# Experimental Validation of Mass Balance Models for *in vitro* Cell-based Bioassays

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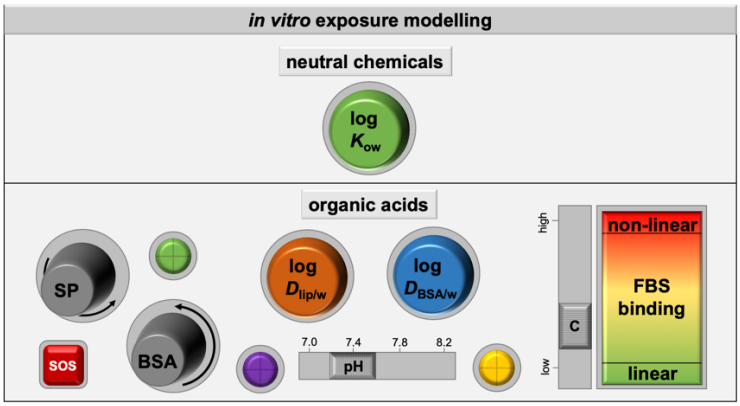




ABSTRACT

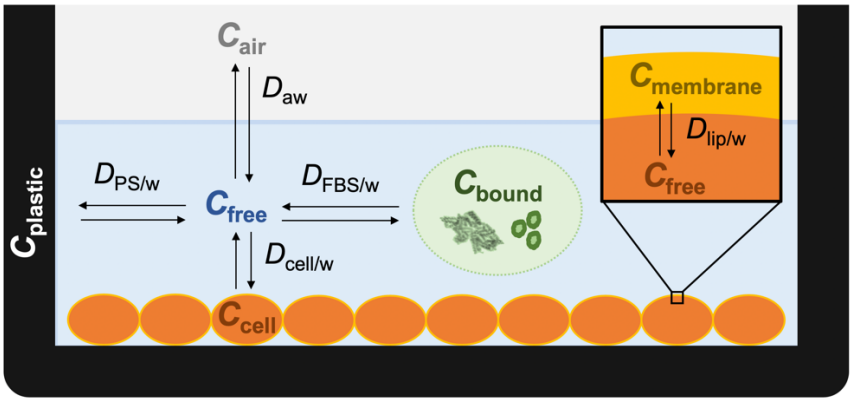
The freely dissolved concentration in the assay medium ($C_{free}$) and the total cellular concentration ($C_{cell}$) are essential input parameters for quantitative *in vitro*-to-*in vivo* extrapolations (QIVIVE), but available prediction tools for $C_{free}$ and $C_{cell}$ have not been sufficiently validated with experimental data. In this study, medium-water distribution ratios ($D_{FBS/w}$) and cell-water distribution ratios ($D_{cell/w}$) for four different cells lines were determined experimentally for 12 neutral and five ionizable chemicals. Literature data for seven organic acids were added to the dataset, leading to 24 chemicals in total. A mass balance model based on bovine serum albumin-water ($D_{BSA/w}$) and liposome-water distribution ratios ($D_{lip/w}$) of the chemicals was used to calculate $D_{FBS/w}$ and $D_{cell/w}$. For all neutral and basic test chemicals, the mass balance model predicted $D_{FBS/w}$ and $D_{cell/w}$ within a factor of 3 and 3.4, respectively, indicating that existing models can reliably predict $C_{free}$ and $C_{cell}$ for these chemicals. For organic acids a further refinement of the model will be required as large deviations between modelled and measured binding to assay medium and cells of up to a factor of 370 were found. Furthermore, saturation of medium proteins should be further explored for organic acids and neutral chemicals with moderate hydrophobicity.

# Introduction

Understanding the exposure to chemicals in *in vitro* cell-based bioassays is a prerequisite for quantitative *in vitro-to-in vivo* extrapolation (QIVIVE). Previous studies have suggested to use the freely dissolved concentration in the assay medium ($C_{free}$), the total cellular concentration ($C_{cell}$) or the concentration in the cellular membranes ($C_{membrane}$) as improved metrics of effective concentrations.[1-4] $C_{free}$ can be determined experimentally in *in vitro* test systems,[1, 3, 5, 6] and is the main metric for QIVIVE, while $C_{cell}$ and $C_{membrane}$ cannot easily be derived by measurements but are useful to estimate critical membrane concentrations and the degree of specificity of the effect.[7]

Models have been developed to calculate the different concentration metrics either from nominal or from measured freely dissolved or total concentrations based on the partition constants (neutral chemicals) or distribution ratios (ionizable chemicals) of the test chemicals between air and water ($D_{aw}$), medium and water (which is essentially the partitioning between fetal bovine serum (FBS) and water ($D_{FBS/w}$)), polystyrene (PS) and water ($D_{PS/w}$), cells and water ($D_{cell/w}$) and membrane lipids and water ($D_{lip/w}$, Figure 1).

**Figure 1.** Distribution of chemicals in *in vitro* test systems between water, air, medium proteins and lipids from fetal bovine serum (FBS), well plate plastic (polystyrene) and cells and within the cells between the intracellular water phase and the cell membranes.



Most models assume that equilibrium between all phases in the *in vitro* test system is attained during the exposure time. Evaporation is a continuous loss process for volatile chemicals but also leads to cross-contamination on the well plates,[7] therefore standard *in vitro* assays should only be performed with non-volatile chemicals. Cellular uptake is typically fast and equilibrium is reached within hours.[8] Diffusion into well plate plastic is very slow and was shown to have a minor influence on *in vitro* exposure when protein-rich media are applied.[9] Therefore, the equilibrium assumption is justified unless chemicals are strongly metabolized during the experiment.

