

# Quantification of a Male Sea Lamprey Pheromone in Tributaries of Laurentian Great Lakes by Liquid Chromatography–Tandem Mass Spectrometry

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**S** Supporting Information

**ABSTRACT:** We developed an assay for measuring 7 $\alpha$ ,12 $\alpha$ ,24-trihydroxy-5 $\alpha$ -cholan-3-one-24-sulfate (3kPZS), a mating pheromone released by male sea lampreys (*Petromyzon marinus*), at low picomolar concentrations in natural waters to assess the presence of invasive populations. 3kPZS was extracted from streamwater at a rate of recovery up to 90% using a single cation-exchange and reversed-phase mixed-mode cartridge, along with [<sup>2</sup>H<sub>5</sub>]3kPZS as an internal standard, and quantified using ultrahigh performance liquid chromatography–tandem mass spectrometry. The limit of detection was below 0.1 ng L<sup>-1</sup> (210 fM), which was the lowest concentration tested. Intra- and interday coefficients of variation were between 0.3–11.6% and 4.8–9.8%, respectively, at 1 ng 3kPZS L<sup>-1</sup> and 5 ng 3kPZS L<sup>-1</sup>. This assay was validated by repeat measurements of water samples from a stream spiked with synthesized 3kPZS to reach 4.74 ng L<sup>-1</sup> or 0.24 ng L<sup>-1</sup>. We further verified the utility of this assay to detect spawning populations of lampreys; in the seven tributaries to the Laurentian Great Lakes sampled, 3kPZS concentrations were found to range between 0.15 and 2.85 ng L<sup>-1</sup> during the spawning season in known sea lamprey infested segments and were not detectable in uninfested segments. The 3kPZS assay may be useful for the integrated management of sea lamprey, an invasive species in the Great Lakes where pheromone-based control and assessment techniques are desired.



## INTRODUCTION

Pheromones are chemical signals released by animals in minute amounts and rapidly disperse in the natural environment<sup>1</sup> diluting their concentration rendering their detection difficult. These chemicals often serve as signature signals for a species or a group of related species.<sup>2</sup> Pheromones released by riverine animals serve as a good target for developing assays to detect invasive or native species. A pheromone released in a stream environment is confined within a largely unidirectional flow as it mixes, allowing assessment of pheromone release levels based on the pheromone concentration and stream discharge. In addition, sensitive assays could be used to characterize the pheromone landscape encountered by animals in their natural environment.<sup>3,4</sup>

We assessed the utility of measuring 7 $\alpha$ ,12 $\alpha$ ,24-trihydroxy-5 $\alpha$ -cholan-3-one 24-sulfate (3kPZS) as a population indicator of an invasive species, the sea lamprey (*Petromyzon marinus*), in the Laurentian Great Lakes.<sup>5</sup> 3kPZS has been reported to be released by mature male sea lampreys<sup>6</sup> at a rate of about 0.5 mg 3kPZS h<sup>-1</sup> male<sup>-1</sup>.<sup>7</sup> Practical application of this analytical method at

biologically relevant concentrations would allow detection of less than 1 ng of 3kPZS in 1 L of streamwater, since discharge in spawning streams can approach 50 m<sup>3</sup> s<sup>-1</sup> and accommodate hundreds of mature animals.<sup>8</sup>

Environmental steroids or steroid-like compounds have been measured using gas chromatography–mass spectrometry (GC/MS), GC-tandem MS,<sup>9–13</sup> and liquid chromatography–tandem mass spectrometry (LC-MS/MS).<sup>14–17</sup> GC-MS has often been used for detecting airborne pheromones,<sup>4</sup> but LC-MS/MS may be most effective for waterborne pheromones and is particularly appropriate for sulfate conjugates. For analysis of environmental 3kPZS, LC-MS/MS does not require derivatization or hydrolysis, both of which can reduce recovery of the analyte.<sup>18,19</sup> A potential limitation of LC-MS/MS is that constituents of natural waters may cause matrix

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suppression and isobaric interference in electrospray ionization (ESI).<sup>20,21</sup> Ion suppression in (ESI) LC-MS/MS has been reported to vary by a factor of 8–10 between and within runs for various analytes.<sup>22</sup> Therefore, quantifying 3kPZS in stream-water matrices by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) requires efficient extraction of 3kPZS that minimizes matrix interferences and an appropriate internal standard to correct for losses during sample preparation and as a result of matrix effects.<sup>23</sup>

Here we report the development and initial application of a UHPLC-ESI-MS/MS detection of picomolar concentrations of 3kPZS in streamwater. This assay could identify streams infested with spawning sea lampreys.

