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Combining Headspace Solid-Phase Microextraction with Internal Benchmarking to Determine the Elimination Kinetics of Hydrophobic UVCBs

Roxana Sühring,* Karina Knudmark Sjøholm,* Philipp Mayer, and Matthew MacLeod

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ABSTRACT: Substances classified as unknown or variable composition, complex reaction products or biological origin (UVCB) present a challenge for environmental hazard and risk assessment. Here, we present a novel approach for whole-substance bioconcentration testing applied to cedarwood oil—an essential oil composed of volatile, hydrophobic organic chemicals. The method yields whole-body elimination rate constants for a mixture of constituents. Our approach combines in vivo dietary fish exposure with internal benchmarking and headspace solid-phase microextraction (HS-SPME) equilibrium sampling followed by suspect-screening analysis. We quantified depuration rate constants of 13 individual cedarwood oil constituents based on relative peak areas using gas chromatography (GC) coupled with Orbitrap-mass spectrometry (MS) and GC triple-quadrupole (QqQ)-MS. For seven constituents with available analytical standards, we compared the rate constants to the results obtained from solvent extraction, clean-up, and targeted GC–MS analysis. The HS-SPME sampling approach allowed for automated sample extraction and analyte enrichment while minimizing evaporative losses of the volatile target analytes and reducing matrix interferences from low-volatility organics. The suspect-screening analysis enabled the quantification of constituents without available analytical standards, while the internal benchmarking significantly reduced variability from differences in delivered dose and analytical variability between the samples.

KEYWORDS: mixture analysis, kinetic BCF, internal benchmarking, suspect-screening analysis, equilibrium sampling, HS-SPME

1. INTRODUCTION

The potential for a chemical to bioaccumulate is an important criterion for the evaluation of its potential environmental risk and is considered in regulatory risk assessment and chemical registration worldwide.1,2 The bioaccumulation potential of a substance is commonly assessed using bioconcentration factor (BCF), which is defined as the concentration of a chemical substance in an organism (typically fish) compared to the freely dissolved concentration in the surrounding water at steady state.3 Kinetically, the BCF can be described as the quotient of the uptake rate constant \( k_u \) [day\(^{-1}\)] by the depuration rate constant \( k_d \) [day\(^{-1}\)].3 The BCF is most commonly determined using well-established testing protocols for single substances with aqueous exposure. However, when testing highly hydrophobic and volatile substances, for which the exposure concentrations in water are difficult to control, or multiconstituent substances such as unknown or variable composition, complex reaction products or biological origin (UVCBs), determining a reliable BCF remains a challenge.4,5 Recent advances in using dietary exposure and internal benchmarking to determine a kinetic BCF of essential oils—a group of well-characterized hydrophobic UVCBs—have succeeded in addressing some of these challenges.5–9 The developed methods provide a way of measuring multiple constituents of essential oils in parallel and establish quality control criteria for the dietary exposure test and the derived kinetic BCFs.5 However, methods described to date rely on the availability of analytical standards for the individual constituents, which places limits on the number of constituents in a UVCB for which data can be obtained. Many UVCBs have hundreds to thousands of constituents that can vary between batches and for which no analytical standards are commercially available.5 Moreover, the published target analysis methods require solvent extraction and clean-up steps.6,9 For volatile UVCBs such as essential oils or volatile constituents in a UVCB, every sample-processing step has the risk of losing...
target analytes due to evaporation, which could change the relative composition of the mixture.\textsuperscript{4–6}

In this study, we combined the recently developed benchmarking approach with an automated equilibrium sampling methodology and quantitative suspect-screening analysis. Our goal was to develop a method for the quantification of in vivo kinetic BCFs of volatile UVCB constituents based on relative peak areas alone, without extraction, clean-up, or solvents, using an equilibrium head-space solid-phase microextraction (HS-SPME) method coupled with gas-chromatography and mass spectrometry (GC–MS).