Three different mass balance models for the prediction of *in vitro* exposure have been developed that share similar structure and are based on the same set of equations. The first model published by Kramer et al. 2012[3] was based on measured values for $D_{aw}$, $D_{FBS/w}$, $D_{PS/w}$ and $D_{cell/w}$ for the calculation of $C_{free}$ in the assay medium. This approach is applicable to many different classes of chemicals because all input parameters are derived experimentally but to date the model has only been applied to a small number of chemicals. The model published by Armitage et al. 2014[10] was developed for chemicals that are predominantly neutral at the pH value of the assay medium and relied on predicted input parameters. The *in vitro* exposure of large numbers of chemicals can be screened with this approach, because only the air-water ($K_{aw}$) and octanol-water partition constant ($K_{ow}$) of the chemicals are required for the calculation of partitioning between water, air, serum albumin, serum lipids, dissolved organic matter, and cells. The mass balance model of Fischer et al. 2017[4] was an extension of the Armitage model that is applicable to non-volatile chemicals but also extends it to ionizable chemicals. Fischer et al. 2017[4] calculated the distribution between assay medium and cells, and both, medium and cells were assumed to be composed of proteins, lipids, and water (for equations see section "Data evaluation"). All cellular and medium proteins were modelled as albumin and all lipids as phospholipids. This model requires only serum albumin-

72 water distribution ratios ($D_{BSA/w}$) and phospholipid-water distribution ratios ($D_{lip/w}$) of the test
73 chemicals at pH 7.4 for the calculation of distribution. Well plate plastic is not included in this
74 model, because its contribution is minimal for bioassays using protein- and lipid-rich cell culture
75 media.[9, 11] Air-medium partitioning can be easily included in the model, e.g. to define which
76 chemicals can be tested under standard bioassay test set ups.[7]

77 More sophisticated modelling approaches also considered cellular metabolism and other kinetic
78 processes[12] and differences in ionization of the test chemicals between the intracellular and
79 extracellular environment.[13] The main problem of all models mentioned above is that they have
80 rarely been validated with experimental data. Ideally those models should be validated by
81 measuring $C_{free}$ and $C_{cell}$ in the actual test system, but the effort required to do so using currently
82 available analytical methods limits this approach to few chemicals and assay formats.[1, 3, 5]
83 However, to build some confidence into these predictive models, the basic assumptions of the
84 models can be scrutinized. The multimedia equilibrium-based mass balance models from Armitage
85 et al.[10] and Fischer et al.[4] assumed that $D_{FBS/w}$ and $D_{cell/w}$ can be calculated from the distribution
86 ratios of the chemicals to proteins and lipids and the protein and lipid content of assay medium
87 and cells. Comparison of experimental $D_{FBS/w}$ and $D_{cell/w}$ with model predictions for a set of diverse
88 chemicals will allow the validation of these assumptions.

89 In a previous study on the partitioning of organic acids to different biological materials (proteins,
90 lipids, cells etc.)[14] we showed that the model by Fischer et al.[4] can only predict binding to the
91 medium-added fetal bovine serum (FBS) at low concentrations of the organic acids, because
92 binding to medium proteins was found to be saturable, which is not considered in any of the simple
93 partitioning models. Furthermore, cell-water distribution ratios ($D_{cell/w}$) were overestimated, likely
94 because cellular proteins are assumed to be equivalent to serum albumin in the model, but the

majority of cellular proteins are structural protein of the cytoskeleton, which have different binding properties.[15] This study was built on our previous work with organic acids[14] and likewise applied the solid-phase microextraction method based on C18-fibers. The binding to serum albumin, phospholipid liposomes, different cell culture media and cell lines was determined for ten neutral chemicals varying widely in hydrophobicity, four organic bases and one chemical with complex speciation. Additionally, distribution ratios of two superhydrophobic chemicals, benzo[a]pyrene (B[a]P) and benzo[k]fluoranthene (B[k]F), were derived using a passive dosing approach based on PDMS-fibers. The combined data set of both studies, containing 24 chemicals, 12 neutral and 12 ionizable chemicals with diverse physicochemical properties, was used for the validation of the mass balance model of Fischer et al.[4] for the prediction of *in vitro* exposure, especially $C_{free}$ and $C_{cell}$.

# Materials and methods

## *Test chemicals*

Twenty-four organic test chemicals that are not prone to loss by medium-air partitioning (medium-air partition constants ($K_{medium/air}$) >10,000 L/L, see Table S1)[7] were included in this study (Table 1) seven of which had already published literature data. More details on the test chemicals (i.e., CAS No., supplier, chemical class, acidity constant (p$K_a$) and $K_{ow}$ of the neutral chemicals) can be found in Table S1, Supporting Information (SI). The neutral chemicals covered a broad range of hydrophobicity, indicated by their log $K_{ow}$ which was ranging from -0.07 for caffeine to 6.13 for B[a]P. The ionizable chemicals included seven organic acids used in a previous study,[14] four organic bases, and labetalol, which shows a complex speciation (47 % cationic and 53 % zwitterionic) at pH 7.4.

## *Materials*

Two different types of SPME fibers were used. Nitinol-based fibers coated with C18-particles embedded in polyacrylonitrile were used for the majority of the test chemicals. The C18-fibers with 45 µm coating thickness were purchased from Supelco (MilliporeSigma) with three different coating lengths (2, 5, and 15 mm) with calculated coating volumes of 69, 173, and 520 nL, respectively. Type and volume of fiber coating were selected based on the partitioning properties of the test chemicals (Table S2). Shorter coating length were used for chemicals that showed strong binding to the fibers to meet the criterium of a fraction bound >20 % in the samples with biological materials. For B[a]P and B[k]F glass fibers with poly(dimethylsiloxane) coating (coating thickness 30 µm) with a length of 1.5 cm (coating volume approx. 198 nL per fiber) from Polymicro Technologies were used, because the PDMS-water partition constant ($K_{PDMS/w}$) of these chemicals was available from the literature,[16] while the fiber-water distribution for the C18-fibers would have been difficult to measure. For the majority of the test chemicals the samples were prepared in amber glass HPLC vials sealed with screw caps with PTFE-coated silicone septa and the SPME fibers were inserted through the septa using blunt cannulas from Braun (0.8 × 22 mm). For B[a]P, B[k]F, bisphenol A, triclocarban, quinoxyfen, fluoranthene amber glass vials sealed tightly with crimp caps with aluminum septa were used and the fibers were inserted completely. For all experiments the same buffers, basal media, bovine serum albumin (BSA), liposomes, FBS and cell lines were used as in our previous study with organic acids.[14]