## EXPERIMENTAL SECTION

**Chemicals and Solutions.** Methanol (MeOH, HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ), and acetonitrile (ACN, HPLC grade) was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Triethylamine (TEA,  $\geq 99.5\%$  for LC-MS), ammonium hydroxide (AH, for LC-MS), trifluoroacetic acid (TFA, UV-Spectro,  $\geq 99.0\%$ ), and sodium azide ( $\geq 99.5\%$ ) were purchased from Sigma-Aldrich (St. Louis, MO). Distilled water was produced by a Barnstead Nanopure Infinity Ultrapure Water System (Thermo Scientific, Asheville, NC). Sulfuric acid (95–98%) and formaldehyde (37%) were purchased from Mallinckrodt (Haselwood, MO). Oasis cartridges HLB (6 cm<sup>3</sup>/500 mg), MCX (6 cm<sup>3</sup>/500 mg), WCX (6 cm<sup>3</sup>/150 mg), WAX (6 cm<sup>3</sup>/150 mg) and MAX (6 cm<sup>3</sup>/500 mg), and extraction manifold were purchased from Waters (Milford, MA).

3kPZS and 5-deuterated 3kPZS ( $[^2\text{H}_5]3\text{kPZS}$ ) were custom synthesized by Bridge Organics Inc. (Vicksburg, MI) with purity greater than 98%. The deuterium atoms on  $[^2\text{H}_5]3\text{kPZS}$  were located at C5(1D), C23 (2D), and C24 (2D) positions. Two mg mL<sup>-1</sup> stock solutions of synthesized 3kPZS and  $[^2\text{H}_5]3\text{kPZS}$  were prepared in 50% methanol. A 3kPZS stock solution of 2  $\mu\text{g mL}^{-1}$  was then prepared by serial dilution. The 2  $\mu\text{g mL}^{-1}$  3kPZS solution was further diluted with 50% methanol to produce stock solutions of 200 ng mL<sup>-1</sup>, 50 ng mL<sup>-1</sup>, 10 ng mL<sup>-1</sup>, 2 ng mL<sup>-1</sup>, 0.5 ng mL<sup>-1</sup>, and 0.1 ng mL<sup>-1</sup>. The 2 mg mL<sup>-1</sup> stock solution of  $[^2\text{H}_5]3\text{kPZS}$  was serially diluted with 50% methanol to yield a stock solution of 100 ng mL<sup>-1</sup>.

**Sample Collection.** River water samples for assay optimization were collected from the Red Cedar River on the campus of Michigan State University (East Lansing, MI), a stream not inhabited by the sea lamprey. The natural migration and inhabitation of sea lamprey in the Red Cedar River is impeded due to the several dams that are scattered along the Grand River, including the Brenke dam in Lansing, downstream from the Red Cedar River/Grand River confluence and from our sampling site. River water samples collected for assay optimization were 1 L and spiked with 1 ng 3kPZS and 5 ng of  $[^2\text{H}_5]3\text{kPZS}$  immediately after collection. Samples collected for assay validation were 1 L and spiked with 5 ng of  $[^2\text{H}_5]3\text{kPZS}$  as internal standard.  $[^2\text{H}_5]3\text{kPZS}$  was prepared in 1 mL of 50% methanol. Samples were collected by wading into the center of the stream and rinsing all sampling equipment and containers 10 times with river water from the sampling location. After collection, samples were inverted 10 times to achieve mixing. Samples were stored in 1 L high-density polyethylene bottles (Nalgene, Rochester, NY) and placed on ice. Within 12 h after collection, samples were filtered

with GF/B glass microfiber filters of 1.0  $\mu\text{m}$  nominal pore size (Whatman, Piscataway, NJ), followed by GN-6 metrical grid filters of 0.45  $\mu\text{m}$  pore size (Poll Corporation, Ann Arbor, MI).

**Optimization of 3kPZS Preservation.** Stability of 5 ng  $[^2\text{H}_5]3\text{kPZS}$  per mL of 50% methanol was determined when placed on a lab bench and when in an incubator at 36–38 °C to simulate the range of temperatures encountered during sampling. A solution of  $[^2\text{H}_5]3\text{kPZS}$  was prepared, placed on a lab bench at 23 °C, resampled at 2, 3, 4, and 6 days, and analyzed for  $[^2\text{H}_5]3\text{kPZS}$  concentration. A second solution of  $[^2\text{H}_5]3\text{kPZS}$  was placed in an incubator set at 37 °C, resampled at 1, 2, 3, 4, 5, 12, 19, and 26 days, and analyzed for  $[^2\text{H}_5]3\text{kPZS}$  concentration.

