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Short Communication

EXHAUSTIVE STEAM DISTILLATION EXTRACTION OF AROMATIC ORGANICS FROM RAINBOW TROUT AND WATER

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Abstract—Exhaustive steam distillation extracted several intermediate molecular weight organic chemicals effectively from rainbow trout, but a high molecular weight organic, benzo[*a*]pyrene, was not extracted. Fish lipids that do not steam-distill appear to retain extremely lipophilic organics in the boiling flask. Therefore, exhaustive steam distillation should be used cautiously in screening effluents for chemicals that are bioaccumulated.

Keywords—Phenol Naphthalene Anthracene Benzo[*a*]pyrene *Salmo gairdneri*

INTRODUCTION

In evaluating the potential environmental hazards of complex chemical mixtures and effluents, it is often desirable to determine the bioaccumulation potential of a wide range of organic compounds and their metabolites using a relatively rapid screening procedure. Although various techniques have been developed to extract organics from fish, many are specific for individual chemical classes and are relatively labor-intensive for use in initial bioaccumulation screening. One potentially useful technique is exhaustive steam distillation (ESD), which isolates organic compounds based on their vapor pressures rather than their chemical functionalities. Veith and Kiwus [1] originally proposed this procedure to extract pesticides and industrial chemicals from fish and sediments. Despite the ease and rapidity of extraction, without the need for solvent concentration and lipid cleanup steps that are required in many other extraction procedures, ESD has not been used routinely in bioaccumulation studies. To date, most of its applications have been limited to analyses of hydrocarbons in sediment [2]. In this article, we report results for the extraction of aromatic and polycyclic aromatic organic compounds from fish and water using exhaustive steam distillation, and discuss this technique's potential uses and limitations for evaluating bioaccumulation hazards of complex chemical mixtures.

MATERIALS AND METHODS

We evaluated ESD extraction efficiencies for four radiolabeled organic compounds: [U-^{14}C]phenol (New England Nuclear), [9-^{14}C]anthracene (California Bionuclear Corporation), and [1(4,5,8)-^{14}C]naphthalene and [7,10-^{14}C]benzo[*a*]pyrene (Amersham Corporation). The solubilities and octanol/water partition coefficients of these compounds are listed in Table 1. For each compound, extraction efficiencies were determined by extracting (a) aqueous solutions spiked with radiolabeled compound and (b) rainbow trout (*Salmo gairdneri*) that had previously bioaccumulated radiolabeled compound.

To prepare fish for ESD extraction, we exposed rainbow trout to radiolabeled compound in well water for 16 to 18 h and then homogenized the whole fish in a Waring blender. Five subsamples (15 to 20 g) of each homogenate were frozen for subsequent ESD extractions, while 10 smaller aliquots (0.1 g) were transferred to scintillation vials, solubilized in 2 ml of NCS tissue solubilizer (Amersham Corporation), and assayed by liquid scintillation counting to determine ^{14}C uptake. For these fish tissue samples and for the aqueous and organic solvent samples described below, a Beckman LS-100C liquid-scintillation counter was used to assay radioactivity. All samples were counted in scintillation cocktail (8.25 g POP, 0.75 g POPOP and 500 ml Triton X-100 per liter of toluene) into

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Table 1. Physical-chemical characteristics of organic compounds used in extraction experiments

Compound	Molecular weight	Aqueous solubility at 25°C (mg/L)	Octanol/water partition coefficient
Phenol	94.1	82[a]	29[b]
Naphthalene	128.2	31.7[c]	2,350[b]
Anthracene	178.2	0.073[c]	28,000[b]
Benzo[*a*]pyrene	252.3	0.0038[c]	288,000[d]

[a]From ref. 7.
[b]Converted from log *P* values in ref. 4.
[c]From ref. 8.
[d]Estimated from Equation 6 in ref. 9.

which 2 ml of glacial acetic acid was added to reduce chemiluminescence. H_2O_2 (25 μl) was added to fish tissue digests to reduce color quench.

The ESD extraction procedure was modified from Veith and Kiwus [1]. Briefly, it involved boiling each homogenized tissue sample in 400 ml of 1% H_2SO_4 under reflux for 16 h. Condensed vapors descended through an extraction solvent reservoir in the condenser column before returning via an overflow tube to the boiling flask, thus partitioning contaminants into the extraction solvent (see Fig. 1 in ref. 1). After cooling, the volume of boiling flask liquid was measured and 100 ml was filtered. Radioactivity in both the unfiltered and filtered boiling flask liquids was assayed to determine ^{14}C remaining in the boiling flask and ^{14}C not extracted from fish tissue residues. Additionally, the extraction solvent and residual water in the condenser column were analyzed for ^{14}C activity. To compute extraction efficiencies and ^{14}C recoveries, we calculated quantities of ^{14}C in each distillation liquid and divided that value by the total amount of ^{14}C in the homogenized tissue that was initially placed in the boiling flask. Efficiencies calculated for the extractions of the four bioaccumulated compounds from fish were then compared with aqueous extractions of the same compounds. To determine these aqueous extraction efficiencies, we added a known quantity of radiolabeled compound to 400 ml of 1% H_2SO_4 in five replicate ESD flasks with no fish tissue present. These spiked solutions were then boiled and analyzed as described above.