*Solid-phase microextraction with C18-fibers*

The protocol published by Henneberger et al.[14] was used for the determination of fiber uptake kinetics, fiber sorption isotherms, FBS sorption isotherms, fiber-water ($D_{f/w}$), fiber-basal medium ($D_{f/basal\ medium}$), BSA-water ($D_{BSA/w}$), liposome-water ($D_{lip/w}$), and cell-water distribution ratios ($D_{cell/w}$) for all chemicals except B[a]P and B[k]F (see below). The standard operating procedure (SOP) for the C18-SPME experiments can be found in the SI. In short, stock solutions of all

chemicals were prepared in methanol and either directly spiked to the samples (PBS, OptiMEM, DMEM, BSA, liposomes and FBS) or diluted in PBS and mixed with the sample (cell suspensions). As the chemicals showed very different binding affinities for the biological materials, the concentration of BSA, liposomes and FBS and the cell number in the cell suspensions had to be adjusted for each chemical so that the fraction bound in the samples was >20 % and the concentration in the SPME fiber was still quantifiable by HPLC (see below and section S2, SI). The amount of biological material used for each chemical and experiment is indicated in Table S2.

The C18-SPME fibers were conditioned in methanol (2 h) and water (20 min) and added to the samples individually. The samples were incubated at 37°C using either an orbital shaker set to 250 rpm or a vortex shaker operated at 1200 rpm (see also Table S1). Fibers were taken from the samples after different time points: between 15 min to 72 h for the kinetic experiments to derive the fiber uptake kinetics and after a fixed time for all subsequent experiments (24 h for the majority of the experiments, see ref [14] for more details). The fibers were extracted with 180 µL – 1000 µL of desorption solution (composition for the different test chemicals can be found in Table S1) for 2 h using the same shaker and speed as for the equilibration with the samples. For all partition experiments control samples in PBS were run in parallel to determine $D_{f/w}$ and stability (mass balance) of the chemicals. The concentration of test chemicals was quantified in the fiber extracts, the PBS samples and the basal media using either an HPLC system equipped with a UV and a fluorescence detector or an LC-MS/MS system, both from Agilent, as detailed in the SI, Table S3 and S4.

*Passive dosing with PDMS-fibers*

The PDMS-fibers were loaded with the test chemicals in 1.5 mL methanol/water (50/50) containing 5 mg/L B[a]P and 1.2 mg/L B[k]F for 7 days. Four fibers were extracted directly after

loading. Either 10 mL OptiMEM, 10 mL DMEM, 1 mL PBS containing 1% FBS, or 1 mL PBS containing 1 mg/mL BSA (4 replicates each), or 1 mL of cell suspension (approx. $5\times10^5$ cells/mL, 3 replicates per cell line) were prepared under sterile conditions. The BSA solution was filtered (0.2 µm) before the experiments. These samples were filled into autoclaved vials under a clean bench and each sample received one of the loaded PDMS-fibers. After an incubation period of six days on a vortex shaker at 1200 rpm, the fibers were removed from the samples and extracted with 180 – 1000 µL of MeOH for 2 days at 1200 rpm. 500 µL of the remaining sample were transferred to a new vial and extracted with 1000 µL of ethyl acetate for 15 min on an orbital shaker at 150 rpm. To facilitate phase separation the samples were centrifuged at 4000 rpm (Thermo Scientific Multifuge X1R, rotor TX-400) and 600 µL of the supernatant were transferred to HPLC vials. Ethyl acetate was evaporated under a nitrogen stream and the samples were re-dissolved in 100 µL of methanol. The concentration of B[a]P and B[k]F was quantified in the fiber and sample extracts by HPLC-FLD as described in section S2, SI.

*Data evaluation*

Fiber-water distribution ratios ($D_{f/w}$) were calculated and fiber uptake kinetics were fitted as described previously.[14] The log-transformed Freundlich model (eq. 1) was used to fit the fiber and FBS sorption isotherms. Freundlich coefficient and Freundlich exponent are indicated by $K_{Fr}$ and $n_{Fr}$, respectively and $C_f$ and $C_w$ are the concentration of the chemical in the fiber and in water, respectively.

$$\log C_f = \log K_{Fr} + n_{Fr} \cdot \log C_w \qquad \text{eq. 1}$$

Because the SPME experiments were performed under depletive conditions, the full mass balance (eq. 2) was used to calculate the distribution ratios between the biological materials and

water ($D_{i/w}$, *i* refers to BSA, liposome, or the sum of proteins and lipids in FBS) as described by Neale et al.[17] and Henneberger et al.[14]

$$D_{i/w}(\text{pH 7.4})\ [L_w/L_i] = \frac{C_{bound}}{C_w} = \frac{\frac{n_{tot}}{n_f} \cdot D_{f/w} \cdot V_f - V_w - V_f \cdot D_{f/w}}{V_i} \quad \text{eq. 2}$$

The concentration of the test chemical bound to the total amount of proteins and lipids in the solution is indicated by $C_{bound}$ and $V_w$, $V_f$ and $V_i$ are the volumes of water, fiber coating, and of all proteins and lipids in the sample, respectively. For the experiments with the C18-fibers $n_{tot}$ was assumed be the total amount of chemical added to the vial. For the passive dosing experiments with PDMS-fibers $n_{tot}$ was calculated for each sample from the amount of chemicals extracted from the PDMS-fiber and extracted from the corresponding dosed sample. Cell-water distribution ratios ($D_{cell/w}$) were calculated based on the total cell volume:

$$D_{cell/w}(\text{pH 7.4})\ [L_w/L_{cell}] = \frac{C_{cell}}{C_w} = \frac{\frac{n_{tot}}{n_f} \cdot D_{f/w} \cdot V_f - V_w - V_f \cdot D_{f/w}}{V_{cell}} \quad \text{eq. 3}$$

The total volume of cells in the vial ($V_{cell}$) was calculated from the cell count of the cell suspensions using previously published conversion factors (HEK293T $1.38 \times 10^{11}$ cells/L, HEK293H $3.48 \times 10^{11}$ cells/L, MCF7 $9.99 \times 10^{10}$ cells/L and H4lle $2.83 \times 10^{11}$ cells/L).[4, 14] For all experiments, the mass balance was calculated for the samples containing no biological material. Only experiments with a mass balance of 85-115 % were considered for the data evaluation.

