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Predicting the Bioconcentration of Fragrance Ingredients by Rainbow Trout Using Measured Rates of *in Vitro* Intrinsic Clearance

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Supporting Information

	Modified BCFBAF v3.01 model			IVIVE model		OECD 305
Chemical	Regression Method	Arnot-Gobas (without biotrans-formation)	Arnot-Gobas (with biotrans-formation)	BCF_{TOT} f_u calc.	BCF_{TOT} $f_u = 1.0$	*in vivo* BCF
Peonile	non B	non B	non B	non B	non B	non B
δ-Damascone	non B	non B	non B	non B	non B	non B
Cyclohexyl salicylate	non B	B	non B	non B	non B	non B
Agrumex	non B	B	non B	non B	non B	non B
Musk xylene	non B	B	non B	B	B	B
Isolongifolanone	non B-B	B	B	B	non B	non B
Pentachlorobenzene	B	B	B	B	B	B
Opalal	B	B	B	B	non B	non B
Iso E Super	B	B	B	B	non B	non B
Methyl cedryl ketone	B	B	B	B	non B	non B-B

ABSTRACT: Bioaccumulation in aquatic species is a critical end point in the regulatory assessment of chemicals. Few measured fish bioconcentration factors (BCFs) are available for fragrance ingredients. Thus, predictive models are often used to estimate their BCFs. Because biotransformation can reduce chemical accumulation in fish, models using QSAR-estimated biotransformation rates have been developed. Alternatively, biotransformation can be measured by *in vitro* methods. In this study, biotransformation rates for nine fragrance ingredients were measured using trout liver S9 fractions and used as inputs to a recently refined *in vitro-in vivo* extrapolation (IVIVE) model. BCFs predicted by the model were then compared to (i) *in vivo* BCFs, (ii) BCFs predicted using QSAR-derived biotransformation rates, (iii) BCFs predicted without biotransformation, and (iv) BCFs predicted by a well-known regression model. For fragrance ingredients with relatively low (<4.7) log K_{OW} values, all models predicted BCFs below a bioaccumulation threshold of 1000. For chemicals with higher (4.7−5.8) log K_{OW} values, the model incorporating measured *in vitro* biotransformation rates and assuming no correction for potential binding effects on hepatic clearance provided the most accurate predictions of measured BCFs. This study demonstrates the value of integrating measured biotransformation rates for prediction of chemical bioaccumulation in fish.

INTRODUCTION

Bioaccumulation in aquatic species is a critical end point in the regulatory assessment of chemicals by authorities such as the European Chemicals Agency (ECHA) and the United States Environmental Protection Agency (U.S. EPA).[1] In general, this entails the determination of a fish bioconcentration factor (BCF). *In vivo* BCF determination (e.g., using OECD Test Guideline 305) requires the use of large numbers of animals with costly and labor-intensive procedures.[2] One principle of the REACH regulation is that the testing of chemicals on animals should be done as a last resort. Predictive models are commonly applied if no *in vivo* BCF data are available. To this end the great majority of substances are screened on the basis of their octanol−water coefficient (log K_{OW}). However, biotransformation can reduce the degree of passive bioaccumulation of hydrophobic chemicals.[3,4] Recent predictive models acknowledge this possibility. Based on a database of *in vivo* biotransformation rate constants (k_{MET}; equivalent to k_M in the cited work),[5] a screening-level quantitative structure−activity relationship (QSAR) model for estimating k_{MET} was developed.[6] This QSAR is based on the attribution of biotransformation of fragments and has been implemented in the Arnot-Gobas bioaccumulation models[7] within the U.S. EPA's Estimation Program Interface (EPI) Suite.[8]

In vitro systems have been proposed as alternative methods that can be used to provide metabolic data needed to refine modeled BCF estimates. Several groups have obtained *in vitro* data for fish using liver S9 fractions or hepatocytes and then extrapolated this information to the intact animal to predict

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biotransformation impacts on chemical bioaccumulation. Incorporation of *in vitro* biotransformation rates into current models resulted in predicted BCFs that were in better agreement with measured values than predictions obtained assuming no biotransformation.[9−12] To date, however, these studies have provided BCF predictions for relatively large fish (e.g., trout, 1 kg; carp, 375 g)[9] that are commonly used for the isolation of hepatocytes and subcellular fractions (liver S9 fractions or microsomes). An improved *in vitro-in vivo* metabolism extrapolation (IVIVE) model for predicting bioconcentration of chemicals in rainbow trout was recently published.[13] In contrast to previous models, the updated model accounts for the fact that animals used for BCF determinations tend to be relatively small. This is important because rate constants that control chemical uptake and elimination (e.g., the branchial uptake rate constant) are generally assumed to scale to a fractional exponent of body weight. In addition, small fish often contain less whole-body lipid than larger individuals of the same species. Thus, the updated model predicts BCF values for a standardized fish, defined as a 10-g rainbow trout that contains 5% whole-body lipid held at 15 °C. The model also incorporates recent refinements of key extrapolation factors and chemical partitioning relationships.[13]

The development of alternatives for bioaccumulation assessment is particularly pertinent to fragrance ingredients, a diverse class of chemicals comprising over 2000 compounds in regular use. Given this large number, it is impractical to conduct *in vivo* studies for the majority of such compounds. Furthermore, fragrance ingredients tend to be lipophilic with more than half having log K_{OW} values of >4. Therefore, in the absence of *in vivo* data, many are considered to have the potential to bioaccumulate. In this study, we determined the *in vitro* metabolic stability of nine fragrance ingredients (log K_{OW} of 4.0−5.8) with known *in vivo* BCFs using rainbow trout liver S9 fractions. Measured biotransformation rates were incorporated into the IVIVE model to predict a BCF for each chemical. These predicted values were then compared with measured *in vivo* BCFs and BCFs predicted from computer-based QSAR models.