2. THEORY

2.1. Automated HS-SPME Equilibrium Sampling. Solid-phase microextraction (SPME) is an extraction method based on the partitioning of analytes from the sample matrix and into a thin polymer coating of the SPME fiber.\textsuperscript{30} SPME combines analyte enrichment, exclusion of sample matrix and introduces the analytes directly into the GC–MS via thermal desorption.\textsuperscript{30} Due to the matrix exclusion, SPME is an interesting technique to combine with suspect-screening or even nontargeted analytical methods. Headspace solid-phase microextraction (HS-SPME) was recently applied to measure chemical activity ratios of semivolatile hydrophobic organic compounds in wastewater treatment plant (WWTP) sludge.\textsuperscript{11} The polymer coating was equilibrated in the headspace above the sludge sample and nontarget GC–MS analysis was then applied to measure changes in chemical activity during the WWTP processes. The benefits of operating such methods in the equilibrium regime are that chemicals have the same chemical activity in polymer and sample, and concentrations in sample and polymer are well related via partition coefficients.\textsuperscript{12,13} Equilibrium sampling can thus facilitate the comparison across different matrices regardless heterogeneity and quality of the sorptive organic matter in the sample matrix.\textsuperscript{14} The methodology can even be used on unidentified molecules, as long as ratios are calculated for the exact same molecular feature.\textsuperscript{10} This potential for suspect-screening and nontargeted analysis makes the equilibrium sampling HS-SPME approach a promising tool for studying and assessing the environmental fate of UVCBs.

2.2. Internal Benchmarking. Internal benchmarking with a conservative benchmark substance is a method used to correct for covariant sources of variability in in vivo kinetic BCF tests (such as, e.g., differences in feeding behavior and individual growth rates).\textsuperscript{7} Similar to the use of internal standards in analytical chemistry, in internal benchmarking the behavior of a target analyte is measured relative to the behavior of a well-characterized standard. In the case of conservative benchmarking, this “well-characterized standard” is a benchmark substance that will not be eliminated by excretion, biotransformation, or respiration in the duration of a BCF experiment. Therefore, it can be used to correct for growth dilution and other interindividual differences in the chemical concentration in the test organisms.

2.2.1. Determining theBenchmarked Kinetic BCF Using Quantitative Suspect-Screening Analysis. For this study, the depuration rate constant ($k_T$ [day$^{-1}$], benchmarked depuration rate constant ($k_{TG}$) [day$^{-1}$], uptake rate constant ($k_i$) [day$^{-1}$], and derived kinetic BCF as well as benchmarked kinetic BCF (BCF$\textsubscript{BM}$) [L kg$^{-1}$ ww] were calculated following the method described in Chen et al.\textsuperscript{9} with the difference that peak areas rather than concentrations were used in the calculations. Briefly, the depuration rate $k_T$ was calculated from the natural logarithm of the measured peak areas in fish at different time points

$$k_T = \frac{\ln A_{X,t2} - \ln A_{X,t1}}{t_2 - t_1}$$

where $A_X$ denotes the peak areas of test substance $X$ at time $t$, which are proportional to chemical activities and lipid-based concentrations of the substance in the fish tissue.\textsuperscript{12,15}

The depuration rate for hexachlorobenzene (HCB) was used to correct for interindividual variability (for details, see Chen et al.\textsuperscript{9})

$$k_G = \frac{\ln A_{HCB,t2} - \ln A_{HCB,t1}}{t_2 - t_1}$$

where $A_X$ and $A_{HCB}$ are the peak areas of test substance $X$ and conservative benchmark substance HCB at time $t$, respectively, and the benchmarked depuration rate constant $k_{TG}$ could then be calculated as

$$k_{TG} = k_T - k_G = \frac{\ln \left( \frac{A_{X,t2}}{A_{HCB,t2}} \right) - \ln \left( \frac{A_{X,t1}}{A_{HCB,t1}} \right)}{t_2 - t_1}$$

The uptake rate constant $k_i$ was estimated based on a model by Arnot and Gobas\textsuperscript{16} (as described in Chen et al.\textsuperscript{9}).

$$k_i = 10^\left(0.8 + 0.876 \log W + 0.017 \left(\frac{15}{K_{OW}} \right)\right)$$

where $W$ is the weight of the fish in g, $T$ is the water temperature in °C, $K_{OW}$ is the octanol-water partitioning coefficient of the target analyte, and $C_{OX}$ is the dissolved oxygen concentration in water [mg L$^{-1}$]. For high-$K_{OW}$ substances, the term $1.85 + \left(\frac{15}{K_{OW}}\right)$ approaches 1.85. Based on the $C_{OX}$ and average fish weight in this study, $k_i$ was estimated as 120 [day$^{-1}$].

