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Comparison of Pressurized Liquid Extraction and Matrix Solid Phase Dispersion for the Measurement of Semi-Volatile Organic Compound Accumulation in Tadpoles

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Abstract

Analytical methods capable of trace measurement of semi-volatile organic compounds (SOCs) are necessary to assess the exposure of tadpoles to contaminants as a result of long-range and regional atmospheric transport and deposition. The following study compares the results of two analytical methods, one using pressurized liquid extraction (PLE) and the other using matrix solid phase dispersion (MSPD), for the trace measurement of over 70 SOCs, including current-use pesticides, in tadpole tissue. The MSPD method resulted in improved SOC recoveries and precision compared to the PLE method. The MSPD method also required less time, consumed less solvent, and resulted in the measurement of a greater number of SOCs than the PLE method.

Keywords: tadpoles, semi-volatile organic compounds, pesticides, pressurized liquid extraction, matrix phase solid dispersion

Introduction

Declines in amphibian species have been reported worldwide [1-4]. Several factors have been suggested to be responsible for these declines, including climate change, ultraviolet radiation, habitat destruction, introduced species, disease, and contaminants [5-9]. While multiple factors are likely responsible for the declines, among contaminants, pesticide exposure has been suggested to be important [5, 10-20].

Many pesticides are semi-volatile organic compounds (SOCs), and undergo both long-range and regional atmospheric transport and deposition to remote ecosystems [21-26]. Recently, Hageman et al. and Usenko et al. have shown that regional agricultural sources are responsible for a significant portion of the pesticide deposition in remote U.S. mountain ecosystems [22, 25]. Previous studies have linked atmospheric transport and deposition of pesticides in remote areas of the Sierra Nevada Mountains to their proximity to the intensely agricultural Central Valley of California [19, 20, 22, 26-29].

Exposure of amphibians to pesticides and other SOCs occurs in low elevation ecosystems near sources, in high elevation ecosystems, and in other remote ecosystems. Previous studies on amphibian SOC body burdens have focused on measuring a fairly limited number of pesticides in tadpole or frog tissue [17, 19, 20, 27-34]. However, amphibians are likely exposed to a far greater array of pesticides. For example, over 500 different pesticides were applied in 2006 in California alone [35] (http://www.cdpr.ca.gov/docs/pur/pur06rep/06_pur.htm).

In the present study, two analytical methods were compared for the trace measurement of over 70 SOCs, including current-use pesticides and their degradation products, in tadpole tissue. One method used pressurized liquid extraction (PLE)

(referred to as the "PLE Method") and was similar to a PLE method developed for measuring SOCs in fish with a moderate to high lipid content (0.71 – 18 %) [36]. The second method used matrix solid phase dispersion (MSPD) (referred to as the "MSPD Method"). MSPD has been used for the measurement of SOCs in food products, as well as animal samples, including tadpoles and frogs, and is a relatively simple method for the extraction of SOCs from samples with a low to moderate fat content [32, 37, 38]. Because tadpoles have a relatively low lipid content (0.01 – 3.3 %) (unpublished data), MSPD was evaluated as a potential extraction method. In order to assess the current state of tadpole exposure to pesticides at low concentration, the objectives of this research were to develop and validate an analytical method to identify and quantify low concentrations of current-use and historic-use pesticides in tadpole tissue.

Materials and Methods

Chemicals and materials

In the summer of 1999, Pacific chorus frog (*Pseudacris regilla*) and Cascades frog (*Rana cascadae*) tadpoles were collected from lakes, ponds, and creeks in the Cascade Mountain Range in Northern California. In the summer of 2003, *P. regilla* tadpoles were collected from several lakes in Sequoia and Kings Canyon National Park. Tadpoles from both regions were pooled and used for analytical method development and validation.