MATERIALS AND METHODS

Chemicals. Fragrance ingredients were provided by Givaudan Schweiz AG. All other test chemicals and reagents were purchased from Sigma-Aldrich except for NADPH (Enzo Life Sciences). The structures of the chemicals tested in trout liver S9 fractions are shown in the Supporting Information (Figure S1). Additional information for each compound is given in Supplementary Table S1. In addition to the nine fragrance ingredients, pentachlorobenzene was tested as a known bioaccumulative control.

Rainbow Trout Liver S9 Fractions. Liver S9 fractions from rainbow trout were purchased from Life Technologies and stored at −80 °C (Batch 1). Additional S9 fractions (Batch 2) were prepared from rainbow trout as described by Johanning et al.[14] and stored in the same manner. Detailed information pertaining to the two batches, including a listing of compounds tested with each, is given as Supporting Information (Table S2).

Determination of *in Vitro* Biotransformation. The metabolic stability of test chemicals was determined as described by Johanning et al.[14] with slight modifications. Trout liver S9 fractions were preincubated in potassium phosphate buffer at pH 7.8 with alamethicin (25 μg/mL) on ice for 15 min. The following cofactors for phase I and II metabolizing enzymes were added: NADPH (1 mM), uridine 5′-diphosphoglucuronic acid (UDPGA; 2 mM), 3′-phosphoadenosine 5′-phosphosulfate (PAPS; 0.1 mM), and glutathione (GSH; 0.5 mM). Reactions were initiated by adding 20 μL of test compound dissolved in water (final concentration 1 μM for all compounds except 0.4 μM for pentachlorobenzene and 10 μM for Agrumex). The final test volume was 200 μL, and the final S9 protein concentration was 1 mg/mL. Range finding experiments were performed in duplicate to optimize substrate concentrations and incubation times. All final incubations were performed in triplicate at 12 °C under shaking conditions (Thermomixer, Eppendorf) in Hirschmann glass inserts. Closed 1.1 mL HPLC microvials were used to test pentachlorobenzene as well as fragrance ingredients with higher volatility (δ-damascone, Agrumex, isolongifolanone, Opalal). Testosterone biotransformation was determined as a positive control for phase I enzyme activity using NADPH as single cofactor without preincubation with alamethicin. Biotransformation of 7-hydroxycoumarin was determined using UDPGA as single cofactor for UDP-glucuronosyltransferase activity and PAPS as single cofactor for sulfotransferase activity.

Negative controls containing heat-denatured S9 protein (1 mg/mL) were run in parallel to distinguish between enzymatic activity and other processes leading to chemical loss such as abiotic degradation, volatilization, and adsorption to the reaction vessel.[14] In addition, parent chemicals were incubated with active S9 protein in the absence of added cofactors to evaluate the influence of cofactor-independent enzymes such as carboxylesterases. Reactions were stopped at appropriate time intervals (0−120 min) by adding an equal volume of acetonitrile (200 μL) containing methyl laurate (1 μM) as an internal standard. Samples were extracted with *tert*-butyl methyl ether (MTBE, 200 μL) in the same tubes and subjected to GC−MS analysis. Incubations with testosterone and 7-hydroxycoumarin were stopped by adding an equal volume of methanol prior to LC−MS analysis.

Instrumental Analysis. The fragrance ingredients and pentachlorobenzene were analyzed by GC−MS (Agilent MSD 5973 or Finnigan MAT SSQ 7000) with the mass spectrometer operated in selected ion monitoring mode. Analytical details are given in the Supporting Information (Table S3). Testosterone and 7-hydroxycoumarin were analyzed by LC−MS (3200 QTRAP ABSciex). Calibration samples were prepared in potassium phosphate buffer, pH 7.8, in the presence of 1 mg/mL heat inactivated S9 protein and extracted as described above.

Determination of *In Vitro* Intrinsic Clearance Rate. Measured concentrations of test compounds were $\log_{10}$-transformed and regressed against time (h). A first-order reaction rate constant (1/h) was calculated by multiplying the fitted slope term from the regression equation by −2.3. The *in vitro* intrinsic clearance rate ($CL_{IN\ VITRO,INT}$; mL/h/mg S9 protein) was then calculated by multiplying the reaction rate by the S9 protein concentration (mg/mL).[14]

***In Vitro-In Vivo* Extrapolation (IVIVE) Model.** An updated IVIVE model was applied to calculate refined BCF estimates (L/kg) for a "standard fish", defined as a 10 g rainbow trout held at 15 °C that contains 5% whole-body lipid.[13] The model uses the estimated $CL_{IN\ VITRO,INT}$ to calculate a hepatic clearance rate (CL_H; L/d), which is then divided by each compound's estimated volume of distribution (V_D; L) to