A method of preserving 3kPZS in river water prior to extraction was needed to allow sampling at remote sites. Recoveries of 3kPZS spiked into river water were evaluated at 0, 1, 4, 7, and 14 days when unpreserved samples were frozen and stored at 4 °C. The effect of adding sulfuric acid, sodium azide, or formaldehyde as preserving agents and acidifying samples to pH 2 on rate of recovery at day 0 was investigated. Changes in rates of 3kPZS recovery were determined using optimized analysis procedures described in the Results.

**Optimization of Solid-Phase Extraction.** Solid-phase extraction was optimized to maximize 3kPZS recovery. The recovery of 3kPZS spiked in deionized water and river water were compared with five functionally different types of solid-phase extraction cartridges: hydrophilic–lipophilic-balanced (Oasis HLB), cation-exchange and reversed-phase mixed-mode (Oasis MCX), weak cation-exchange and reversed-phase mixed-mode (Oasis WCX), weak-anion-exchange and reversed-phase mixed-mode (Oasis WAX), and strong anion-exchange and reversed-phase mixed-mode (Oasis MAX). Recovery of 3kPZS was optimized for each type of cartridge by investigating different loading speeds (2 mL min<sup>-1</sup> to 20 mL min<sup>-1</sup>), washing solutions (deionized water and 30–60% methanol), and eluting solutions (100% methanol, and methanol containing 1–5% ammonium hydroxide). Extraction eluents were evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco Co, MO). Dry residues were reconstituted with 100  $\mu\text{L}$  of 50% methanol before injection into UHPLC-MS/MS.

**UHPLC-MS/MS Analysis and Optimization.** 3kPZS concentrations in 10  $\mu\text{L}$  of reconstituted eluents were determined with Waters ACQUITY Ultra Performance LC System coupled with the Waters Quattro Premier XE tandem quadrupole mass spectrometer. Separation was achieved using a Waters C18 column (ACQUITY UPLC BEH 1.0  $\times$  50 mm, 1.7  $\mu\text{m}$  particle size) with oven temperature of 50 °C, flow rate of 0.15 mL min<sup>-1</sup>, and the gradient program in Table S1.

The mass spectrometer was operated in the negative electrospray ionization mode using multiple reaction monitoring (MRM). The compound-dependent cone and collision potentials were optimized with Waters QuanOptimize software by injecting standard solution of 3kPZS and  $[^2\text{H}_5]3\text{kPZS}$  into the MS through the column. The optimum conditions were reached by choosing parameters that gave the greatest integrated peak areas.

Optimization of additional source parameters including gas flow rates and temperatures were determined by repeatedly injecting 10  $\mu\text{L}$  of 100 ng mL<sup>-1</sup> 3kPZS and 10  $\mu\text{L}$  of 100 ng mL<sup>-1</sup>  $[^2\text{H}_5]3\text{kPZS}$  (in 50% methanol) with LC/MRM analysis. Before each injection, one parameter was slightly adjusted and resulting changes in integrated peak areas of the summed signals of all precursor/product ion analytes monitored

were recorded. In this manner, each of the parameters was optimized to maximize MRM signal for 3kPZS and [ $^2\text{H}_5$ ]3kPZS.

Calibration curves to determine 3kPZS and [ $^2\text{H}_5$ ]3kPZS concentration were obtained by performing a linear regression analysis on standard solutions of 3kPZS ranging from 200 ng mL $^{-1}$  to 0.1 ng mL $^{-1}$  (see Chemicals and Solutions) using the ratio of standard area (3kPZS) to internal standard area ([ $^2\text{H}_5$ ]3kPZS). The linearity of calibration curves was obtained from analysis of standards ranging from 0.05 ng mL $^{-1}$  to 100 ng mL $^{-1}$ , and all correlation coefficient ( $r^2$ ) values were  $\geq 0.999$ . The range of minimum detection limit for this method was between 0.02 and 0.31 ng mL $^{-1}$ .