Using the estimated $k_i$ and measured $k_T$ and $k_{TG}$, the kinetic BCF [L kg$^{-1}$ ww] and BCF$\textsubscript{BM}$ [L kg$^{-1}$ ww] could be calculated as

$$BCF = \frac{k_i}{k_T}$$

and

$$BCF_{BM} = \frac{k_i}{k_{TG}}$$

3. MATERIALS AND METHODS

3.1. Materials. Virginian cedarwood oil, batch number AS00254371 (provided by Givaudan UK Ltd.) was used as model UVCB for the method development and evaluation. Cedarwood oil was chosen due to the detailed available characterization data (provided by the manufacturer Givaudan UK Ltd.), its comparably high bioaccumulation potential,\textsuperscript{17} and the opportunity to compare the newly obtained results with kinetic BCF data for the same fish from the targeted GC-MSD analysis described in Sühring et al.\textsuperscript{17}
Hexachlorobenzene (HCB) purchased from Sigma-Aldrich was used as a conservative benchmark substance to correct for interindividual variability in test concentrations between the test animals due to, e.g., differences in feeding behavior. The purified plant oil Miglyol 812 (Caesar & Loretz GmbH) is an ultrapure mixture of medium-chain triglycerides that has been found to have similar partitioning properties for hydrophobic target analytes as storage lipids of the fish. It made it a suitable partitioning reference phase for the HS-SPME measurements.

3.2. In Vivo Experiment. The in vivo test used in this experiment was previously published by Sühring et al. In short, 49 adult rainbow trout (Oncorhynchus mykiss) were exposed to a single dose of cedarwood oil and benchmark substances using dietary exposure (at 0.8% of their body weight). In a separate aquarium, 15 control fish were kept as a control group. Following the initial exposure, both exposure group and control group were fed clean food daily at around 0.7% of their body weight.

Six to eight fish from the exposure group and two fish from the control group were sacrificed on days 1, 2, 4, 7, 14, 21, and 28 and stored at −20 °C until analysis. All fish were kept in accordance with the Swedish National Agriculture Board’s guidance on research animals (Statens Jordbruksverks Föreskrifter och Allmanna Råd om Försöksdjur, SJVFS 2015:24). Ethical approval for the experiments was obtained from Stockholms Djurförsöksstifts Nämnd (permit 16938/17). For the present study, at each time point, three fish from the exposure group and two fish from the control group were analyzed.

3.3. Method Development and Quality Assurance. 3.3.1. Samples for Method Development. Ten aliquots (5 g each) from one rainbow trout were used for the GC triple-quadrupole (QqQ) and GC-Orbitrap-MS method development with fish matrix. Following homogenization of the whole frozen fish, the aliquots were sampled directly into headspace vials with magnetic screw caps (18 mm thread, 1.3 mm poly(tetrafluoroethylene) (PTFE)/silicone septum, Sigma-Aldrich). The vials were kept upright at −18 °C until analysis. Saturated sodium chloride (NaCl, 5 mL) solution in ultrapure water was added to each aliquot upon thawing prior to the instrumental analysis.

3.3.2. Instrumental Analysis. Two analytical techniques were used to explore the feasibility, robustness, and limits of the proposed kinetic BCF determination based on equilibrium sampling:

1. HS-SPME-GC-QqQ-MS using targeted multiple reaction monitoring (MRM) and quantification based on relative peak areas.
2. HS-SPME-GC-Orbitrap-MS using high-resolution accurate mass suspect-screening analysis with tentative identification based on spectral library matching and quantification based on relative peak areas.

3.3.3. Method Optimization Parameters. The HS-SPME sampling time was optimized to ensure equilibrium of cedarwood oil constituents between lipid, air, and SPME fiber had been achieved. For equilibrium sampling time optimization, the sampling temperature was held constant at 35 °C while triplicate aliquots of 1 mg L⁻¹ cedarwood oil in miglyol were sampled for 5, 10, 20, 40, 80, and 160 min. A simple first-order equation was fitted to determine the required sampling time to reach 90% equilibrium ($t_{90}$) (Table S1)

$$A = A_{\text{max}} \times (1 - e^{-(kt)})$$

where $A$ is the peak area of the target analyte, $A_{\text{max}}$ is the maximum peak area of the target analyte, $k$ is a fitted constant, and $t$ is the time. $t_{90}$ could then be calculated as

$$t_{90} = \frac{\ln(100 - 90)}{k}$$

For the GC-QqQ-MS MRM method, the collision energy was optimized for the target compounds (Table S4).

3.3.4. Quality Assurance. It is important to minimize and be aware of the risk for evaporative losses when working with essential oil UVCBs since they contain volatile components. To minimize potential evaporation losses, the fish samples were contained in gastight autosampler vials and stored at −20 °C prior to analysis. Empty “blank” vials were stored with the fish samples until the analysis.