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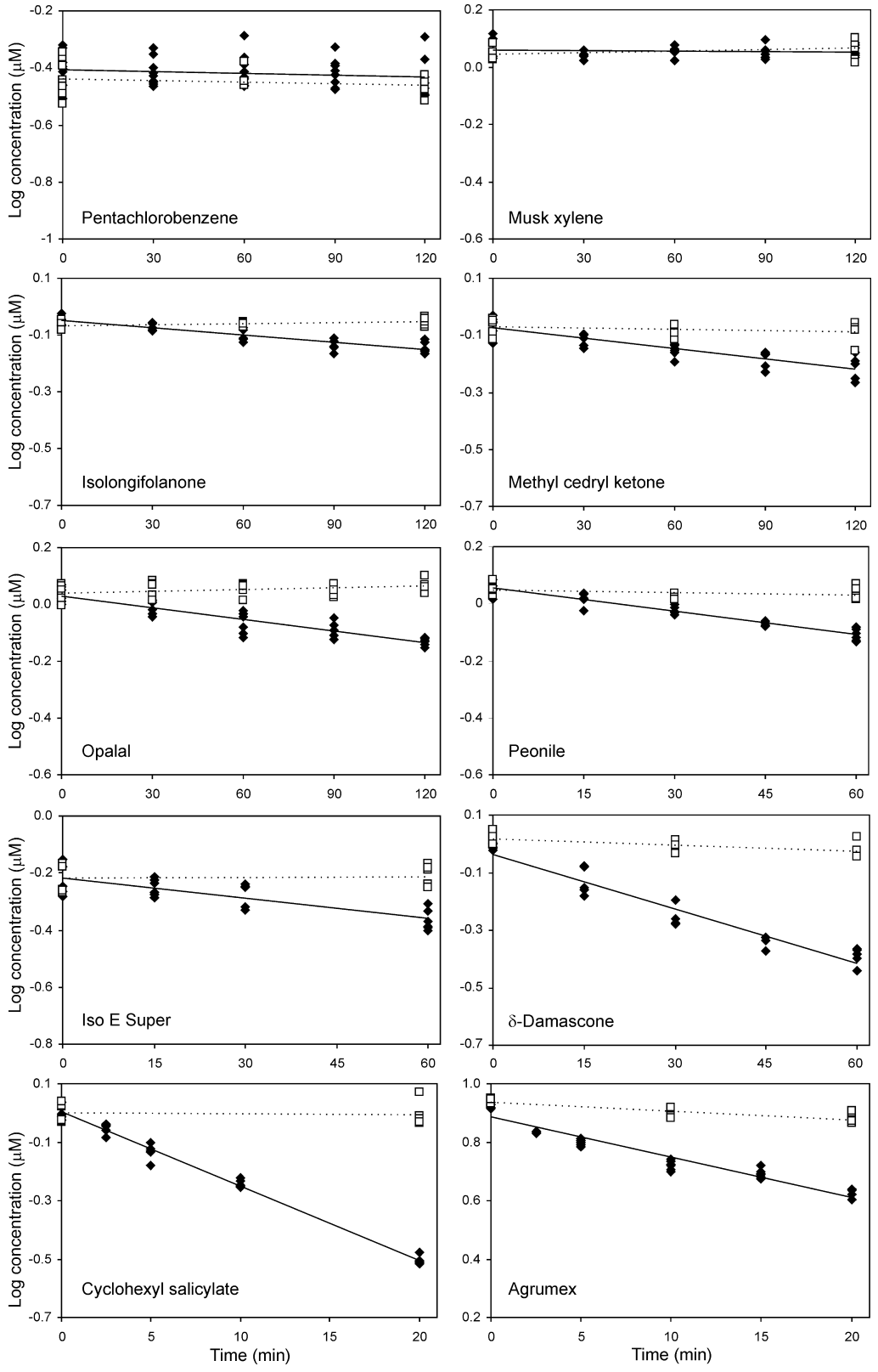

Figure 1. Biotransformation of pentachlorobenzene, musk xylene, isolongifolanone, methyl cedryl ketone, Opalal, Peonile, Iso E Super, δ-damascone, cyclohexyl salicylate, and Agrumex by trout liver S9 fractions. For isolongifolanone (isomer 1), Iso E Super (isomer 2), and Agrumex (isomer 1), only the major isomers are shown. Log-transformed concentrations from at least two independent experiments using triplicates for each time point are shown. Closed diamonds denote active S9 plus cofactors (NADPH, UDPGA, PAPS, and GSH), and open squares denote inactive S9.

calculate a whole-body biotransformation rate constant (k_{MET}; 1/d). The model includes a term (f_U; unitless) that corrects for potential binding effects on clearance. Two possibilities were explored in regard to this term: (a) f_U was calculated ("f_U calc") as the ratio of free chemical fractions in blood plasma and the *in vitro* S9 system, and (b) f_U was set to 1.0 ("f_U = 1.0").[13]

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Table 1. Comparison of Estimated BCFs Using the BCFBAF Model, *in Vitro* Intrinsic Clearance Rates by Trout Liver S9 Fractions ($CL_{IN\ VITRO,\ INT}$), Predicted BCFs Calculated with the IVIVE Model (BCF_{TOT}), and Measured *in Vivo* BCFs (OECD 305)

			BCFBAF v3.01 model[a]			S9 *in vitro* assay	IVIVE model[b]		*in vivo* BCF[c]
chemical	CAS no.	log K_{OW}[d]	regression method	Arnot-Gobas (without biotransformation)	Arnot-Gobas (with biotransformation)	$CL_{IN\ VITRO,\ INT}$ (mL/h/mg protein)	BCF_{TOT} (L/kg) f_U calc	BCF_{TOT} (L/kg) f_U = 1.0	measured BCF (L/kg)
Peonile	10461-98-0	4.0	202	494	349	0.38	347	102	365−406
δ-damascone	57378-68-4	4.2	274	778	534	1.05	332	105	50−56
cyclohexyl salicylate	25485-88-5	4.7	586	2361	144	3.49	312	140	320−480
Agrumex[e]	88-41-5	4.8 (I1)	682 (I1)	2928 (I1)	429 (I1)	2.72 (I1)	390 (I1)	151 (I1)	128−156
		4.7 (I2)	586 (I2)	2361 (I2)	393 (I2)	9.33 (I2)	205 (I2)	137 (I2)	
musk xylene	81-15-2	4.9	794	3618	176	0	3618	3618	5950−9720
isolongifolanone[e]	23787-90-8	4.9 (I1)	794 (I1)	3618 (I1)	1478 (I1)	0.12 (I1)	2578 (I1)	348 (I1)	381
		5.1 (I2)	1076 (I2)	5441 (I2)	1845 (I2)	0.10 (I2)	3759 (I2)	435 (I2)	
pentachlorobenzene	608-93-5	5.17	1197	6238	4018	0	6238	6238	6452−8570
Opalal	62406-73-9	5.3	1459	7958	3231	0.19	3778	343	85−137
Iso E Super[e]	54464-57-2	5.7 (I2)	2678 (I2)	14854 (I2)	6221 (I2)	0.66 (I1)	1766 (I1)	241 (I1)	366−381
		5.6 (I1, I3, I4, I5)	2301 (I1, I3, I4, I5)	12991 (I1, I3, I4, I5)	5821 (I1, I3, I4, I5)	0.33 (I2)	3121 (I2)	285 (I2)	
						0.71 (I3)	1669 (I3)	238 (I3)	
						0.64 (I4, I5)	1809 (I4, I5)	243 (I4, I5)	
methyl cedryl ketone	68039-35-0	5.8	3118	16677	5634	0.17	5892	397	867−3920[f]