Tadpoles were placed in cryovials and in liquid nitrogen or on dry ice after collection and during shipment and were stored at -20ºC to -80ºC until analysis. A liquid nitrogen – cooled mortar, CoorsTek 99.5% alumina pestles (100 mm) and sodium sulfate

(Na_2SO_4) was purchased from VWR (West Chester, PA, USA). Octadecylsilyl (C_{18}) (bulk sorbent), empty 60 ml solid phase extraction (SPE) columns, and silica SPE columns (Mega Bond-elut 20 g) were purchased from Varian, Inc. (Palo Alto, CA, USA). Non-labeled SOC standards (**Table 1**) were purchased from Chemical Services (West Chester, PA, USA), Restek (Bellefonte, PA, USA), Sigma-Aldrich (St. Louis, MO, USA), and AccuStandard (New Haven, CT, USA), or obtained from the U.S. Environmental Protection Agency repository [39]. Isotopically labeled chemical standards, including 24 surrogate standards, were purchased from CDN Isotopes (Pointe-Claire, QC, Canada) and Cambridge Isotope Laboratories (Andover, MA, USA) and used for quantificaiton [39]. All chemical standards were stored at 4°C until use. Optima grade solvents (acetonitrile, dichloromethane, hexane, and ethyl acetate) were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Pressurized Liquid Extraction (PLE) Method

The PLE method was used to extract SOCs from tadpole tissue as described in Ackerman et al. 2008 for extracting SOCs from fish tissue [36]. Briefly, 2 grams of frozen, ground tadpole tissue was further ground with 65 g Na_2SO_4 (enough to fill the PLE cell) and the mixture was packed into a 66 ml PLE cell (Dionex, Salt Lake City, UT, USA). In the case of SOC spike and recovery experiments, non-labeled SOC standards (Table 1) were added to the ground sample at the top of the PLE cell prior to extraction to assess SOC recoveries over the entire analytical method. In order to measure and subtract the background SOC concentration in the tadpole tissue (tissue blanks) used in the spike and recovery experiments, the isotopically labeled surrogates were added to the ground sample at the top of the PLE cell prior to extraction. Lab blank experiments

consisted of 65 g Na_2SO_4 without tadpole tissue packed into the PLE cell and spiked with the isotopically labeled surrogates at the top of the PLE cell prior to extraction. The standards, both non-labeled and labeled, were spiked at approximately 150 ng and the PLE conditions used dichloromethane (DCM) at 100ºC, 1500 psi, 2 cycles of 5 min, and 150% flush volume [36] (see **Table 2** for PLE method details). Additional Na_2SO_4 was added to the extracts to remove any remaining water. The extracts were reduced in volume (TurboVap II, Caliper Life Sciences, Hopkinton, MA, USA; 12 psi N_2, 30 ˚ C), solvent exchanged to hexane, purified with silica gel, solvent exchanged to DCM and further purified using gel permeation chromatography (Waters, Milford, MA, USA) [36].

Matrix Solid Phase Dispersion (MSPD) Method

The ground tadpole tissue (2 g) was further ground with C_{18} and Na_2SO_4 in proportions of 1:5:17.5 by weight, respectively. The tadpole to C_{18} ratio was similar to a previously published MSPD method [38] and the Na_2SO_4 ratio was adjusted so that the mixture filled the solid phase extraction column within approximately 2 cm of the top of the column. This tadpole mixture was packed into a 60 ml solid phase extraction column containing 30 g Na_2SO_4. In the case of SOC spike and recovery experiments, non-labeled SOC standards (Table 1) were added to the tadpole mixture on the top of the MSPD column to assess SOC recoveries over the entire analytical method. Tissue blanks and lab blanks were analyzed as described in the PLE method, by spiking the isotopically labeled surrogates on the top of the MSPD column prior to extraction. The standards, both non-labeled and labeled, were spiked at approximately 150 ng. The MSPD column containing the ground tadpole sample, was placed on a vacuum manifold (Supelco, Bellefonte, PA, USA), a vacuum was applied, and the sample was eluted with 300 ml