In equilibrium sampling, negligible depletion must be confirmed to ensure that the sampling does not lower the chemical activity of the analytes in the sample. Negligible depletion sampling was confirmed by reextracting two samples three times each.

Due to the high viscosity of the fish tissue, we were concerned whether the HS sampling would be representative of the entire sample or merely the sample surface. To facilitate diffusive exchange with the whole of the sample, the samples were submerged in saturated NaCl solution.

The linear range of the QqQ-MS and Orbitrap-MS was tested by analyzing a dilution series of cedarwood oil in miglyol that covered the expected concentration range of the samples. Method blanks using empty HS vials, HS vials filled with 2 mL of saturated NaCl solution as well as vials filled with 1 mL of miglyol only were analyzed after every fifth sample. The limit of detection (LOD) was calculated as the average relative peak area (relative to HCB) of the blank (method blank or detection in fish from the control group) + 3× standard deviation. The limit of quantification (LOQ) was calculated as the average relative peak area of the blank + 10× standard deviation. For compounds without a detectable signal in the blanks, the LOD was calculated based on the relative peak area of detectable target analytes in exposed fish corresponding to a signal-to-noise ratio of 3 and the LOQ based on a signal-to-noise ratio of 10.

All tests were performed in triplicate to evaluate the precision of the measured peak areas. For the evaluation of accuracy, HS-SPME-GC-QqQ-MS MRM and HS-SPME-GC-Orbitrap-MS depuration rate constant results for $\alpha$-cedrene, $\beta$-cedrene, thujaopene, and cedrol were compared to the target analysis results (by GC-MSD) for cedarwood oil using the same fish. The three analysis techniques were performed by two different members of staff at different institutes.

3.4. Final Instrumental Analysis. Based on the method optimization and quality control, the following parameters were chosen for the instrumental analysis.

3.4.1. GC-QqQ-MS. The HS-SPME-GC-QqQ-MS analysis was performed using a PAL RTC 120 autosampler (CTC, Zwingen, Switzerland). Separation and detection of the cedarwood oil constituents were done using an Agilent Technologies 7890B GC system coupled with a 7010B QqQ-MS (Agilent Technologies, Denmark).

An SPME fiber with 30 µm poly(dimethylsioxane) (PDMS) coating (Supelco, Bellafonte, PA) was used for the HS-SPME extraction. The SPME fiber was conditioned in accordance

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with the manufacturer’s instructions prior to use. The vial was transferred to the agitator and given 10 min to reach the sampling temperature of 35 °C. The fully automated HS-SPME sampling was then conducted for 40 min at an agitation speed of 250 rpm. Following HS-SPME sampling, the SPME fiber was desorbed for 10 min in the split/splitless GC inlet at 275 °C. The injection inlet was operated in splitless mode with a septum purge flow of 3 mL min⁻¹ for 10 min, followed by a 100 mL min⁻¹ purge flow to the split vent for an additional 10 min. In between samples, the fiber was cleaned in the conditioning station at 300 °C for 5 min.

The GC was fitted with an Agilent 122-5562UI DB5-ms 60 m × 0.25 mm, 0.25 μm thickness column using 1.2 mL min⁻¹ helium as carrier gas. The GC oven program was as follows: initial column temperature of 50 °C for 10 min, 30 °C min⁻¹ increase to 200 °C, followed by an increase at 5 °C min⁻¹ to 300 °C, and an increase at 30 °C min⁻¹ to 310 °C, held for 1 min. The total GC cycle was 37 min.

The QqQ-MS was operated in multiple reactions monitoring mode (MRM), the ion source in electron ionization (EI) mode with an electron energy of 70 eV. General parameters for MRM were as follows: the EMV was 1409, filament current 35 μA, and dwell time 50 ms. The MS transfer line was held at 250 °C, the ion source temperature was 230 °C and the quadrupole temperature was 150 °C. In the collision cell, nitrogen was used as collision gas at a flow of 2.25 mL min⁻¹ and helium as quench gas at 1.5 mL min⁻¹. Target compounds, retention times, transitions, and collision energies are presented in Table S4 for the MRM method.

3.4.2. GC-Orbitrap-MS. The HS-SPME-Orbitrap-MS analysis was performed using a Triplus RSH autosampler with an Arrow SPME, a Thermo Scientific Trace 1310 Series GC coupled to a Thermo Scientific Orbitrap-MS. An Arrow SPME fiber with 100 μm poly(dimethylsiloxane) (PDMS) was used for the HS-SPME extraction. The SPME fiber was conditioned in accordance with the manufacturer’s instructions prior to use.