[a]BCFBAF v3.01 model, which is part of EPI Suite™ v4.10.[8] BCF predictions were obtained using the regression model and the model by Arnot and Gobas,[6] modified for a standard fish (10 g, 5% lipid, 15 °C). The Arnot and Gobas model was run assuming no biotransformation ("without biotransformation") or by using the QSAR-estimated biotransformation rate for the compound of interest ("with biotransformation"). [b]IVIVE, *in vitro-in vivo* extrapolation model. BCFs expressed on a total chemical basis (BCF_{TOT}) were calculated on the basis of measured *in vitro* biotransformation rates under two assumptions with regard to the chemical binding term f_U: (a) f_U is calculated as the ratio of free chemical fractions *in vivo* in blood plasma and *in vitro* in the S9 reaction system ("f_U calc");[13] (b) f_U is set to 1.0, assuming equal availability of the test chemical to biotransformation enzymes *in vitro* and *in vivo*.[13,9] [c]*In vivo* BCFs determined using OECD Test Guideline 305.[2] BCFs were normalized to 5% lipid except for isolongifolanone and methyl cedryl ketone, as lipid contents were not reported; see Supporting Information (Table S5) for details. [d]Log K_{OW}, experimental values, internal results, Givaudan, except for pentachlorobenzene;[39] see Supporting Information (Table S1) for details. [e]Intrinsic clearance rates were determined for the two isomers of Agrumex (I1, I2), two isomers of isolongifolanone (I1, I2), and five different isomers of Iso E Super (I1−I5). [f]*In vivo* BCFs for methyl cedryl ketone were determined for separate body parts: 3920 L/kg for internal organs including intestine, heart, liver, and dorsal kidney; 1159 L/kg for head with gills; 867 L/kg for the remaining edible part.

The first correction assumes that biotransformation enzymes operate against the free or unbound chemical fraction *in vivo* (in plasma) and *in vitro*. The second correction assumes that chemical availability to the enzymes *in vitro* and *in vivo* is effectively the same, either because binding does not limit activity or because the fraction available in both systems is identical. Binding values for trout plasma and the S9 system were calculated using log K_{OW}-based algorithms given by Nichols et al.[13]

The IVIVE model uses the estimated value of k_{MET} as an input to a one-compartment bioaccumulation QSAR model provided by Arnot and Gobas,[7] assuming a water-only exposure and no organism growth. All other model inputs (e.g., the branchial exchange constants k_1 and k_2 and the fecal egestion constant k_E) are calculated using log K_{OW}-based algorithms given by the original authors.[7] Parameters used as input and parameters calculated by the IVIVE model are shown in the Supporting Information (Table S4).

BCF Prediction Using BCFBAF v3.01. The BCFBAF program v3.01 (EPI Suite™ v4.1)[8] predicts BCFs using two models. The first is a revised version of the regression-based model included in the original BCFWIN program ("regression method"). Log BCF is estimated from log K_{OW} and a series of correction factors, if applicable. These correction factors were identified on an empirical basis[15] but, in certain cases, may be rationalized on the basis of known biotransformation reactions or likely reactivity. In the present study, none of the chemicals investigated contained structural fragments associated with correction factors given by the authors (i.e., the predicted BCF was based only on log K_{OW}). Additionally, the BCFBAF program contains the Arnot-Gobas QSAR model.[7] Within EPI Suite, this model is configured to accept biotransformation rate constants calculated by a fragment-based estimation program.[6] The model estimates BCFs in three general trophic levels of fish (lower, middle, and upper) at a default temperature of 10 °C and assumes default lipid contents of 5.98%, 6.85%, and 10.7%, respectively. To achieve greater comparability with the IVIVE model, we modified the Arnot-Gobas model in EPI Suite to predict BCFs for a "standard fish" (i.e., 10 g, 5% lipid content, 15 °C). The result of this modification is that the Arnot-Gobas model (as implemented here) and IVIVE model differ only with respect to the source of estimated k_{MET} values.

In Vivo BCFs. *In vivo* BCFs determined in different fish species using OECD Test Guideline 305 were compared to BCFs predicted by the different models. Details on the fish species and BCF determination are shown in the Supporting Information (Table S5).

RESULTS AND DISCUSSION

Positive Controls. The metabolic stability of testosterone and 7-hydroxycoumarin were determined to evaluate the two batches of trout liver S9 fractions (Supporting Information, Table S2). Although the two batches differed somewhat, particularly with respect to phase I activity (i.e., a 2.5-fold

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difference in testosterone clearance), both batches exhibited robust activity toward these substrates.

Determination of *in Vitro* Biotransformation of Fragrance Ingredients. Depletion curves for the nine fragrance ingredients are shown in Figure 1. The resulting *in vitro* clearance rates are given in Table 1 along with measured log K_{OW} values for each compound. Additional depletion studies were performed with a known bioaccumulative chemical (pentachlorobenzene). No biotransformation by trout liver S9 fractions was observed for pentachlorobenzene or musk xylene. Slow biotransformation was detected for isolongifolanone, methyl cedryl ketone, and Opalal ($CL_{IN\ VITRO,\ INT}$ = 0.10−0.19 mL/h/mg). Moderate biotransformation was seen for Peonile, Iso E Super, and δ-damascone ($CL_{IN\ VITRO,\ INT}$ = 0.33−1.05 mL/h/mg). Cyclohexyl salicylate and Agrumex were more rapidly biotransformed compared to the other fragrance ingredients ($CL_{IN\ VITRO,\ INT}$ = 2.72−9.33 mL/h/mg).