The mass balance model of Fischer et al.[4] was used to predict $D_{FBS/w}$ and $D_{cell/w}$ (eq. 4 + 5). Experimental values from previous studies were used for the required volume fractions of proteins ($VF_{prot,FBS}$) and lipids ($VF_{lip,FBS}$) in FBS[4] and of proteins ($VF_{prot,cell}$), lipids ($VF_{lip,cell}$) and water ($VF_{w,cell}$) in the cells.[4, 14]

$$D_{FBS/w}(\text{pH 7.4})[L_w/L_{prot+lip}] = VF_{prot,FBS} \cdot D_{BSA/w} + VF_{lip,FBS} \cdot D_{lip/w} \quad \text{eq. 4}$$

$$D_{cell/w}(\text{pH 7.4})[L_w/L_{cell}] = VF_{prot,cell} \cdot D_{BSA/w} + VF_{lip,cell} \cdot D_{lip/w} + VF_{w,cell} \quad \text{eq. 5}$$

# Results and discussion

## *Fiber-water distribution*

Kinetics of fiber uptake and reproducibility between fibers (i.e., $D_{f/w}$ calculated for ten replicate extractions from PBS) were measured for 22 chemicals using the C18-fibers (Table 1), including the data for eight chemicals from this study and from two previous studies for seven organic acids[14] and for bisphenol A, quinoxyfen, coumarin, caffeine, metoprolol, propranolol and labetalol.[5] As expected, the time to reach equilibrium with the C18-fibers ($t_{95\%}$, Table 1 and Figure S1) increased with increasing hydrophobicity of the test chemicals and $D_{f/w}$ (Figure S2). For the hydrophilic chemicals caffeine, coumarin, zingerone, and lamotrigine $t_{95\%}$ was below 1 h at a shaking speed of 250 rpm compared to the more hydrophobic gingerols for which $t_{95\%}$ at 250 rpm was 6 – 7.8 h. For even more hydrophobic chemicals the samples were equilibrated using a vortex shaker set to 1200 rpm. Increased shaking speed decreases the thickness of the unstirred water layer on the surface of the fibers that controls the kinetics of fiber uptake for hydrophobic chemicals and therefore decreases $t_{95\%}$ (<9 h for the tested chemicals). $D_{f/w}$ also increased with increasing hydrophobicity. For neutral chemicals there was nearly a 1:1 relationship between log $D_{f/w}$ and log $K_{ow}$ (Figure S3B).

**Table 1.** Equilibration times ($t_{95\%}$) and logarithmic fiber-water distribution ratios (log $D_{f/w}$) determined at a constant nominal concentration ($C_{nom}$, n = 10). Fiber sorption isotherms in PBS at pH 7.4 and 37°C were fitted with the log-transformed Freundlich model (eq. 1) and compared to linear sorption ($n_{Fr}$ = 1) using the extra sum-of-squares F test. Significant deviation from linear sorption is indicated by a P value <0.05.

| Chemical | $t_{95\%}$ [h] | log $D_{f/w}$ (pH 7.4) [$L_w/L_f$] | SD | log $C_{nom}$ [M] | Isotherm | P value | $n_{Fr}$ [-] | log $K_{Fr}$ [(mmol/$L_f$)·(mmol/$L_w$)$^{-nFr}$] |
|---|---|---|---|---|---|---|---|---|
| Benzo[a]pyrene (B[a]P) | n.a. | 5.24[a] | 0.02[a] | n.a. | n.a. | n.a. | n.a. | n.a. |
| Benzo[k]fluoranthene (B[k]F) | n.a. | 5.23[a] | 0.03[a] | n.a. | n.a. | n.a. | n.a. | n.a. |
| Bisphenol A | 1.2[b] | 3.10[b] | 0.21[b] | -5.05[b] | n.a. | n.a. | n.a. | n.a. |
| Quinoxyfen | 5.0[b] | 5.16[b] | 0.16[b] | -6.70[b] | n.a. | n.a. | n.a. | n.a. |
| Triclocarban | 8.1 | 5.06 | 0.06 | -6.70 | n.a. | n.a. | n.a. | n.a. |
| Fluoranthene | 5.7 | 4.86 | 0.05 | -6.70 | n.a. | n.a. | n.a. | n.a. |
| 6-Gingerol | 6.0 | 3.99 | 0.25 | -5.15 | non-linear | 0.0008 | 0.79 | 3.31 |
| 8-Gingerol | 7.8 | 4.68 | 0.05 | -5.22 | linear | 0.95 | 0.98 | 4.57 |
| Caffeine | <1[b] | 1.42[b] | 0.03[b] | -4.30[b] | linear | 0.70 | 0.99 | 1.35 |
| Coumarin | <1[b] | 1.71[b] | 0.05[b] | -4.52[b] | linear | 0.56 | 0.99 | 1.80 |
| Zingerone | <1 | 1.92 | 0.06 | -4.52 | non-linear | 0.001 | 0.91 | 1.81 |
| Lamotrigine | 0.5 | 1.72 | 0.12 | -4.70 | linear | 0.39 | 1.02 | 1.80 |
| Metoprolol [B] | 2.3[b] | 2.45[b] | 0.08[b] | -5.52[b] | non-linear | <0.0001 | 0.83 | 2.04 |
| Propranolol [B] | 8.1[b] | 3.10[b] | 0.10[b] | -5.15[b] | non-linear | <0.0001 | 0.76 | 2.48 |
| Diphenhydramine [B] | 1.6 | 3.11 | 0.10 | -5.15 | non-linear | <0.0001 | 0.73 | 2.46 |
| Venlafaxine [B] | 2.4 | 2.66 | 0.12 | -5.22 | non-linear | <0.0001 | 0.78 | 2.24 |
| Diclofenac [A] | 3.5[c] | 2.47[c] | 0.04[c] | -4.80[c] | linear[c] | 0.1[c] | 0.96[c] | 2.53[c] |
| 2,4-Dichlorophenoxyacetic acid (2,4-D) [A] | 0.3[c] | 1.50[c] | 0.06[c] | -5.05[c] | non-linear[c] | <0.0001[c] | 0.79[c] | 1.10[c] |
| Ibuprofen [A] | 0.8[c] | 2.52[c] | 0.13[c] | -5.05[c] | non-linear[c] | <0.0001[c] | 0.74[c] | 1.90[c] |
| Naproxen [A] | 1.5[c] | 2.23[c] | 0.06[c] | -5.05[c] | non-linear[c] | 0.02[c] | 0.95[c] | 2.16[c] |
| Torasemide [A] | 2.4[c] | 3.16[c] | 0.06[c] | -5.22[c] | linear[c] | 0.67[c] | 1.01[c] | 3.16[c] |
| Warfarin [A] | 6.0[c] | 1.96[c] | 0.05[c] | -5.22[c] | non-linear[c] | <0.0001[c] | 0.91[c] | 1.73[c] |
| Genistein [A] | 3.3[c] | 2.62[c] | 0.03[c] | -5.15[c] | linear[c] | 0.05[c] | 0.95[c] | 2.51[c] |
| Labetalol [C] | 6.0[b] | 3.04[b] | 0.09[b] | -5.30[b] | non-linear | 0.006 | 0.88 | 2.61 |