**Validation of Optimized 3kPZS Assay.** *Analysis of 3kPZS-Spiked River Water Samples.* To determine the accuracy, precision, and reproducibility of the 3kPZS streamwater assay, four water samples were collected from the Red Cedar River each day over the course of 3 days. River water samples collected were 1 L and spiked with 1 ng 3kPZS and 5 ng of [ $^2\text{H}_5$ ]3kPZS. Water samples were assayed the day of collection using the optimized extraction and UHPLC-MS/MS procedures, and the intra- and interday coefficients of variation (CVs) were calculated.

*Analysis of Water Samples Collected from a 3kPZS-Spiked Stream.* Synthesized 3kPZS was spiked into the Upper Ocqueoc River, MI, to activate the entire stream discharge at 4.74 ng L $^{-1}$  ( $1 \times 10^{-11}$  M,  $n = 1$ ) or 0.24 ng L $^{-1}$  ( $5 \times 10^{-13}$  M,  $n = 5$ ) to further validate the assay in natural conditions at two concentrations. Stream discharge was estimated using the velocity-area method.<sup>24</sup> To determine variation in discharge estimates, the Ocqueoc River discharge on 11Aug10 was estimated six times, resulting in a mean estimate of 0.84 m $^3$  s $^{-1}$  with standard deviation of 0.03 m $^3$  s $^{-1}$ . A stock solution of 73.3  $\mu\text{g}$  3kPZS L $^{-1}$  was applied with a peristaltic pump (Masterflex 7553-70, Cole-Parmer, Vernon Hills, IL) at a rate of 167 mL min $^{-1}$  ( $\pm 5$  mL min $^{-1}$ ) to achieve an in-stream concentration of  $5 \times 10^{-13}$  M over 3 h. A stock solution of 1.48 mg 3kPZS L $^{-1}$  was applied over 3 h to achieve a concentration of  $1 \times 10^{-11}$  M. 3kPZS application occurred from 2300 to 0200 h. A lamprey blocking mechanism downstream of the experimental site prevents lamprey infestation, and hence no natural 3kPZS released by sea lampreys was expected in sampled waters. Application of synthesized 3kPZS to the Ocqueoc River was approved by United States Environmental Protection Agency through experimental use permit 75437-EUP-2.

Triplicate water samples were collected 20 m upstream and 250 m downstream of the 3kPZS application location. On the evenings of June 9th, 10th, 12th, and 19th 2009, streamwater samples were collected during 3kPZS application. On the evening of 11 June 2009, water samples were collected before and during the administration of 3kPZS. On the evening of 18 June 2010, water samples were collected before ( $\sim 2030$  h), during ( $\sim 0100$  h), and after ( $\sim 0500$  h) administration of 3kPZS. Each sample was immediately spiked with 5 ng of [ $^2\text{H}_5$ ]3kPZS, inverted several times, and placed on a bed of ice until they could be transferred to a  $-20$  °C freezer, which occurred no later than 24 h after the sample was collected.

*Analysis of Naturally Released 3kPZS in Sea Lamprey Infested Streams.* To determine the utility of the assay to detect 3kPZS released by sea lampreys under diverse stream conditions, water samples were collected and analyzed from Laurentian Great Lakes tributaries in Michigan. Six of the selected streams contained populations of sea lamprey, and one stream was not infested with sea lampreys (Table S2). Release of 3kPZS has only

been reported in sexually mature male sea lamprey,<sup>6</sup> so samples were collected before (27Apr10), during (30Jun10), and after the mating season (24Aug10). Three of the sea lamprey infested streams sampled contained a barrier that prevented sea lamprey establishment in the upper reaches, while the lower reaches remained infested. Therefore, the general sampling strategy was to collect water upstream and downstream of the sea lamprey barrier or dam on each stream. 3kPZS was expected to be detected downstream of sea lamprey barriers during the spawning season. One sample per site was collected in April, five samples per site were collected in June, and two samples per site were collected in August. All samples were collected between 0700 and 2200 h. Samples were stored as those collected from the Upper Ocqueoc River.

## RESULTS AND DISCUSSION

**Stability of Synthesized [ $^2\text{H}_5$ ]3kPZS and 3kPZS.** [ $^2\text{H}_5$ ]3kPZS internal standard prepared in 50% methanol was stable in a laboratory environment at ambient conditions for at least 6 d and at 36 to 38 °C for at least 26 d (Table S3). Unpreserved 3kPZS-spiked stream samples that were frozen at  $-20$  °C showed no decline in 3kPZS recoveries over 14 d, whereas 3kPZS in unpreserved samples stored at 4 °C quickly declined over time (Table S4). Degradation was presumed to be due to microbial activity.<sup>25</sup> Rates of 3kPZS recovery declined to approximately 60%, 69%, and 69% when sulfuric acid, sodium azide, or formaldehyde was added, respectively, as preserving agents (data not shown). Sterilized controls were not examined during stability assays of [ $^2\text{H}_5$ ]3kPZS and 3kPZS and should be examined in the future when assessing biodegradation of lamprey pheromone components.