185 acetonitrile, followed by 100 ml DCM at a flow rate of approximately 25 ml/min (see
186 Table 2 for MSPD method details). The DCM fraction was reduced and stored as an
187 archive fraction. To determine if additional SOCs were eluted from the MSPD column
188 with the DCM, this fraction was analyzed and contained no spiked SOCs. Acetonitrile
189 was chosen as the MSPD column elution solvent because of its ability to simultaneously
190 elute SOCs with a wide range of polarities. The acetonitrile fraction was reduced to 0.5
191 ml using a TurboVap II (12 psi N_2, 30 ˚ C), approximately 1.0 ml hexane was added, and
192 silica cleanup was performed. The 20 g silica solid phase extraction column was
193 preconditioned as described in [36] and the SOCs were eluted from the column using 100
194 ml ethyl acetate. Different silica column elution solvents were tested and it was
195 determined that ethyl acetate successfully eluted the target SOCs with minimal co-elution
196 of matrix interferences.

197 ***Instrumental Analysis***

198 Just prior to instrumental analysis, the triplicate recovery extracts were reduced
199 and spiked with the isotopically labeled surrogates and internal standards to assess spiked
200 SOC recoveries over the entire method. In the case of the tissue and lab blanks, the
201 internal standards were spiked into the extract just prior to instrumental analysis.

202 Semi-volatile organic compounds were identified and quantified using an Agilent
203 6890 gas chromatograph (Santa Clara, USA) coupled to an Agilent 5973N mass selective
204 detector. Briefly, 1 µl of the extract was injected using an HP 7683 autosampler, a pulsed
205 splitless injection was performed, and 30 m x 0.25 mm inner diameter x 0.25 um film
206 thickness DB-5 column (J&W Scientific, Palo Alto, CA, USA) was used for separation of
207 the SOCs [39]. Standard calibration curves were prepared prior to instrumental analysis

of samples. Selective ion monitoring mode was used to identify and quantify the SOCs. Either electron impact ionization or electron capture negative ionization was used based on the mode of ionization with the lowest instrumental detection limit for a given SOC [39].

For quality assurance and quality control, one lab blank was included with each batch of samples. Calibration curves were monitored throughout using check standards run for every 3 to 4 samples. Ion abundances were considered a match if they were within ± 20% of the standard or National Institute of Standards and Technology mass spectra library. A signal to noise ratio of 3:1 was used in identification of target analytes and retention times were monitored such that identified target analytes matched check standards within ± 0.05 minutes. Sample specific estimated detection limits were calculated using Environmental Protection Agency method 8280A [40] (**Table 3**). The instrumental limits of detection, ions monitored, and gas chromatograph oven parameters for electron impact mode and negative chemical ionization mode have previously been published [39].

Statistical Analysis

Average analyte recoveries were compared using a two-sided, two-sample t-test in SPLUS (version 8.0). A p value < 0.01 was considered significant. Individual SOC average recoveries greater than 180 % or less than 20 % were excluded from statistical analysis, including average and standard deviation calculations, as these recoveries were outside the acceptable range.

Results and Discussion

Comparison of PLE Method for Fish and Tadpoles

231 The PLE method resulted in higher average SOC recoveries from fish tissue (54.8
232 ± 15.5 % [standard deviation]) than from tadpole tissue (46.8 ± 15.3 %) (ref. [36] and
233 Table 1). This was especially true for the DDXs (DDTs, DDDs, and DDEs), and PCBs
234 ($p < 0.01$) (Table 1). The additional SOC losses from tadpole tissue in the PLE method
235 may have been due to higher SOC losses during extract evaporation and solvent
236 exchanges.
237 The precision for the PLE method, as indicated by the percent relative standard
238 deviations of the SOC recoveries, was higher for the fish tissue (ranged from 0.46% to
239 21.6%, with an average of 5.88%) than for the tadpole tissue (ranged from 17.4% to
240 96.9%, with an average of 34.1%) (ref. [36] and Table 1). This may also be due to
241 additional SOC losses during tadpole extract evaporation and solvent exchange.
242 ***Comparison of PLE and MSPD Methods for Tadpoles***
243 The MSPD method had significantly higher average SOC recoveries for tadpole
244 tissue (80.6 ± 25.9 %) than the PLE method (46.8 ± 15.3 %) (Table 1) ($p < 0.01$). In
245 addition, the average MSPD recoveries of organochlorine pesticides, organophosphorous
246 pesticides, PCBs, and PAHs were significantly higher than the average PLE recoveries of
247 these same SOCs ($p < 0.01$). However, the MSPD average recoveries for dieldrin and
248 endrin were above the acceptable range (Table 1) and may be the result of these target
249 analytes not behaving in the same manner as the labeled surrogate standards they were
250 quantified against (d_4-endosulfan I and d_4-endosulfan II, respectively). The average
251 tadpole PLE recoveries of acenaphthylene, acenaphthene, parathion, and endrin aldehyde
252 were below the acceptable range (Table 1) and may be a result of losses during solvent
253 evaporation.