Many fragrance ingredients exist as isomeric mixtures, and in this study we evaluated the metabolic stability of the individual isomers of isolongifolanone, Iso E Super, and Agrumex. A similar, slow turnover was found for both isomers of isolongifolanone. For Iso E Super, a 2-fold difference in biotransformation rates for the different isomers was found, while rates determined for the two isomers of Agrumex differed by a factor of 3.4 (Table 1, Figure 2). These differences in activity translate to modest differences in predicted BCFs for individual isomers. For example, predicted BCFs for the two isomers of Agrumex differed by 1.9-fold when using the full binding assumption (i.e., f_U calc) and 1.1-fold when assuming f_U = 1.0. Similar differences in predicted BCFs were observed for individual isomers of isolongifolanone and Iso E Super. *In vivo* studies provide BCF estimates for individual isomers only if an isomer-specific analysis of chemical residues is performed, while BCF predictions based on log K_{OW} and QSAR-estimated biotransformation rates (i.e., the Arnot-Gobas model) do not presently distinguish between different stereoisomers except if there are differences in measured octanol−water partition coefficients. Although in principal it would be possible to amend the Arnot-Gobas model to account for isomer-specific differences, existing QSAR methods for estimating log K_{OW} and biotransformation rates are based on 2-D molecular structures.

In all experiments, heat-inactivated S9 fractions served as negative controls. The results confirmed in all cases no loss due to abiotic processes. As a second control, all incubations were performed in the presence of active S9 fractions without added cofactors (data not shown). In all cases except for δ-damascone and Agrumex, activity was completely dependent on added cofactors. For δ-damascone, a slow S9-dependent decrease in substrate concentration was noted in the absence of cofactors; however, this decrease was substantially slower than that observed in the presence of added cofactors. In contrast, the rate of substrate depletion for both isomers of Agrumex was similar in the presence and absence of added cofactors (50% and 46% decrease in 20 min for isomer 1, with and without added cofactors; 62% and 60% decrease, respectively, for isomer 2). Because Agrumex is an ester, hydrolysis catalyzed by one or more carboxylesterases may be involved. Carboxylesterases do not require any additional cofactors, and high activity has been detected in rainbow trout.[16,17]

Assessment of Modeled and Measured BCFs Using the U.S. EPA Threshold Criterion for Bioaccumulation. Measured substrate depletion rates were used as inputs to the IVIVE model to calculate BCF values for each test compound (Table 1). These modeled BCF values were then compared to (i) *in vivo* BCFs, (ii) BCF predictions based on log K_{OW} and QSAR-derived biotransformation rates ("Arnot-Gobas, with

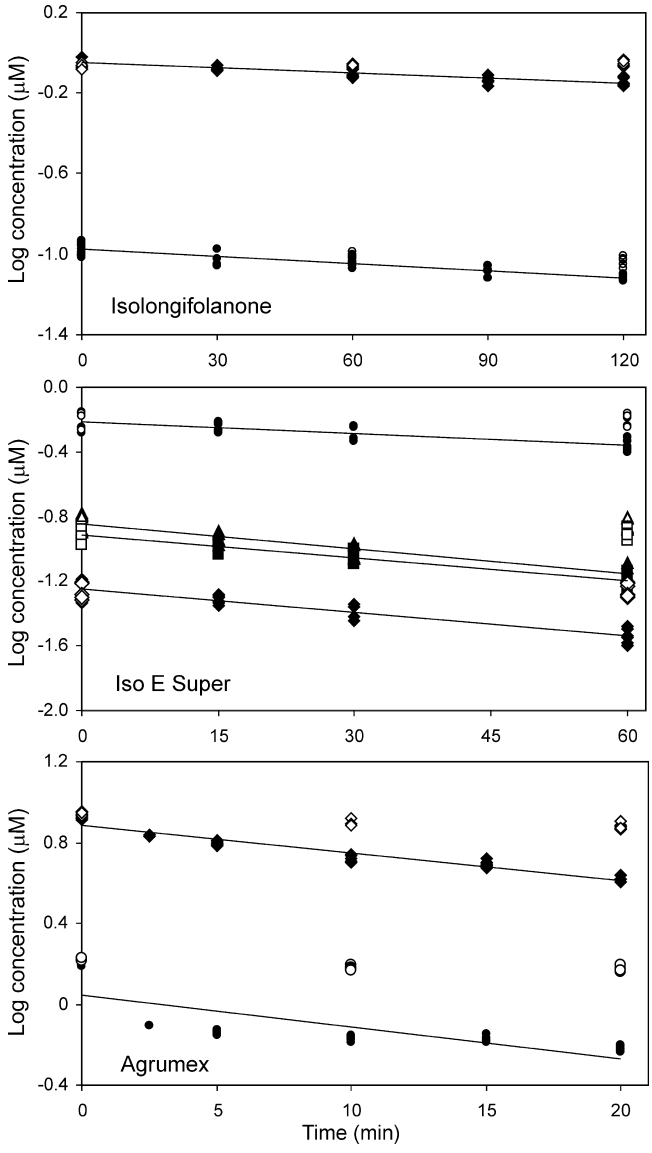

Figure 2. Biotransformation of the individual isomers of isolongifolanone, Iso E Super, and Agrumex by trout liver S9 fractions. For isolongifolanone and Agrumex, isomers 1 (I1; major isomer) and 2 (I2) were analyzed separately, while five isomers were analyzed for Iso E Super: isomer 1 (I1), isomer 2 (I2; major isomer), isomer 3 (I3), isomers 4 and 5 (I4, I5, which were not separated and thus quantified together). Log-transformed concentrations from two independent experiments using triplicates for each time point are shown. Initial concentrations were 1 μM for isolongifolanone and Iso E Super resulting in initial concentrations of 0.89 μM (I1) and 0.11 μM (I2) for isolongifolanone isomers and 0.06 μM (I1), 0.60 μM (I2), 0.14 μM (I3), and 0.12 μM (I4 and I5) for Iso E Super isomers. The initial concentration of Agrumex was 10 μM, which resulted in initial concentrations of 8.39 μM (I1) and 1.58 μM (I2) for the two isomers. Closed diamonds denote active S9 plus cofactors (NADPH, UDPGA, PAPS, and GSH) for I1, and open diamonds denote inactive S9 for I1; closed circles denote active S9 plus cofactors for I2, and open circles denote inactive S9 for I2; closed triangles denote active S9 plus cofactors for I3, and open triangles denote inactive S9 for I3; closed squares denote active S9 plus cofactors for I4 and I5, and open squares denote inactive S9 for I4 and I5.