**Solid-Phase Extraction Conditions.** Among five functional types of solid-phase cartridges examined, the cation-exchange and reversed-phase mixed-mode (MCX) was found to achieve maximum recovery of 3kPZS from river water while minimizing matrix interference (Table 1). All samples were spiked with 5 ng of [ $^2\text{H}_5$ ]3kPZS internal standard before solid-phase extraction. MCX cartridge was preconditioned with 8 mL of methanol followed by 8 mL of deionized water at 8 mL min $^{-1}$  and then loaded with a prefiltered sample at 14 to 17 mL min $^{-1}$ . After loading, the cartridge was washed with 8 mL of deionized water at 8 mL min $^{-1}$ , and analytes were eluted with 8 mL of methanol at 4 mL min $^{-1}$ . This procedure was subsequently used to extract all stream samples.

While it was expected that 3kPZS in deionized water would not be extracted effectively by MCX (rate of recovery of 3.2%; Table 1), it was unexpected that 90% of 3kPZS in streamwater was recovered. Since the cation exchange site failed to compete effectively for 3kPZS in deionized water, it was likely that 3kPZS interacted with substances in streamwater, resulting in a neutrally or slightly positively charged complex. Since the MCX is a mixed-mode cartridge, it is effective at retention of both hydrophobic and electrostatic interaction and therefore has a broad affinity for a wide range of compounds. 3kPZS in deionized water may not form a complex of high enough polarity to gain affinity for the resin on the cartridge; however, we have not yet determined exactly why this occurs. Nonetheless, the 3kPZS in all ten streams selected for this study was extracted efficiently. 3kPZS rate of recovery with MCX in river water was nearly unchanged when loading speed increased from 4 to 20 mL min $^{-1}$  (data not shown), further suggesting that the presumed 3kPZS complex

**Table 1. Optimal Extraction Conditions for 1 ng of 3kPZS<sup>a</sup> in 1 L of Deionized Water and 1 L of River Water Using Different Extraction Cartridges**

	HLB <sup>b</sup>	MCX <sup>c</sup>	WCX <sup>d</sup>	WAX <sup>e</sup>	MAX <sup>f</sup>
loading speed (ml min <sup>-1</sup> )	14–17	14–17	14–17	3–5	3–5
washing solution	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O
eluting solution	MeOH <sup>g</sup>	MeOH	MeOH	2%AH <sup>h</sup> /MeOH	2%TFA <sup>i</sup> /MeOH
recovery in H <sub>2</sub> O (%)	100	3.2	11.4	93	0
recovery in stream (%)	76	90	69	53	0

<sup>a</sup> 7 $\alpha$ ,12 $\alpha$ ,24-Trihydroxy-5 $\alpha$ -cholan-3-one-24-sulfate. <sup>b</sup> HLB: hydrophilic–lipophilic-balanced. <sup>c</sup> MCX: cation-exchange and reversed-phase mixed-mode. <sup>d</sup> WCX: weak cation-exchange and reversed-phase mixed-mode. <sup>e</sup> WAX: weak-anion-exchange and reversed-phase mixed-mode. <sup>f</sup> MAX: strong anion-exchange and reversed-phase mixed-mode. <sup>g</sup> MeOH: methanol. <sup>h</sup> AH: ammonium hydroxide. <sup>i</sup> TFA: trifluoroacetic acid.

**Table 2. Intra- and Interday Variation of 3kPZS and [<sup>2</sup>H<sub>5</sub>]3kPZS Concentrations (ng L<sup>-1</sup>) Measured in 1 L Water Samples Collected from the Red Cedar River,<sup>a</sup> MI, Using Cation-Exchange and Reversed-Phase Mixed-Mode Optimized Solid-Phase Extraction and UHPLC-MS/MS Methods**

compound	intraday assay						interday assay days 1, 2, and 3 (n = 12)	
	day 1 (n = 4)		day 2 (n = 4)		day 3 (n = 4)		mean $\pm$ SD	CV (%)
	mean $\pm$ SD	CV <sup>b</sup> (%)	mean $\pm$ SD	CV (%)	mean $\pm$ SD	CV (%)		
3kPZS	1.21 $\pm$ 0.14	11.5	1.1 $\pm$ 0.06	5.5	1.06 $\pm$ 0.017	1.6	1.12 $\pm$ 0.11	9.8
[ <sup>2</sup> H <sub>5</sub> ]3kPZS	5.08 $\pm$ 0.017	0.3	5.18 $\pm$ 0.42	8.01	5.21 $\pm$ 0.09	1.7	5.16 $\pm$ 0.25	4.8