Based on preliminary studies of several potential extraction solvents, we selected a mixture containing 75% *n*-butyl ether:20% cyclohexane:5% *n*-octanol to be used in the ESD solvent reservoir. This solvent mixture provided an acceptable compromise among (a) specific gravity (must be less dense than water for condensate to descend through the solvent and return to the boiling flask), (b) high extraction efficiency for phenolic compounds and for less polar polycyclic aromatic organics and (c) compatibility with high pressure liquid chromatography (HPLC). We also determined that ESD extraction of phenol was maximized after 6 h of refluxing. Therefore, 16-h overnight distillations provided a convenient work schedule and sufficient time for complete removal of extractable compounds from fish tissue.

During extractions of ^{14}C-labeled phenol and anthracene tissue, we additionally fractionated the steam distillation reservoir solvent layers, reservoir water layers and filtered boiling flask liquids using HPLC. Samples of each liquid were injected onto a μ-C_{18} reversed-phase HPLC column, eluted isocratically at a rate of 2.0 ml/min with 40% MeOH:60% H_2O for phenol fractionations and 60% CH_3CN:40% H_2O for anthracene fractionations, and monitored at 254 nm UV absorbance. The mobile phase eluting prior to the retention time of the parent compound was collected and designated the precontaminant fraction; the mobile phase containing the parent compound was also collected. We assayed each fraction for radioactivity to determine the purity of the original radiolabeled compound, the presence of possible metabolites in the precontaminant fraction and the recovery of total ^{14}C.

Additional ESDs were conducted with spiked aqueous solutions and with control and previously exposed fish to investigate low benzo[a]pyrene (BAP) extraction efficiencies. First, fish exposed to [^{14}C]BAP were digested at room temperature in 2 N KOH, neutralized to pH 7 with acetic acid to prevent foaming during boiling and steam-distilled for 16 h to determine if the pH of the boiling flask liquid affected extraction efficiency. Also, [^{14}C]BAP was spiked into boiling flasks containing (a) uncontaminated fish tissue and 1% H_2SO_4 or (b) uncontaminated fish tissue that had previously been digested in 2 N KOH and neutralized to pH 7 with acetic acid to determine if the presence of uncontaminated fish tissue affected extraction efficiency. Finally, [^{14}C]BAP was spiked into boiling flasks containing only pH 7 water or 2 N KOH. All of these treatments were boiled and analyzed as in the ESDs described above.

RESULTS

Phenol, naphthalene and anthracene were extracted with high efficiency from rainbow trout and from 1% H_2SO_4 with no fish tissue present (Table 2). However, BAP was not extracted from

Table 2. Efficiencies of extracting radiolabeled compounds by exhaustive steam distillation from spiked acidic solution and from rainbow trout[a]

Chemical	Aqueous extractions		Tissue extractions	
	Extraction efficiency[b] (%)	^{14}C recovery[c] (%)	Extraction efficiency[b] (%)	^{14}C recovery[c] (%)
Phenol	61 (±2)	99 (±2)	47 (±2)	87 (±2)
Naphthalene	100 (±1)	113 (±4)	99 (±1)	106 (±1)
Anthracene	97 (±4)	97 (±3)	92 (±2)	108 (±5)
Benzo[*a*]pyrene	77 (±4)	112 (±10)	<1 (±<1)	93 (±3)

[a]See text for details of extraction procedures. Numbers in parentheses are 1 SD of the mean value ($n = 5$).
[b]Extraction efficiency is expressed as the percentage of total ^{14}C activity that partitioned into the extraction reservoir solvent.
[c]Recovery is expressed as the percentage of ^{14}C activity initially in the boiling flask that was accounted for in the system after exhaustive steam distillation extraction.

fish. Even though we accounted for 93% of the original [^{14}C]BAP after boiling in 1% H_2SO_4, greater than 99% of that ^{14}C activity remained in the boiling flask (Table 2). Of this unextracted ^{14}C activity, 85% could be filtered from the solution as ^{14}C either still associated with the tissue residue or adsorbed onto the filter. Less than 1% of the ^{14}C activity was extracted when control fish homogenates were spiked with [^{14}C]BAP and refluxed in 1% H_2SO_4 (Table 3), again demonstrating low BAP extraction efficiencies in the presence of fish tissue. Furthermore, digesting fish in 2 N KOH and neutralizing the boiling flask liquid did not improve extraction efficiencies. Yet [^{14}C]BAP was extracted with high efficiency from acidic, neutral and basic aqueous solutions that contained no fish tissue, indicating that BAP did codistill with water in this apparatus (Table 3). Increasing the pH of the aqueous solution increased the efficiency of extracting BAP.

