.015 ± 0.008 (0.008–0.025)
p,p'-DDD	10.471 ± 19.126 (n/d–44.219)	2.751 ± 4.737 (n/d–14.724)	0.082 ± 0.087 (n/d–0.204)
Endrin aldehyde	6.043 ± 9.719 (n/d–22.317)	4.049 ± 10.023 (n/d–30.699)	n/d
Endosulfan sulfate	2.685 ± 4.079 (0.052–9.828)	1.144 ± 1.371 (0.007–3.514)	0.010 ± 0.012 (n/d–0.022)
Endrin ketone	7.496 ± 12.645 (n/d–29.183)	3.362 ± 6.517 (n/d–20.092)	0.065 ± 0.129 (n/d–0.258)

Mean ± SD. Range is presented between parentheses. Extreme values are shown in bold. n/d, not detected.

higher concentration levels than others. That should be carefully monitored because most bird species studied are important in the food webs of the desert of Baja California Sur. Most studied species are insectivorous but also seedeaters and raptor predators were included. It is significant that individuals of all species in and around cultivated areas showed the presence of pesticides notwithstanding the small areas for agriculture existing in the desert of Baja California. Small passerines and medium sized wild birds could be used as indicators of habitat contamination because they will be exposed to the great array of these pesticides at a local and regional scale. These birds are also important in food webs as they are prey of third order predators. It is in top order predator species where biomagnification can be more evident and affect the persistence of populations (Henny et al., 2003). Changes in functional biological systems due to potential effects of OC pesticides can be monitored and then action can be taken to mitigate the effects of OC pesticides in the system.

5. Conclusions

Organochlorine pesticides in low pg/μl concentrations were efficiently extracted from avian plasma with 1cc/30 mg Oasis® HLB SPE cartridges. Dilution of sub-100 μl plasma samples to 1 ml with deionized water enhanced plasma desorption and subsequent partitioning of the OC pesticides to the HLB sorbent. The selectivity and sensitivity of SIM quantification in the NCI mode together with the low background HLB extracts eliminated the necessity of extract clean up and yielded high recoveries of degradation less stable OCs. Even after 2 months of intensive use (>1000 injections of standards and samples) the injection port liner was only slightly discolored and the Rtx-CLPesticides2 column performance had not degraded as measured by the NCI response of the standards, peak shape and retention times. The results show that the method can be successfully applied to the analyses of OC pesticides in wild bird plasma volumes as low as 15 μl. This nonlethal method solves the problems that appear when studying the levels of OCs pesticides in small vertebrates like birds, mammals and reptiles (unpublished data), and enhance population studies in ecotoxicology. OC pesticides are currently used in Baja California Sur. Monitoring of OCs pesticides in this state and in the region will be important because of expected increasing use. Terrestrial wild birds could be used as indicators of habitat quality in relation to chemical contamination regardless of their body size.

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