<sup>a</sup> The Red Cedar River did not contain any naturally occurring 3kPZS, so 3kPZS and [<sup>2</sup>H<sub>5</sub>]3kPZS were spiked into the sample at 1 ng L<sup>-1</sup> and 5 ng L<sup>-1</sup>, respectively. <sup>b</sup> CV = coefficient of variation.

**Table 3. Quantified 3kPZS Concentrations in the Upper Ocqueoc River, Presque Isle County, MI, from Water Samples Collected When 3kPZS at 0.24 ng L<sup>-1</sup> (5  $\times$  10<sup>-13</sup> M) and 4.74 ng L<sup>-1</sup> (1  $\times$  10<sup>-11</sup> M) Was Applied to the Stream**

date	3kPZS administered (ng L <sup>-1</sup> )	3kPZS measured <sup>a</sup> (ng L <sup>-1</sup> )	StDev concentration <sup>b</sup> (ng L <sup>-1</sup> )
09Jun10	0.24	0.38	0.09
10Jun10	0.24	0.34	0.08
11Jun10	0.24	0.22	0.02
12Jun10	0.24	0.26	0.02
18Jun10	0.24	0.50	0.07
19Jun10	4.72	5.29	0.14

<sup>a</sup> Triplicate samples were collected each day 250 m downstream of the application location. <sup>b</sup> Standard deviations of the average concentration are reported.

was mainly captured by the lipophilic reverse-phase site and not the cation exchange site. Factors such as pH or ionic strength in river water samples, which may explain the differences in recovery rates of different river and laboratory water samples (Table 1), were not examined.

The effective and one-step extraction of 3kPZS by MCX from streamwater, while unexpected, yielded samples ready for UHPLC-MS/MS injection without further clean up. Methanol was an efficient eluting agent for 3kPZS but is presumed to not release positively charged compounds captured on cation exchange sites. Moreover, these sites repel negatively charged matrices, thus further reducing interferences. This is highly advantageous over solid-phase extractions of steroid hormones from environmental matrices using either octadecyl (C18)-bonded silica adsorbent<sup>26–31</sup> or HLB cartridge. Extracts of

C18 or HLB often require further clean up by liquid–liquid extraction, solid phase purification on C18/NH<sub>2</sub> columns,<sup>32,33</sup> silica gel column chromatography,<sup>34,35</sup> gel permeation on Biobeads SX-3 columns, high performance liquid chromatography (HPLC) fractionation,<sup>27,36</sup> or a combinations of these methods.<sup>26,37,38</sup> Intense extraction and clean up procedures increase the potential for mechanical losses of the analyte of interest.<sup>39</sup>

**UHPLC-MS/MS Optimization.** Optimized source-dependent parameters were capillary voltage –3.50 kV, multiplier voltage 650 V, desolvation gas flow 600 L h<sup>-1</sup>, cone gas flow 50 L h<sup>-1</sup>, desolvation 350 °C, source temperature 130 °C, and RF lens' at 0.2 V. The anionic 3kPZS and [<sup>2</sup>H<sub>5</sub>]3kPZS displayed fragmentation patterns with dominant daughter ions at *m/z* 97 (HSO<sub>4</sub><sup>-</sup>) from [M-H]<sup>-</sup> of 3kPZS (parent *m/z* 471.3, in dissolved form with the disassociation of an ammonium group) and at *m/z* 98 (DSO<sub>4</sub><sup>-</sup>) from [M-H]<sup>-</sup> of [<sup>2</sup>H<sub>5</sub>]3kPZS (parent *m/z* 476.3; Table S5). LC/MS analysis indicated that both labeled and unlabeled forms were not resolved on the C18 column.