[a] data from Ter Laak et al.[16]; [b] data from Henneberger et al.[5]; [c] data from Henneberger et al.[14], [B] organic bases, [A] organic acids, [C] chemical with complex speciation.

Fiber sorption isotherms were measured for six of the neutral and for all ionizable chemicals and fitted using the Freundlich model (Figure S4). The determined Freundlich exponents were significantly different from one (extra sum-of-squares F test) for two neutral chemicals (6-gingerol and zingerone) and nine of the 12 ionizable chemicals tested, indicating non-linear and consequently concentration-dependent sorption to the C18-fibers (Table 1). For bisphenol A, quinoxyfen, triclocarban, and fluoranthene no isotherms were measured, because the concentration range that could be tested was very limited, determined on the upper end by the aqueous solubility of the chemicals and on the lower end by the detection limit of instrumental analysis. To account for the non-linear sorption to the C18-fiber when evaluating the data for binding to BSA, liposomes, FBS and cells, control samples in PBS were prepared for all chemicals at fiber

concentrations similar to the samples with the biological materials to derive the corresponding $D_{f/w}$ required in eq. 2 and 3.

**Table 2.** Experimentally determined distribution ratios at pH 7.4 and 37°C between bovine serum albumin (BSA) and water ($D_{BSA/w}$), liposomes and water ($D_{lip/w}$), fetal bovine serum (FBS) and water ($D_{FBS/w}$, normalized to the total lipid and protein content of FBS), medium and water ($D_{medium/w}$, calculated from $D_{FBS/w}$ using eq. 6 for a medium with 10 % FBS) and cells and water ($D_{cell/w}$, normalized to the total volume of cells; the average value of all cell lines tested was calculated for each chemical, individual $D_{cell/w}$ are given in the SI, Table S6).