Table 2. Assessment of Modeled and Measured BCFs of Fragrance Ingredients and Pentachlorobenzene Using the U.S. EPA Threshold Criterion for Bioaccumulation[a]

	Modified BCFBAF v3.01 model[b]			IVIVE model[c]		OECD 305
Chemical	Regression Method	Arnot-Gobas (without biotrans-formation)	Arnot-Gobas (with biotrans-formation)	BCF_{TOT} f_u calc.	BCF_{TOT} $f_u = 1.0$	*in vivo* BCF
Peonile	non B	non B	non B	non B	non B	non B
δ-Damascone	non B	non B	non B	non B	non B	non B
Cyclohexyl salicylate	non B	B	non B	non B	non B	non B
Agrumex	non B	B	non B	non B	non B	non B
Musk xylene	non B	B	non B	B	B	B
Isolongifolanone	non B-B	B	B	B	non B	non B
Pentachlorobenzene	B	B	B	B	B	B
Opalal	B	B	B	B	non B	non B
Iso E Super	B	B	B	B	non B	non B
Methyl cedryl ketone	B	B	B	B	non B	non B-B

[a]Chemicals were categorized on the basis of measured or predicted BCFs using the threshold criterion employed by U.S. EPA (non bioaccumulative (non B): BCF <1000 L/kg; bioaccumulative (B): BCF ≥1000 L/kg).[1] Non B−B classifications (cross-hatched, orange-green boxes) indicate that predictions for individual isomers of the same compound were above and below the criterion value or that *in vivo* BCFs determined in multiple studies ranged from below the criterion to above the criterion. [b]BCFs were predicted using models provided as part of BCFBAF v3.01, which is part of EPI Suite™ v4.10[8] (see text for details). For the present effort, the model given by Arnot and Gobas[7] was modified to predict BCFs for a standard fish (10 g, 5% lipid, 15 °C). [c]BCFs were predicted for a standard fish by using the *in vitro* intrinsic clearance rate determined in the S9 assay as an input to the IVIVE model.[13]

biotransformation"), (iii) BCF predictions obtained using the Arnot-Gobas model assuming no biotransformation (i.e., k_{MET} = 0.0; "Arnot-Gobas, without biotransformation"), and (iv) the regression method. For a binary yes/no bioaccumulation assessment, we began by applying the threshold criterion employed by U.S. EPA. This criterion, which holds that a substance is bioaccumulative (B) if BCF ≥1000, is the most stringent in widespread regulatory use (Table 2).[1] Additional assessments were then performed using other well-known criteria values (see below).

***In Vitro* Biotransformation Has Little Impact on BCF Predictions for Compounds with Lower log K_{OW} Values.** For Peonile and δ-damascone, two fragrance ingredients with relatively low log K_{OW} values (4.0 and 4.2, respectively), all model-predicted BCFs were below the bioaccumulation threshold of 1000 L/kg, which is in agreement with *in vivo* data (Table 1, 2). For such compounds, the rate constant that controls chemical elimination across the gills generally exceeds modeled rates of whole-body biotransformation, even at high rates of $CL_{IN\ VITRO,INT}$. Under these conditions, biotransformation has relatively little impact on predicted steady-state BCFs.[13]

***In Vitro* Biotransformation Reduces Predicted BCFs for Chemicals with Higher log K_{OW} Values, Depending on the Rate of Intrinsic Clearance and Assumed Value of f_U.** For fragrance ingredients with higher log K_{OW} values (≥4.7), the Arnot-Gobas model without biotransformation predicted a high bioaccumulation potential (Table 1). Similarly, the regression method predicted BCF values >1000 for substances with a log K_{OW} ≥5.1. However, the *in vivo* data show that most of these substances are not bioaccumulative, suggesting that they undergo biotransformation.

For cyclohexyl salicylate and Agrumex, the two substances with the highest *in vitro* intrinsic clearance rates, BCFs predicted by the IVIVE model or the Arnot-Gobas model with predicted biotransformation were similar and below the B threshold, which is in agreement with measured *in vivo* BCFs (Tables 1 and 2). As such, these modeled predictions tend to reinforce one another. Within a regulatory context, however, greater confidence may be given to BCFs predicted by the IVIVE model since these values are based on measured rates of biotransformation.

Slow to moderate biotransformation rates were observed for isolongifolanone, Opalal, Iso E Super, and methyl cedryl

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ketone. Incorporation of these *in vitro* turnover rates into the IVIVE model resulted in BCFs ranging from 1669 to 5892 L/kg when f_U was calculated from estimated binding values. These BCF predictions and those predicted by the Arnot-Gobas model with biotransformation correlate poorly with *in vivo* BCF values. In contrast, BCF predictions for isolongifolanone (348 and 435 L/kg for isomers 1 and 2, respectively), Opalal (343 L/kg), and Iso E Super (238−285 L/kg for the different isomers) matched *in vivo* values much better when f_U was set equal to 1.0. All of these values are below the B criterion. Measured BCFs for methyl cedryl ketone were in between the BCFs predicted by the IVIVE model using the two different binding assumptions. However, these *in vivo* BCFs were determined for separate body parts (edible part, 867 L/kg; head, 1159 L/kg; internal organs, 3920 L/kg), and information required to calculate a whole body BCF is not reported making comparisons with predicted BCFs difficult. The *in vivo* BCFs determined for the internal organs were 4-fold higher compared to the head with gills and the remaining edible part. The edible part comprises by weight a larger fraction of total body weight than the other tissues; however, substantial accumulation could have occurred in tissues (e.g., adipose fat) not represented by any of these samples.