The fully automated HS-SPME sampling was conducted at 35 °C for 90 min at an agitation speed of 250 rpm. Following HS-SPME sampling, the SPME fiber was desorbed for 10 min in the split/splitless GC inlet at 275 °C. The injection inlet was operated in splitless mode with a septum purge flow of 5 mL min⁻¹ for 10 min, followed by a 100 mL min⁻¹ purge flow to the split vent for an additional 10 min.

In between samples, the fiber was cleaned in the conditioning station at 300 °C for 5 min. The GC was fitted with a 30 m × 0.25 mm ID, TG-5SilMS capillary column (Thermo), with a 0.25 μm film thickness. Helium was used as carrier gas at a constant flow of 1.2 mL min⁻¹.

The GC oven temperature program started at 40 °C (held for 12 min), followed by an increase by 10 °C min⁻¹ to 250 °C and 30 °C min⁻¹ to 300 °C. The final temperature was held for 2 min. The total GC cycle was 42 min. The MS transfer line was set to 280 °C and the ion temperature source to 230 °C. The MS instrument was operated in EI mode (70 eV). Full scan MS acquisition using an m/z range of 50–500 was performed. The resolution was at 60 000 and the AGC target at 1 × 10⁶.

3.5. Data Processing. Agilent MassHunter Qualitative and Quantitative Analysis for QQQ (vers. B.09.00/Build 9.0.647.0) was used for peak identification, integration, and quantification of compounds included in the MRM method. For the Orbitrap analysis, peak identification and integration were done using Thermo Scientific Xcalibur Qualitative Analysis (version 3.1.6.10). Identification of the detected compounds was based on standards (where possible), comparison with suspect spectra from a custom suspect-screening spectral library based on the characterization data provided by Givaudan UK Ltd., calculated retention indices, and NIST spectral searches for the tentative identification of detected constituents that were not part of the suspect library. Further data processing was performed using RStudio (version 1.1.456) and GraphPad Prism (version 7.03). Data points below the LOD were treated as 1/2 LOD in the statistical analysis.

4. RESULTS

4.1. Results of the Method Optimization and Quality Control. A total of 12 cedarwood oil constituents with retention times across the entire chromatogram were selected to optimize the HS-SPME sampling time (Figures S1 and S2). Using the HS-SPME-GC-QqQ-MS, most target analytes reached equilibrium within sampling times between 30 and 45 min. The only exemptions were β-himachalene (t₉₀ of 74 min), widdrol, and cedrol that both had t₉₀ of over 3 h (Table S1). From the characterization data of the cedarwood oil and its previous analysis in rainbow trout, we knew that cedrol is metabolized rapidly. Moreover, we found that both widdrol and β-himachalene are comparably minor constituents in Virginian cedarwood oil. Therefore, we chose a sampling time of 40 min, accepting that β-himachalene, widdrol, and cedrol would not be at equilibrium at this point.

For the HS-SPME-GC-Orbitrap-MS, equilibrium times of the tentatively identified target analytes were longer than in the HS-SPME-GC-QqQ-MS analysis (Table S2), because the Arrow SPME had a thicker coating (100 μm) than the SPME fiber applied in the HS-SPME-GC-QqQ-MS analysis (30 μm). Most target analytes had equilibration times around 90 min. Similar to the HS-SPME-GC-QqQ-MS equilibration times, cedrol and widdrol needed significantly longer to reach equilibrium. Due to their high metabolism rate in fish, the SPME sampling time was selected based on the remaining target analytes and set to 90 min.

Repeated measurements of the same sample resulted in similar peak areas (<10% deviation), confirming negligible depletion on both analytical platforms. Method blanks based on empty vials, vials with NaCl solution, vials with miglyol, and carryover were found to be negligible in all analyzed blank samples. α-Cedrene, β-cedrene, thujopsene, and cedrol were detected in fish from the control group with relative peak areas of at least 4 orders of magnitude below the relative peak areas in the exposed fish. Blank levels of the remaining analytes were below a signal-to-noise ratio of 3. The resulting LOD ranged from a relative peak area of 0.079 for 9-cedranone to a relative peak area of 9.2 for α-cedrene. The LOQ ranged from a relative peak area of 0.26 for 9-cedranone to a relative peak area of 25 for α-cedrene. A full list of the detected blanks in the control fish, the LODs, and LOQs is presented in Table S3.