| Chemical | bovine serum albumin | | | liposomes | | | fetal bovine serum | | | medium | cells | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | log $D_{BSA/w}$ (pH 7.4) [$L_w/L_{BSA}$] | SD | n | log $D_{lip/w}$ (pH 7.4) [$L_w/L_{lip}$] | SD | n | log $D_{FBS/w}$ (pH 7.4) [$L_w/L_{prot+lip}$] | SD | n | log $D_{medium/w}$ (pH 7.4) [$L_w/L_{medium}$] | log $D_{cell/w}$ (pH 7.4) [$L_w/L_{cell}$] | SD | n |
| B[a]P | 4.98 | 0.04 | 4 | 7.05[c] | - | - | 5.31 | 0.10 | 4 | 3.05 | 4.46 | 0.15 | 12 |
| B[k]F | 4.74 | 0.06 | 4 | 6.92[c] | - | - | 5.18 | 0.11 | 4 | 2.92 | 4.34 | 0.16 | 12 |
| Bisphenol A | 3.01[a] | - | - | 3.50[d] | - | - | 2.86 | 0.17 | 12 | 0.69 | 1.66 | 0.37 | 24 |
| Quinoxyfen | 3.96 | 0.11 | 4 | 5.32 | 0.31 | 12 | 3.59 | 0.18 | 10 | 1.35 | 3.90 | 0.33 | 15 |
| Triclocarban | 4.73 | 0.04 | 4 | 6.29 | 0.09 | 12 | 4.24 | 0.08 | 12 | 1.98 | 3.91 | 0.26 | 21 |
| Fluoranthene | 4.41[a] | - | - | 5.41[c] | - | - | 4.01 | 0.14 | 12 | 1.75 | 3.71 | 0.32 | 12 |
| 6-Gingerol | 3.08 | 0.16 | 4 | 3.45 | 0.30 | 12 | 2.92 | 0.13 | 12 | 0.74 | 2.81 | 0.58 | 12 |
| 8-Gingerol | 3.80 | 0.34 | 4 | 4.70 | 0.14 | 8 | 3.09 | 0.23 | 12 | 0.89 | 3.52 | 0.35 | 12 |
| Caffeine | 1.66 | 0.05 | 4 | 0.08[e] | - | - | 1.80 | 0.33 | 11 | 0.13 | n.a. | - | - |
| Coumarin | 2.05 | 0.02 | 4 | 2.09 | 0.16 | 12 | 1.59 | 0.12 | 16 | 0.08 | 1.19 | 0.55 | 12 |
| Zingerone | 2.54 | 0.27 | 4 | 1.97 | 0.21 | 12 | 2.67 | 0.22 | 12 | 0.55 | 1.04 | 0.40 | 12 |
| Lamotrigine | 2.16 | 0.13 | 4 | 2.06 | 0.16 | 12 | 1.50 | 0.18 | 16 | 0.07 | 0.59 | 0.06 | 3 |
| Metoprolol [B] | 1.51 | 0.20 | 4 | 1.42[f] | 0.06 [f] | 4 | 1.46 | 0.33 | 16 | 0.06 | 0.80 | 0.13 | 3 |
| Propranolol [B] | 1.78 | 0.10 | 4 | 2.73 | 0.15 | 12 | 1.30-2.42[h] | - | 16 | 0.04-0.38[h] | 0.80 | 0.30 | 6 |
| Diphenhydramine [B] | 1.99 | 0.20 | 4 | 2.17 | 0.28 | 12 | 1.90 | 0.23 | 16 | 0.15 | n.a. | - | - |
| Venlafaxine [B] | 1.58 | 0.15 | 4 | 1.64[f] | 0.05[f] | 4 | 1.69[i] | 0.23 | 12 | 0.10[i] | n.a. | - | - |
| Diclofenac [A] | 4.40[b] | 0.08[b] | 4 | 2.64[g] | - | - | 2.74-3.40[b,h] | - | 30 | 0.60-1.17[b,h] | 1.46[b] | 0.25[b] | 11 |
| 2,4-D [A] | 3.56[b] | 0.02[b] | 4 | 2.02[b] | 0.16[b] | 6 | 2.04-3.01[b,h] | - | 20 | 0.20-0.82[b,h] | 1.15[b] | 0.25[b] | 12 |
| Ibuprofen [A] | 4.02[b] | 0.09[b] | 4 | 1.81[g] | - | - | 2.36-3.37[b,h] | - | 21 | 0.35-1.14[b,h] | 1.31[b] | 0.40[b] | 12 |
| Naproxen [A] | 5.21[b] | 0.11[b] | 4 | 2.17[b] | 0.08[b] | 6 | 2.80-4.85[b,h] | - | 16 | 0.65-2.59[b,h] | 1.55[b] | 0.57[b] | 12 |
| Torasemide [A] | 3.81[b] | 0.07[b] | 4 | 2.05[b,f] | 0.05[b,f] | 4 | 3.26[b] | 0.13[b] | 25 | 1.04[b] | 1.67[b] | 0.67[b] | 15 |
| Warfarin [A] | 3.46[b] | 0.08[b] | 4 | 1.62[b] | 0.30[b] | 6 | 2.15-2.90[b,h] | - | 20 | 0.25-0.73[b,h] | 1.19[b] | 0.57[b] | 11 |
| Genistein [A] | 2.83[b] | 0.03[b] | 4 | 3.32[b] | 0.11[b] | 6 | 2.71[b] | 0.22[b] | 25 | 0.58[b] | 1.08[b] | 0.15[b] | 12 |
| Labetalol [C] | 1.24 | 0.08 | 4 | 3.26 | 0.12 | 12 | 1.79 | 0.28 | 16 | 0.12 | 0.67 | 0.33 | 4 |

[a] data from Endo et al.[18]; [b] data from Henneberger et al.[14]; [c] data from van der Heijden et al.[19]; [d] data from Kwon et al.[20]; [e] no measurable binding, predicted with UFZ-LSER database[21]; [f] determined with equilibrium dialysis, four replicate dialysis cells measured at three different time points; [g] data from Avdeef et al.[22]; [h] non-linear sorption isotherm, log $D_{FBS/w}$ were concentration-dependent and the ranges were reported; [i] highest concentration excluded from calculation; n.a. - not analyzed, [B] organic bases, [A] organic acids, [C] chemical with complex speciation.

## *Bovine serum albumin- and liposome-water distribution ratios*

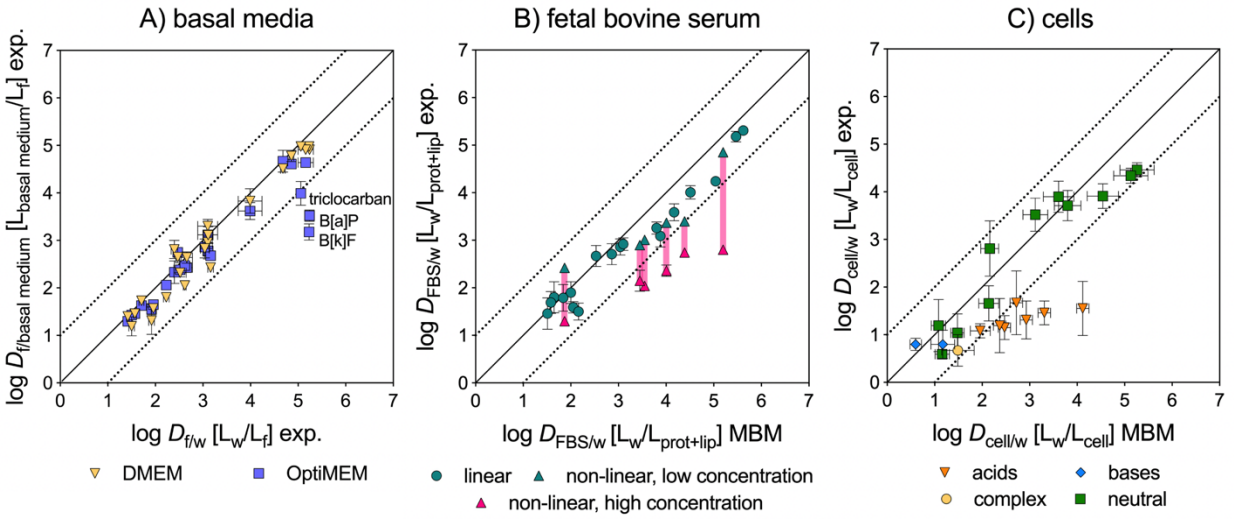
For the validation of the mass balance model reliable data for BSA-water and liposome-water distribution were required for all chemicals as input parameters. Data from literature were used, if available (e.g., for bisphenol A and fluoranthene) or from a previous study for the seven organic acids.[14] In this study $D_{BSA/w}$ of 15 chemicals and $D_{lip/w}$ of ten chemicals were additionally measured (Table 2). For caffeine the binding to liposomes was too weak for experimental determination (fraction bound <20% at a liposome concentration of 49.5 g/L) and $D_{lip/w}$ was predicted using a polyparameter linear free energy relationship (PP-LFER).[21] For metoprolol and venlafaxine the $D_{lip/w}$ determined with the C18-SPME method had very high standard deviations (up to 0.6 log-units), and therefore $D_{lip/w}$ was determined with equilibrium dialysis as described by Henneberger et al.[14] For neutral chemicals $D_{BSA/w}$ and $D_{lip/w}$ were linearly correlated, while no correlation was observed for the charged chemicals (see SI section S7 for further discussion).