Overall, these observations suggest that BCFs generated by the IVIVE model using predicted binding values for blood plasma and the S9 system tend to overpredict measured *in vivo* values. A better correlation between predicted and measured BCFs was obtained by setting f_U = 1.0. Similar results were reported previously by Escher et al.[18] In this earlier study, the authors used a PDMS depletion method to measure binding of several organic compounds (log K_{OW} 4.88−5.76) in rainbow trout plasma and liver S9 fractions. This information was then combined with published *in vitro* biotransformation data to estimate a set of BCF values. As in the current effort, predicted BCFs based on calculated f_U values tended to overestimate measured BCFs by a substantial margin. Much better predictions of measured BCFs were obtained by setting f_U = 1.0.

Using a model-based approach, it can be shown that the potential for chemical binding to impact *in vitro-in vivo* metabolism extrapolations varies with the rate of *in vitro* clearance.[13] When *in vitro* activity is high, hepatic clearance is rate-limited by blood flow to the liver and modeled predictions of hepatic clearance (and by extension biotransformation impacts on the BCF) become insensitive to errors in the value of f_U. Evidence for this can be seen in BCFs predicted by the IVIVE model for the two most well-metabolized fragrance ingredients, Agrumex and cyclohexyl salicylate. BCFs predicted for these compounds assuming f_U = 1.0 are lower than those predicted under the full binding assumption. However, the difference in predicted BCFs is small (approximately 2-fold). Much larger differences in predicted BCFs (up to 15-fold) were obtained for compounds that exhibited low rates of clearance and also possess high log K_{OW} values (e.g., Opalal, Iso E Super, or methyl cedryl ketone).

In a recent study, Nichols et al.[19] used a solid-phase microextraction (SPME) method to directly characterize the binding of six polycyclic aromatic hydrocarbons in trout liver S9 fractions and in solutions used to perfuse isolated trout livers. Measured levels of *in vitro* activity were extrapolated to the intact liver and compared to measured levels of hepatic clearance as a means of directly evaluating chemical binding effects on *in vitro-in vivo* metabolism extrapolations. Although both binding assumptions (f_U calc and f_U = 1.0) provided good estimates of hepatic clearance for well-metabolized compounds (due to flow limitations on clearance), measured levels of clearance for more slowly metabolized compounds were most accurately predicted when f_U was set equal to 1.0.

A mechanistic explanation for these findings remains to be established. One possibility, however, is that chemical desorption from binding sites in protein and lipid is functionally instantaneous. Under these circumstances, bound chemicals would be able to desorb rapidly becoming similarly available to biotransformation enzymes *in vivo* and in the presence of S9 proteins. This hypothesis is supported by the study from Han et al., who observed even higher clearance values for compounds with log K_{OW} values >4 from serum incubations with trout hepatocytes compared to incubations in protein-free media.[20] As noted previously,[13,19] the effect of chemical binding on hepatic clearance remains one of the principal sources of uncertainty in metabolism extrapolations with fish. The effect of protein binding on prediction of hepatic clearance for drugs also remains under active discussion.[21−24]

Bioconcentration of Non-biotransformed Chemicals Is Correctly Predicted. Musk xylene was not metabolized *in vitro* by trout liver S9 fractions. Consequently, the BCF predicted by the IVIVE model was identical to that given by the Arnot-Gobas model with no biotransformation. This BCF prediction is close to the value determined in fish by Rimkus et al.[25] and reported in the EURAS BCF Gold Standard Database (http://ambit.sourceforge.net/euras/) and is well above the B threshold (≥1000 L/kg). In contrast, BCFs for musk xylene predicted by the regression method or the Arnot-Gobas model with estimated biotransformation (794 and 176 L/kg, respectively) significantly underpredicted measured values (Table 1). This finding indicates that determination of *in vitro* biotransformation rates may be useful for chemical classes not well represented in the training sets of existing QSAR models with estimated biotransformation. Moreover, by collecting *in vitro* data for strategically selected chemicals it may be possible to refine and improve existing biotransformation QSARs.

Pentachlorobenzene, a metabolically stable compound that is known to accumulate in fish, was used in this study as a negative benchmark. No biotransformation of pentachlorobenzene was detected *in vitro*, which resulted in a predicted BCF well above the B threshold (Table 1). Similar BCFs were predicted by the Arnot-Gobas model with and without biotransformation. The BCF predicted by the regression method was somewhat lower (1197 L/kg) than that obtained using the IVIVE or Arnot-Gobas models but still exceeded the B criterion. These findings are in agreement with *in vivo* BCFs for pentachlorobenzene as reported by Chaisuksant et al.[26] and Yakata et al.[27] and in the EURAS BCF Gold Standard Database (http://ambit.sourceforge.net/euras/) (Table 1).

Assessment of the Bioaccumulation Potential of Fragrance Ingredients Using Other Regulatory Criteria. Additional bioaccumulation assessments were performed using B criteria developed by the European Commission and Environment Canada (Supporting Information, Table S6).[1] Results similar to those described above were obtained when using the B criterion applied by the European Commission (non B, BCF <2000 L/kg; B, BCF ≥2000 L/kg). The exceptions include modeled predictions for isolongifolanone, Opalal, and pentachlorobenzene. All three chemicals were predicted by the regression method to be "non-B." *In vivo* data

for isolongifolanone and Opalal are consistent with this prediction. In contrast, *in vivo* BCFs for pentachlorobenzene exceeded this B criterion value. This finding is in agreement with BCFs predicted by the other models. The application of this higher B criterion value also resulted in correct classification of the modeled BCF for isolongifolanone when using the Arnot-Gobas model with biotransformation (i.e., a classification consistent with that based on the *in vivo* BCF).

All fragrance ingredients were classified as "non-B" by the regression method when using the BCF criterion (non-B, BCF <5000 L/kg; B, BCF ≥5000) applied by Environment Canada. With the exception of methyl cedryl ketone, similar results were obtained using the IVIVE model, regardless of which binding assumption was employed (for methyl cedryl ketone, a "non-B" classification was obtained only when f_U = 1.0). In each case, these B classifications were consistent with those based on *in vivo* BCFs. In contrast, the Arnot-Gobas model without biotransformation yielded incorrect classifications ("B", when *in vivo* BCFs indicated "non-B") for all compounds with log K_{OW} values greater than 5.17. The incorporation of QSAR-estimated biotransformation rates into the Arnot-Gobas model improved its performance relative to *in vivo* BCF values, although predicted BCFs for Iso E Super and methyl cedryl ketone remained above the criterion value.