All analyzed samples were within the linear range of the detectors. Triplicate analyses were in good agreement with less than 10% standard deviation. Details on the accuracy and robustness of the method are presented in Section 5.

4.2. Application on In Vivo Cedarwood Oil Samples. 4.2.1. Measured Cedarwood Oil Constituents. A total of 15 cedarwood oil constituents were identified in the pure cedarwood oil accounting for around 83% of the instrument response peak area of the whole mixture. Six constituents (α-funebrene, α-cedrene, β-cedrene, thujopsene, cuparene, and...
cedrol) were confirmed using analytical standards. The remaining nine (α-chamigrene, β-chamigrene, β-himachalene, α-longipinene, β-funebrene, 4-epi-α-acoradiene, α-cuprenene, γ-himachalene, and widdrol) were tentatively identified using suspect-screening with a custom spectral library based on the characterization data provided by Givaudan UK Ltd., a matching factor >800, and matching retention indices (Tables S5, S6, and 1).

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<th>constituents</th>
<th>pure oil</th>
<th>day 1</th>
<th>day 7</th>
<th>day 14</th>
<th>day 28</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>α-cedrene</td>
<td>x</td>
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<tr>
<td>β-cedrene</td>
<td>x</td>
<td></td>
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<td></td>
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<tr>
<td>thujopsene</td>
<td>x</td>
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<tr>
<td>cuparene</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cedrol</td>
<td>x</td>
<td></td>
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<tr>
<td>α-chamigrene</td>
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<td>β-chamigrene</td>
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<tr>
<td>α-cuprenene</td>
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<td>9-cedranone</td>
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</table>

“Detected constituents (>LOD) are marked with “x”.

In the fish samples collected at the day of exposure, a total of 13 cedarwood oil constituents were detected using HS-SPME-GC-Orbitrap-MS analysis with a suspect-screening using the custom library and a matching factor >800, as well as a NIST spectral library search using accurate mass, and matching retention indices for compounds that could not be identified through the suspect-screening (Table S7). The tentatively identified constituents in fish sampled on day 1 after the exposure were the six main cedarwood oil constituents (α-funebrene, α-cedrene, β-cedrene, thujopsene, cuparene, and cedrol), constituents in the custom suspect library: 4-epi-α-acoradiene, β-chamigrene, α-longipinene, β-funebrene, and widdrol, as well as thujopsadiene and 9-cedranone, which were not present in the characterization data of the pure oil and had not been detected in the pure oil. α-Cuprenene and β-himachalene were not detected in any of the fish samples even though they were present in the pure oil (Table S7). 9-Cedranone was not detectable in fish sampled after day 4 after the exposure. In fish sampled on day 14 after he exposure 10 constituents were detected (α-longipinene and widdrol were not detectable). This was further reduced to 9 constituents on day 28 after the exposure (cedrol was not detectable at that point) (Table 1).

4.2.2. Depuration Rate Constants and Derived Kinetic BCFs. Using MRM data and HCB as internal benchmark, average kTG ranged from 0.014 day⁻¹ for β-cedrene to 0.18 day⁻¹ for cedrol, which correspond to median kinetic BCfBM between 660 L kg⁻¹ ww for cedrol and 8900 L kg⁻¹ ww for β-cedrene when the Arnot and Gobas model is used to estimate k1. The derived half-lives (t1/2) for the cedarwood oil constituents in fish ranged from 4 days for cedrol to 51 days for β-cedrene (Table S8).

Using Orbitrap suspect-screening analysis and HCB as internal benchmark, average kTG for the tentatively identified cedarwood oil constituents ranged from 0.016 day⁻¹ for α-cedrene to 0.19 day⁻¹ for cedrol (Figure S3), which meant that the benchmarked peak areas of the individual constituents declined by <10% for α-cedrene to 100% for cedrol (Figure 1). The derived median kinetic BCfBM ranged from 650 L kg⁻¹ ww for cedrol to 7500 L kg⁻¹ ww for β-cedrene (Table S9).

In addition to the six major cedarwood oil constituents, kTG and BCfBM could be quantified for seven additional cedarwood oil constituents in the exposed fish. The kTG for these tentatively identified constituents ranged from 0.02 day⁻¹ for β-chamigrene to 0.79 day⁻¹ for 9-cedranone. The resulting median kinetic BCfBM ranged from 150 L kg⁻¹ ww for 9-cedranone to 3300 L kg⁻¹ ww for β-chamigrene (Figure S3 and Table 2).