## *Distribution in cell culture media*

The basal media OptiMEM and DMEM had very small sorption capacity for ionizable and neutral chemicals and the fractions bound to the proteins and lipids of the basal media were often too small to be calculated. This can best be visualized by comparing $D_{f/w}$ and the distribution ratio between the C18-fibers and the basal medium ($D_{f/basal\ medium}$). For both basal media and nearly all tested chemicals $D_{f/basal\ medium}$ was very close to $D_{f/w}$ (Figure 2A). Sorption of the chemicals to the proteins and lipids of the basal medium would lead to a decrease of $D_{f/basal\ medium}$ compared to $D_{f/w}$. This was only the case for the three chemicals with the highest $D_{lip/w}$ (triclocarban, B[a]P, B[k]F) and only for the medium OptiMEM, indicating that this basal medium did indeed contain small amounts of lipids. Based on the protein and lipid content of the cell culture basal media OptiMEM and DMEM measured in a previous study[4] a significant binding to the colloids in the basal media (fraction bound >20 %) was expected for 19 of the 24 chemicals. As already stated in our previous

study with organic acids,[14] the detected proteins and lipids in the basal media might be small peptides and fatty acids that do not offer attractive binding sites for the test chemicals. We therefore recommend to determine protein and lipid content of the complete assay medium (e.g., as described in reference 5) and not of the individual medium constituents. Because of the minimal contribution of the basal medium to the sorption capacity of the complete cell culture medium (basal medium plus FBS supplement), the medium-water distribution ratio is essentially the same as the FBS-water distribution ratio ($D_{FBS/w}$) of the test chemicals.

**Figure 2.** Comparison of (A) fiber-water ($D_{f/w}$) and fiber-basal medium distribution ratios ($D_{f/basal\ medium}$) for two cell culture basal media (OptiMEM and DMEM), (B) modelled (mass balance model (MBM) of Fischer et al.[4]) and measured fetal bovine serum-water distribution ratios ($D_{FBS/w}$) and (C) modelled (MBM, Fischer et al.[4]) and measured cell-water distribution ratios ($D_{cell/w}$). Error bars are plotted for all experimental values (exp.) but are often hidden by the data points.



299 The proteins and lipids of FBS showed a high sorption capacity for neutral and ionizable
300 chemicals. For all neutral and basic chemicals, as well as for labetalol linear binding to FBS was
301 measured in the tested concentration range (see FBS sorption isotherms in SI, Figure S6) and all
302 measured data were combined to calculate a single FBS-water distribution ratio ($D_{FBS/w}$). The
303 experimental log $D_{FBS/w}$ were ranging from 1.46 for metoprolol to 5.31 for B[a]P (Table 2). Note
304 that medium-water distribution ratios ($D_{medium/w}$ or $D_{FBS/w}$) can be expressed in different units.
305 Fischer et. al[4] defined $D_{medium/w}$ as the ratio of the concentration in the complete medium (protein,
306 lipid and water phase) and in a hypothetical pure water phase. The resulting $D_{medium/w}$ had the unit
307 $L_w/L_{medium}$. In this study $D_{FBS/w}$ was calculated as the ratio of the concentration bound to medium
308 proteins and lipids and the freely dissolved concentration in the water phase of the medium (unit:
309 $L_w/L_{prot+lip}$) derived from the Freundlich isotherms. If the total medium volume ($V_{medium}$), the
310 volume of water ($V_w$) and of medium proteins and lipids ($V_{prot+lip}$) are known both distribution
311 ratios are easily convertible (eq. 6). Table 2 also lists $D_{medium/w}$ of the test chemicals calculated
312 from the experimental $D_{FBS/w}$ for a medium containing 10 % FBS.

313 $$D_{\mathrm{medium/w}}(\mathrm{pH\ 7.4})[\mathrm{L_w/L_{medium}}] = \frac{D_{\mathrm{FBS/w}} \cdot V_{\mathrm{prot+lip}} + V_{\mathrm{w}}}{V_{\mathrm{medium}}} \qquad \text{eq. 6}$$

314 Saturation of FBS binding was observed for the organic acids (for sorption isotherms see ref [14])
315 and for the base propranolol (Figure S6L). Therefore, the range of $D_{FBS/w}$ (highest concentration
316 tested to lowest concentration tested) is depicted in Table 2. For the neutral chemicals $D_{FBS/w}$ was
317 linearly correlated with log $K_{ow}$ (Figure S3E).

318 The mass balance model was able to predict the measured $D_{FBS/w}$ for the chemicals that showed
319 linear binding to FBS (petrol blue circles in Figure 2B) and $D_{FBS/w}$ at low concentrations for the
320 chemicals that showed non-linear binding (petrol blue triangles in Figure 2B) very well (RMSE =
321 0.48 log-units). As expected, $D_{FBS/w}$ was overestimated by up to 2.4 log-units (naproxen) at high

concentrations for the chemicals that showed non-linear binding, because saturation of binding sites was not included in the model (pink triangles in Figure 2B). For all chemicals with linear FBS sorption isotherms $D_{FBS/w}$ given in Table 2 can be used to calculate the medium-water distribution ratio for any FBS-containing medium, if the total protein and lipid content of the medium is known, assuming that the ratio between protein and lipid content is similar among different FBS types.