HPLC fractionation of phenol and anthracene ESD liquids supported the reliability of this technique for extracting these two compounds and their metabolites from fish. For phenol extractions, all ^{14}C activity in the extraction solvent layer appeared in the parent phenol peak. In the boiling flask liquid, however, only 56% of the ^{14}C activity appeared in the phenol peak. On the

Table 3. Efficiencies of extracting [^{14}C]-benzo[*a*]pyrene (BAP) by exhaustive steam distillation from spiked aqueous solution, spiked rainbow trout and rainbow trout previously exposed to [^{14}C]BAP[a]

Treatment	Boiling flask liquid		
	1% H_2SO_4	Neutralized 2 N KOH	2 N KOH
Spiked aqueous solution	77 (±4)	91 (±1)	99 (±1)
Spiked fish homogenate	<1 (±<1)	2 (±<1)	—
Exposed fish homogenate	<1 (±<1)	<1 (±<1)	—

[a]See text for details of extraction procedures. Extraction efficiency is expressed as the percentage of total ^{14}C activity that partitioned into the extraction reservoir solvent; numbers in parentheses are 1 SD of the mean value ($n = 5$). (—), value not determined because of excess foaming during boiling.

basis of total ^{14}C activity present in the distillation apparatus after boiling, 76% of the ^{14}C activity was in the extractable phenol and only 24% was in the unextractable metabolites or parent compound. In the anthracene extractions, 92% of the ^{14}C activity appeared in the extraction solvent layer. Within this layer, though, 22% of the ^{14}C activity appeared in the precontaminant fraction. Because of its shorter retention time, we assume that this ^{14}C activity in the precontaminant fraction represented anthracene metabolites that were liberated from fish tissue and partitioned into the extraction solvent layer. However, we did not chemically identify either compound's metabolites.

DISCUSSION

Bioaccumulation potentials of organic chemicals can be predicted from octanol/water partition coefficients [3] and from reversed-phase HPLC retention times [4,5]. But not all organics bioaccumulate as intensively as predicted from surrogate physical-chemical parameters. For example, Southworth et al. [6] demonstrated that the predicted uptake of azaarenes from water was up to one order of magnitude greater than measured bioaccumulation in fathead minnows (*Pimephales promelas*). In that study, the sum total of metabolites of dibenz[*a*,*h*]acridine accumulated to levels four times greater than did the parent compound, suggesting that initial metabolic transformation of parent compound was not the rate-limiting step in the depuration process. Given the complexity of some organic mixtures and the potential for persistent metabolites to be formed (e.g., DDE from DDT), bioaccumulation hazards may be difficult to estimate a priori for some effluents. Therefore, rapid screening techniques are needed to confirm bioaccumulation of organic compounds from complex chemical mixtures.

In this study, we found that three intermediate molecular weight aromatic organic compounds (phenol, naphthalene and anthracene) and their metabolites were extracted effectively from rainbow trout using ESD. But a high molecular weight, low-solubility aromatic organic (BAP) was not extracted from water to which we added homogenized fish containing no BAP, or from fish that had previously bioaccumulated the chemical. Thus, fish tissue acted as a "keeper" and did not allow BAP to codistill with water. This may have been caused by adsorption of BAP to protein residues. More likely, fatty acids from fish tissue retained BAP in hydrophobic micelles. Since lipids do not distill in the ESD apparatus [1], BAP probably remained with the lipids in the boiling flask because of BAP's high lipophilicity and low water solubility (Table 1).

The ease and rapidity of ESD make it a promising technique for bioaccumulation screening of effluents. In fact, steam distillation has been shown in this study and previous studies to extract low and intermediate molecular weight aliphatic, aromatic and chlorinated hydrocarbons and their metabolites effectively from water, sediment and fish [1,2]. For these chemical classes, we believe that ESD is superior to techniques such as solvent homogenization and Soxhlet extraction because (a) it extracts phenolics more efficiently (Meyer, unpublished data) and (b) it does not require tedious cleanup steps to prepare samples for liquid or gas chromatography. However, ESD does not extract extremely high molecular weight, lipophilic organics from fish, even though they can be extracted from water and sediments using this technique. Since extremely lipophilic organics are often a major concern in bioaccumulation screening, another extraction technique that recovers those compounds might be used in conjunction with steam distillation to encompass the range of potentially bioaccumulated chemicals in an effluent.

In this study, we boiled fish for 16 h in a relatively strong acid solution. Under those conditions, aromatic amines and other organic bases may be hydrolyzed and hence not recovered quantitatively. Distilled H_2O at neutral pH can be substituted for 1% H_2SO_4 as the boiling flask liquid if such reactions are a concern; however, recoveries of phenolic compounds and other weak acids may decline as the pH of the boiling flask liquid is increased. Thus, the extraction technique can be optimized differently for various complex chemical mixtures. Judicious use of ESD can save considerable time and effort during preliminary screening of effluents, as long as the results are interpreted in light of the technique's limitations.

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