5. Discussion

The depuration rate constants and derived kinetic BCFs obtained from the Purge and Trap GC-MSD, HS-SPME-GC-QqQ-MS, and HS-SPME-GC-Orbitrap-MS were in very good agreement when using internal benchmarking. Without internal benchmarking, on the other hand, the results differed considerably (Figures 2 and S4 and Table S10). The average % differences between the results from the different analytical techniques were 229% for k1, 21% for kTG, and 19% for kinetic BCfBM. The relative standard error [%] of the non-benchmarked HS-SPME-based measurements was significantly lower than the relative standard error of the targeted Purge and Trap measurements. The relative standard errors of the depuration rate ranged from 8% for cedrol to 2406% for α-cedrene in HS-SPME-Orbitrap-MS measurements (Figure S3) and from 25% for cedrol and 5628% for α-cedrene in targeted Purge and Trap GC–MS measurements.16 The relative standard errors of the benchmarked depuration rates, on the other hand, were comparable for HS-SPME-based measurements and Purge and Trap measurements, with relative standard errors ranging from 15 to 111% (median: 50%) for HS-SPME-Orbitrap-MS measurements (Figure S3) and from 16 to 57% (median: 41%) for targeted Purge and Trap GC–MS measurements.17 The measurements were done on aliquots of the same fish samples; therefore, the higher variability of the Purge and Trap method has to be linked to the analytical method itself rather than differences between the uptake among individual fish.

These observations indicate that (a) there is a high interindividual variability of cedarwood oil concentrations among the exposed fish, (b) analyte can be lost in the extraction and clean-up process, and (c) internal benchmarking is a powerful tool to correct for both interindividual and analytical variabilities such as analyte losses, thereby enabling a quantification based on relative peak areas for constituents without available analytical standards.

The good agreement between results obtained from analysis performed at different times, in different labs, by different people, and on different aliquots of the fish highlight the robustness and repeatability of the Purge and Trap GC–MSD, the HS-SPME-GC-QqQ-MS, and the HS-SPME-GC-Orbitrap-MS methods. All three methods are clearly well suited to...
quantify $k_{TG}$ and kinetic BCF$_{BM}$ of the volatile, hydrophobic constituents of cedarwood oil in rainbow trout following in vivo dietary exposure. However, the HS-SPME-GC-QqQ-MS and HS-SPME-GC-Orbitrap-MS methods have a number of advantages compared to the Purge and Trap GC-MSD method.

First, the GC-HS-SPME-QqQ-MS and HS-SPME-GC-Orbitrap-MS analysis and quantification of $k_{TG}$ were performed entirely based on the peak areas of the detected analytes—thereby enabling the quantification of constituents without available analytical standards. This was possible because of the SPME sampling under (near) equilibrium conditions. The high agreement of the obtained results with the targeted GC-MSD analysis confirmed that the benchmarking approach corrected well for possible matrix effects on the HS-SPME sampling and that quantification of $k_{TG}$ based on peak areas alone was possible. Using HS-SPME sampling with internal benchmarking and suspect-screening analysis for the quantification of $k_{TG}$ of a UVCB like cedarwood oil offers the opportunity to determine the kinetic BCF of numerous UVCB constituents without restrictions due to the availability (or lack) of analytical standards. It should be noted that the method can also be applied for semivolatile UVCBs. For more complex UVCBs than cedarwood oil, the methodology can be adapted to quantify the BCF of unknown constituents in a mixture using nontargeted analysis and facilitate semiquantitation of the actual amounts taken up by the organism by using averaged response factors for tentatively identified or unknown compounds that do not have standards.$^{20,21}$

![Figure 1. Change of the relative peak areas ($A_{relative}$) over time for the main cedarwood oil constituents benchmarked with HCB compared to benchmarked peak areas at the day of exposure.](image)

![Table 2. Median $t_{1/2}$ [Days], Average ± Standard Deviation $k_T$ [Day$^{-1}$], $k_{TG}$ [Day$^{-1}$], Median, 5th Percentile and 95th Percentile Kinetic BCF [L kg$^{-1}$ ww], and Kinetic BCF$_{BM}$ [L kg$^{-1}$ ww] of the Tentatively Identified Cedarwood Oil Constituents Measured with HS-SPME-GC-Orbitrap-MS in Full Scan](table)
Furthermore, the use of the fully automated HS-SPME sampling technique reduces the sample handling to homogenization and sampling into a vial, compared to a solvent extraction, evaporation, and elution of the Purge and Trap GC-MSD method. The minimalized sample handling reduces the risk of analyte losses as well as the risk for errors during the extraction and clean-up process. The HS-SPME-GC-MS method therefore offers a high level of robustness.