**3kPZS Assay Validation.** When river water samples were spiked with 1 ng L<sup>-1</sup> 3kPZS and 5 ng L<sup>-1</sup> [<sup>2</sup>H<sub>5</sub>]3kPZS, the intraday average concentration determined by optimized assay procedures ranged between 1.06 and 1.21 ng L<sup>-1</sup> for 3kPZS and 5.08 and 5.21 ng L<sup>-1</sup> for [<sup>2</sup>H<sub>5</sub>]3kPZS (Table 2 and herein). The 3 day interday average concentration measured was 1.12 ng L<sup>-1</sup> for 3kPZS and 5.16 ng L<sup>-1</sup> for [<sup>2</sup>H<sub>5</sub>]3kPZS. Coefficients of variation (CVs) ranged between 0.3 and 11.5% for 3kPZS and 0.3 and 8.1% for [<sup>2</sup>H<sub>5</sub>]3kPZS. Interday CV for 3kPZS was 9.8% and for [<sup>2</sup>H<sub>5</sub>]3kPZS was 4.8%.

The Ocqueoc River discharge was spiked with 3kPZS at 0.24 ng L<sup>-1</sup> for a total of five nights, and the average 3kPZS concentration of triplicate samples collected downstream of the source each night was 0.38, 0.34, 0.22, 0.26, and 0.50 ng L<sup>-1</sup>, with an

**Table 4. Concentration of 3kPZS, Recovery Rates of [<sup>2</sup>H<sub>5</sub>]3kPZS (IS Loss %), and 3kPZS Limit of Detection (LOD) in Stream Water Samples Collected from Michigan Streams above and below Sea Lamprey Barriers in Late April, June, and August 2009**

river	location	3kPZS ng L <sup>-1</sup>	IS loss	LOD	3kPZS ng L <sup>-1</sup>	IS loss	LOD	3kPZS ng L <sup>-1</sup>	IS loss	LOD
		27Apr09	%	(ng L <sup>-1</sup> )	30Jun09 (StDev)	%	(ng L <sup>-1</sup> )	24Aug09	%	(ng L <sup>-1</sup> )
Betsie	above	ND <sup>b</sup>	42	0.06	0.63 (0.07)	54	0.09	ND	50	0.08
Betsie	below	ND	43	0.05	0.68 (0.07)	53	0.09	ND	48	0.08
Carp Lake Outlet	above	ND	45	0.15	0.95 (0.39)	50	0.08	ND	48	0.1
Carp Lake Outlet	below	ND	45	0.13	0.16 (0.18)	43	0.08	ND	43	0.11
Cheboygan	above	ND	51	0.09	1.09 (1.10)	34	0.10	ND	50	0.08
Cheboygan	below	ND	52	0.08	2.85 (1.96)	32	0.10	ND	48	0.09
Little Manistee	above	ND	62	0.16	0.09 (0.18)	60	0.05	ND	60	0.12
Little Manistee	below	ND	62	0.16	0.04 (0.06)	64	0.02	ND	58	0.14
Manistee	above	ND	61	0.12	ND	76	0.20	ND	65	0.15
Manistee	below	ND	41	0.10	0.25 (0.03)	43	0.11	ND	61	0.14
Nagel <sup>a</sup>	above	ND	58	0.23	ND	49	0.06	ND	50	0.1
Ocqueoc	above	ND	58	0.28	0.39 (0.05)	71	0.20	ND	64	0.24
Ocqueoc	below	ND	44	0.18	2.31 (1.99)	65	0.24	ND	64	0.2

<sup>a</sup>Nagel Creek contains no lampreys and served as a negative control. <sup>b</sup>ND = 3kPZS not detected.

overall mean of the five nights equaling 0.34 ng L<sup>-1</sup> (Table 3). The river discharge was spiked with 3kPZS at 4.7 ng L<sup>-1</sup> one night, and the mean 3kPZS concentration from triplicate samples was 5.3 ng L<sup>-1</sup> (Table 3). 3kPZS was not detected in samples collected upstream of the 3kPZS application location or in samples collected downstream of the application location when 3kPZS was not applied (Table S6).

These results demonstrated the 3kPZS assay reliably measures the presence of 3kPZS. Minor discrepancies between quantified and expected 3kPZS are likely due to errors in discharge estimates, 3kPZS application rates, and the stratified nature in which pheromones disperse. In turbulent streamwater, pheromone plumes are mixed into smaller and smaller stratified plumes resulting in variable concentrations at a sampling site through time.<sup>40</sup> Multiple samples collected from several locations will provide the most reliable assessment of pheromone concentration.