The MSPD method also had higher precision, as indicated by the percent relative standard deviation of the SOC recoveries, (ranging from 0.86 % to 40.7 %, with an average of 11.3 %) than the PLE method (ranging from 17.4 % to 96.9 %, with an average of 34.1 %) for tadpole tissue (Table 1). Instrumental precision was assessed using replicate injections of extracts and standards on an intra- and inter-day basis for both MS ionization modes. Intra-day instrumental precision ranged from 0 % to 20.6 % relative standard deviation for extracts (all SOCs detected; $n = 20$) and 0.025 % to 13.1 % for standards (all SOCs; $n = 10$). Inter-day instrumental precision ranged from 0 % to 38.6 % relative standard deviation for extracts ($n = 20$) and 0.63 % to 15.9 % for standards ($n = 13$).

The PLE and MSPD estimated detection limits were not significantly different and ranged from 0.19 to 2900 pg/g wet weight (Table 3). Both the PLE and the MSPD methods were capable of detecting, but not quantifying, carbaryl and carbofuran. However, the MSPD method was capable of detecting 15 additional current-use pesticides and their degradation products, including the triazine herbicides, over the PLE method (Table 1). The ability to measure current-use pesticides in tadpole tissue is particularly important because some have been reported to cause sublethal effects in amphibians at low concentrations and are among the pesticides implicated in population declines [16, 18, 20, 41].

In addition to significantly higher recoveries for several SOC classes, better precision, and detection of a larger number of SOCs, the MSPD method resulted in shorter extract preparation time and less solvent consumption (Table 2). The MSPD

method also resulted in reduced use of dichloromethane, a chlorinated solvent and probable human carcinogen (Table 2) [42] (http://www.epa.gov/iris/subst/0070.htm).

Analytical Variability vs. Tadpole SOC Concentration Variability

The MSPD method was used to measure SOC concentrations in tadpole samples collected from several site in the Cascades Mountains, California, USA. Comparisons of the relative standard deviation of intra-day injections of the same tadpole extract (injection replicates), subsamples of the same tadpole sample processed using the MSPD method (analytical replicates), and different tadpole samples collected from the same site and processed using the MSPD method (site replicates) are shown in **Figure 1**. For most SOCs measured in these samples, the site variability (25 to 100% average relative standard deviation) was greater than the analytical (5 to 45%) and instrumental (1 to 5%) variability. This indicates that the MSPD method is precise enough to study intra- and inter-site variability in tadpole SOC concentrations. This method will be used in future studies to understand the accumulation of SOC in tadpoles collected throughout the California Cascade and Sierra Nevada Mountains.

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421
422
423 Figure 1. SOC concentration variability among tadpole samples collected from the
424 Cascade Mountains, California, USA using the MSPD method. "Injection
425 replicates" are intra-day injections of the same tadpole extract (n = 9); "analytical
426 replicates" are subsamples of a tadpole sample processed using the MSPD method
427 (n = 8); "site replicates" are different tadpole samples, collected from the same
428 site, processed using the MSPD method (n = 8). "< detection limit" indicates

concentrations were below the estimated detection limit in greater than 50 % of replicates. Only replicate sets with at least 50% detections are shown and values below the estimated detection limit (EDL) were substituted with ½ EDL.