Finally, the elimination of a solvent extraction and clean-up reduces lab time for the analysts and increases the safety both from environmental and workplace points of view by eliminating the need for organic solvents.

In the case of cedarwood oil, using HS-SPME-GC-Orbitrap-MS enabled us to measure the depuration rate and determine the bioconcentration for >80% of the constituents in the mixture (based on the composition analysis provided by Givaudan), compared to around 75% using purge and trap GC-MSD with analytical standards. The tentatively identified compounds included substances that were expected to be present in cedarwood oil based on the available characterization data (widdrol, β-funebrene, β-chamigrene, α-longipinene, 4-epi-α-acoradiene), thuipsadiene that was not detected in the pure oil but has previously been reported in essential oils, including cedarwood oil, as well as a possible derivative of the target analytes (9-cedranone). Of the tentatively identified constituents, 9-cedranone and widdrol were eliminated most effectively with median kinetic BCFBM = 500, while β-chamigrene had a kinetic BCFBM > 2000 at a 95% confidence level (Table 1).

Overall, we observed that aliphatic sesquiterpenes such as the cedrenes, thuipsene, or chamigrene were eliminated slower from the fish than the mixed aliphatic and aromatic sesquiterpene cuparene. The fastest elimination rates were observed for the hydroxylated sesquiterpenes cedrol and widdrol as well as the oxidized sesquiterpene 9-cedranone. The rapid elimination of cedrol, widdrol, and 9-cedranone, respectively, is likely due to their hydroxyl-groups and carbonyl group (in the case of 9-cedranone) that allow for Phase II metabolic reactions. Chen et al. reported that terpenes present in pine oil were readily eliminated in a similar dietary exposure in vivo test. It should be noted that the use of 1/2 LOD for nondetected concentrations means that the derived BCF for cedrol, widdrol, and 9-cedranone should be seen as a conservative “worst case” BCF.

The presented HS-SPME-GC mass spectrometry method offers the possibility to identify and quantify potentially bioaccumulative constituents in different essential oils based on commercially available essential oil mixtures without available analytical standards, thereby providing the opportunity to develop databases of constituent structures and their respective kTG or kinetic BCFBM which could be used to develop a quantitative structure–activity relationship model for essential oils. This information will improve the understanding of the structural features that drive the bioaccumulation potential of different essential oil constituents and potentially reduce the need for further animal testing. The quantitative analysis of the kinetic BCF of suspect or even unknown constituents is a powerful tool to better understand the potential environmental impact of UVOCBs.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c00179.

Equilibrium sampling time for the cedarwood oil constituents identified and tentatively identified with GC-QqQ-MS and GC-Orbitrap-MS; average detected blanks; limit of detection, and limit of quantification; target compounds, retention times, transitions, and collision energy of the GC-QqQ-MS MRM method; nonisothermal Kovats retention index; confirmed and tentatively identified constituents in pure cedarwood oil measured by HS-SPME-GC-QqQ-MS and HS-SPME-GC-Orbitrap-MS; confirmed and tentatively identified cedarwood oil constituents in fish measured by HS-SPME-GC-QqQ-MS and HS-SPME-GC-Orbitrap-MS; median t1/2 [days], average ± standard deviation kT [day−1], kTG [day−1], median, 5th percentile and 95th percentile kinetic BCF [L kg−1 ww], and kinetic BCFBM [L kg−1 ww] of the cedarwood oil constituents measured with HS-SPME-GC-QqQ-MS in MRM and HS-SPME-GC-Orbitrap-MS in full scan; depuration kinetics for the main cedarwood oil constituents in different essential oils based on the presented HS-SPME-GC mass spectrometry method.

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**Figure 2.** Depuration rate constant (kT) [day−1] (left) and benchmarked depuration rate constant (kTG) [day−1] (right) for the main cedarwood oil constituents measured by HS-SPME-GC-Orbitrap-MS (circles) and HS-SPME-GC-QqQ-MS (triangles) with quantification based on relative peak areas in comparison to kT and kTG of the same cedarwood oil constituents measured by GC-MSD with quantification using analytical standards and external calibration. A perfect fit line (1:1) is included as a visual reference.
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