To the best of our knowledge, there was only one other study that attempted to characterize the possible occurrence of a bile acid in sea lamprey spawning streams.<sup>3</sup> This bile acid, petromyzonol sulfate (PS), was analyzed with C18 extraction and LC-ESI-MS, with a detection limit in streamwater of about 0.47 ng L<sup>-1</sup>. The analyte was estimated based on chromatographic profiles of 10 *m/z* units centered on the expected *m/z* value of the molecular ion.<sup>3</sup>

**3kPZS Assay Application.** Samples collected below sea lamprey barriers during the spawning season contained detectable levels of 3kPZS that ranged from 0.04 ng L<sup>-1</sup> in the Little Manistee River to 2.85 ng L<sup>-1</sup> in the Cheboygan River (Table 4 and herein). Samples collected in June above barriers on the Cheboygan, Little Manistee, and Ocqueoc Rivers also had detectable levels of 3kPZS, but those rivers also have a history of sea lamprey infestation above their barriers. Samples collected in late April above and below sea lamprey barriers did not contain detectable levels of 3kPZS, even though adult and larval sea lampreys were present below barriers. Samples collected in August above and below sea lamprey barriers did not contain detectable levels of 3kPZS. The average limit of 3kPZS detection in the samples reported was 0.13 ng L<sup>-1</sup> (St. Dev. = 0.07 ng L<sup>-1</sup>). The average loss of [<sup>2</sup>H<sub>5</sub>]3kPZS during storage and analysis was

52% (St. Dev. = 15%). The 3kPZS limit of detection in stream-water samples was dependent on extraction efficiency of the internal standard ([<sup>2</sup>H<sub>5</sub>]3kPZS) and noise level differences among streams and sampling dates.

Results from samples collected from sea lamprey infested streams validate the utility of the assay to detect spawning populations of sea lampreys in diverse stream conditions. Spermiating male sea lampreys were the most plausible source of 3kPZS in water samples collected below sea lamprey barriers, as expected from previous studies,<sup>6</sup> since 3kPZS was not detected below barriers prior to the maturation of male sea lampreys, and 3kPZS was not detected above effective sea lamprey barriers (Table 4). Assayed water samples show that spermiating male sea lampreys release sufficient 3kPZS to induce behavioral responses in ovulated female sea lampreys, where responses have been observed at 0.005 ng L<sup>-1</sup>.<sup>41</sup> Larval sea lampreys, although present below sea lamprey barriers in April and August, did not release detectable amounts of 3kPZS during the adult migration in the streams sampled. Since sea lampreys die after mating, the 3kPZS contained in the corpus of male sea lampreys was washed away two months after the peak of spawning season.

Interesting exceptions occurred on the Betsie River and Carp Lake Outlet where 3kPZS was detected above the barrier, yet these streams were presumed to not have sea lamprey upstream of the barrier in 2009. American Brook lamprey (*Lampetra appendix*) are highly prevalent above the Betsie River barrier,<sup>42</sup> and it is possible that they may also employ 3kPZS as a mating pheromone. However, successful embryonic development of the sea lamprey is more restricted during low stream temperatures compared to American brook lamprey.<sup>42</sup> Stream temperatures may be the key in deciphering whether 3kPZS plumes are a result from native or invasive lamprey, based upon which life history phase, and thermal range, each species released the compound. It is necessary to fully characterize the release of 3kPZS from lamprey species native to the Great Lakes to determine the full utility of the 3kPZS assay to detect spawning populations of sea lampreys versus spawning populations of lamprey species. At the Carp Lake Outlet, one of the five samples collected upstream of the barrier at Carp Lake Outlet did not

contain detectable levels of 3kPZS. It is possible that minute amounts of synthesized 3kPZS from unrelated behavioral studies occurring at Hammond Bay Biological Station may have contaminated samples collected upstream of the barrier at Carp Lake Outlet.

In conclusion, the 3kPZS assay is suitable for quantifying 3kPZS in streamwater with good reproducibility, high accuracy, and low intra- and interday variation. 3kPZS is a robust modifier of ovulated female behavior in experimental streams and shows promise for improved integrated sea lamprey management through enhanced trapping of females.<sup>41</sup> Quantification of 3kPZS in streamwater may provide an affordable and noninvasive method of identifying streams infested with spawning sea lampreys.<sup>43</sup> Deployment of this method in sea lamprey management is expected to enhance detection of spawning populations and facilitate future research.

## ■ ASSOCIATED CONTENT

**S Supporting Information.** Six tables that describe in greater detail the operating conditions of the UHPLC-MS/MS, stability of [<sup>2</sup>H<sub>5</sub>]3kPZS and 3kPZS, streamwater sampling locations, and 3kPZS concentration in streamwater samples. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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