Taken together, these observations suggest that the accuracy of a BCF prediction model, evaluated in terms of its ability to predict whether *in vivo* BCFs are likely to exceed a given criterion value, depends to some extent on the criterion value itself. If this value is set high enough, all measured and modeled values will suggest a "non-B" classification. Differences in model performance emerge as the criterion value is reduced, in large part because biotransformation impacts on bioaccumulation become relatively more important.

Predictability and Possible Limitations of the IVIVE Model. Previous efforts to extrapolate *in vitro* biotransformation data for fish to the intact animal have been performed using a variety of different chemicals including pesticides,[9,12,20] surfactants,[10,28] and pharmaceutical substances.[11] Some of these chemicals possess relatively low log K_{OW} values (<3). As indicated previously, biotransformation is unlikely to have a significant impact on the bioaccumulation of chemicals with log K_{OW} values in this range.[13] Due to these physicochemical properties, these compounds thus provide a relatively poor test of the extrapolation procedures.

In the present study, we performed *in vitro-in vivo* extrapolations for nine fragrance ingredients (e.g., esters, ketones, nitriles, ethers) that exhibit higher log K_{OW} values (4.0−5.8). Indeed, the log K_{OW} range of fragrance ingredients is particularly suitable to investigate the impact of measured biotransformation on bioaccumulation assessments for hydrophobic chemicals. Furthermore, this is the first study to apply the improved IVIVE model[13] to an external test set. BCF predictions based on measured *in vitro* turnover rates determined in trout liver S9 fractions can be considered as a conservative assessment. The model presently assumes that the liver is the only site of biotransformation. Gomez et al. observed a reduction of predicted BCFs for ibuprofen and propranonol by trout gill biotransformation.[11] Intestinal biotransformation also may serve as a major route of clearance for orally ingested chemicals. For example, it has been shown that cytochrome P450 3A subfamily isoforms (CYP3A27) are localized in the gastrointestinal tract of rainbow trout.[29] Phase II enzymes such as UDP-glucuronosyltransferase and glutathione-transferease are also known to be active in subcellular fractions from rainbow trout gut.[30]

The use of liver S9 fractions to measure *in vitro* activity may also result in overprediction of BCFs for high log K_{OW} substances that are very slowly metabolized. As discussed by Nichols et al.,[13] there are practical limitations on the lower limit of activity (about 0.05/h) that can be measured using this system due to the working lifetime of the preparation. In such cases it may be more appropriate to use isolated hepatocytes or spheroid cultures of hepatocytes,[31−33] although this remains to be demonstrated. From a regulatory perspective, however, a conservative assessment of metabolic rate and therefore of the bioaccumulation potential of fragrance ingredients would be favored to guarantee environmental safety.

Intra- and interspecies differences in biotransformation result in some uncertainty regarding the regulatory use of BCF predictions based on *in vitro* rates in S9 fractions from rainbow trout. Due to the limited availability of data, we compared these predicted BCFs with *in vivo* BCFs from different fish species such as carp, bluegill, and zebrafish (see Supporting Information, Table S5). Depending on the individual enzyme, there can be substantial differences in activity for different fish species.[34,35] Variability even between different strains of rainbow trout was found by Koponen et al.[36] A rigorous evaluation of the *in vitro-in vivo* extrapolation procedure would require, therefore, that *in vivo* BCF data be collected using the same strain of fish used for *in vitro* testing. This type of study, utilizing well-matched experimental animals, represents a clear research need.

In conclusion, compared to BCFs calculated with the BCFBAF v3.01 models, BCFs generated by the IVIVE model were in better agreement with those measured in fish. This was especially true for well-metabolized fragrance ingredients with higher log K_{OW} values (≥4.9) such as Agrumex, cyclohexyl salicylate, and Iso E Super. At the same time, for a chemical with no detectable *in vitro* biotransformation (musk xylene), the IVIVE model also better predicted the high measured BCFs as compared to a model that utilizes QSAR-predicted biotransformation rates. Consistent with earlier reports, the IVIVE model performed particularly well when the model term (f_U) used to account for chemical binding effects was set equal to 1.0. For fragrance ingredients with lower log K_{OW} values (4.0−4.8), similar BCFs were predicted using either binding assumption, with the trend that measured BCFs were slightly underpredicted when assuming f_U = 1.0. However, the extent of this underprediction (2- to 4-fold) is comparable to the variability expected among *in vivo* BCF studies for a given chemical. Thus, even high quality experimental BCF data differ by >0.5 log units for at least 35% of chemicals tested and >1 log unit for at least 10% of chemicals.[37] Key sources of uncertainty and variability of BCFs were reviewed by Arnot and Gobas.[38]

A mechanistic rationale for setting f_U = 1.0 remains to be established, and further research is needed to better understand binding effects on chemical biotransformation. Presently, however, it may be reasonable to use the two different binding assumptions (i.e., f_U calc vs f_U = 1.0) to estimate upper and lower limits of hepatic clearance to predict the upper and lower limits of bioconcentration.[13] Improvements to the *in vitro* methods and IVIVE models are expected, and as more data accumulate on *in vitro* biotransformation rates as well as *in vivo* BCFs, it will become easier to judge which assumption is more correct. Nevertheless, current approaches still have substantial value in a weight-of-evidence approach for bioaccumulation

assessment and as a way to prioritize chemicals for *in vivo* BCF testing. Thus, this study demonstrates the use of *in vitro* biotransformation data to refine the estimation of a partitioning-based BCF and validates the IVIVE approach for a class of chemicals (fragrance ingredients) that has not been used in building the model.

■ ASSOCIATED CONTENT

Supporting Information

Chemical structures, information on chemicals, activities of the two batches of trout liver S9 fractions, GC−MS methods, parameters calculated by the IVIVE model, and *in vivo* BCF data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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