*Estimation of protein saturation*

Mass balance models should be used with caution for the prediction of $C_{free}$ at high concentrations of the test chemicals as strong saturation effects were observed for organic acids,[14] but also for the base propranolol in this study. In theory, protein saturation can be estimated using mass balance models by calculating the molar ratio of chemical to BSA (ν in [$mol_{chemical}/mol_{BSA}$]), which is the number of test molecules bound to one molecule of BSA. This calculation only requires $C_{free}$ in the assay medium (modelled or measured in [$mol/L_w$]) and $D_{BSA/w}$ [$L_w/L_{BSA}$] of the test chemical:

$$\nu = C_{\mathrm{free}} \cdot D_{\mathrm{BSA/w}} \cdot V_{\mathrm{m,BSA}} \qquad \text{eq. 7}$$

The molar volume of BSA ($V_{m,BSA}$ = 48.87 $L_{BSA}/mol_{BSA}$) can be derived by dividing the molar mass of BSA (66.463 kg/mol) by a protein density of 1.36 kg/L. For chemicals that have only one specific binding site (e.g., organic acids like naproxen) saturation can be expected if ν is >0.1 (i.e., more than 10 % of the binding sites are occupied). For neutral chemicals the threshold is probably much higher, as the binding is likely happening at multiple non-specific binding sites.[18, 23] We estimated ν of the neutral chemicals at the solubility limit ($C_{free}$ = water solubility, SI, Table S5). For the neutral hydrophilic chemicals coumarin and caffeine ν was >200. However, the determined $D_{FBS/w}$ of this study and experimentally determined $C_{free}$ in *in vitro* cell-based bioassays from a

345 previous study[5] suggest that the free fraction of neutral hydrophilic chemicals and bases in assay
346 medium is high (>50 %) in any case and even if saturation of the medium proteins occurs this will
347 not lead to a significant change in $C_{free}$. For hydrophobic chemicals like B[a]P and B[k]F saturation
348 of the water phase is expected to be reached before saturation of the proteins, since the ν was well
349 below 0.1 for these chemicals at $C_{free}$ = water solubility. For neutral chemicals with moderate
350 hydrophobicity (e.g., bisphenol A, fluoranthene and 6-gingerol) ν was up to 600. Therefore, for
351 organic acids that show very strong and specific binding to serum albumin and neutral chemicals
352 with moderate hydrophobicity saturation phenomena should be further explored in the future. The
353 most important question is, whether saturation of medium proteins happens at the concentrations
354 typically tested in *in vitro* bioassays. First results from our group indicate that this is indeed the
355 case for organic acids like diclofenac, but not for neutral and basic chemicals.[5] We suggest to
356 consider saturation of binding if ν is >0.1 for all chemicals that are known to have only one specific
357 binding site on albumin (e.g., the majority of organic acids) and for all other chemicals if ν is >10,
358 which means that more than 10 molecules of the test chemical are bound to one molecule of
359 albumin. This threshold allows no quantitative assessment of protein saturation, but can be used
360 for prioritizing chemicals for experimental exposure assessment.

361 *Cell-water distribution ratios*

362 The cell-water distribution ratios at pH 7.4 (log $D_{cell/w}$) were measured for 21 chemicals for up
363 to four different cell lines (Table S6). When the data for the individual cell lines were plotted
364 against the average of all cell lines, the data agreed within a factor of ten and no systematic
365 differences between cell lines were visible (Figure S7), which was confirmed by statistical tests
366 (SI section S10, Table S8-S10). Therefore, all measured data were combined to calculate a single
367 $D_{cell/w}$ for each chemical (Table 2). These average values of $D_{cell/w}$ were compared with the $D_{cell/w}$
368 predicted by the mass balance model (Figure 2C). Protein and lipid contents were available from

the literature[4, 14] for seven cell lines and the average of all cell lines was used for the comparison (see also Table S7). For all chemicals except the organic acids (orange triangles in Figure 2C) the model predicted $D_{cell/w}$ very well (RMSE = 0.53 log-units). Again, larger deviations from the model prediction of up to 2.57 log-units were found for the organic acids.

In a previous study, the prediction of $D_{cell/w}$ could be improved for organic acids by using structural proteins as surrogates for cellular proteins.[14] For all neutral test chemicals of this study the structural protein-water distribution ratios ($D_{SP/w}$) could be predicted using poly-parameter linear free energy relationships (pp-LFER) included in the UFZ-LSER database.[21, 24] For four of the ionizable test chemicals (propranolol, diclofenac, ibuprofen, naproxen) $D_{SP/w}$ was available from the literature. For the other chemicals, $D_{SP/w}$ was estimated using different models (see SI Section S11), because there is no generally applicable prediction tool available so far. The $D_{cell/w}$ predicted using structural proteins to represent cellular proteins agree much better with the measured data for all test chemicals (RMSE = 0.57, Fig. S8), supporting the results of our earlier study.[14]

### *Recommendations for future improvements of mass balance models and* in vitro *exposure assessment*

The results of this study indicate that multimedia equilibrium-based mass balance models can reliably predict $C_{free}$ and $C_{cell}$ for neutral chemicals and organic bases. Because the model gives reliable predictions for organic bases the use of serum albumin as surrogate for medium proteins is likely to be sufficient and other proteins like $\alpha_1$-acid glycoprotein do not need to be considered.

For organic acids there is definitely a need for further improvements, because both, $D_{FBS/w}$ (at high concentrations of the test chemicals) and $D_{cell/w}$ were overestimated by the model.[14] Quantitatively predicting protein saturation is difficult, but the estimation method presented in this study can be used to assess whether protein saturation can be relevant for the tested concentrations.

For a better prediction of $D_{cell/w}$ a better surrogate than BSA for cellular proteins would be required and structural proteins appear to be the best available alternative. However, considering the fact that $C_{free}$ is not linearly related to $C_{nom}$ for organic acids and that purification of structural proteins is at least as labor-intense as cell culture, measuring $C_{free}$ in the actual *in vitro* bioassay medium at different concentrations and $D_{cell/w}$ for the cell line of interest might be more pragmatic for organic acids than generating the sorption isotherms for the medium and the binding data for structural proteins that would be required for improving the mass balance model.

Mass balance models are also not applicable to chemicals that undergo loss processes like cellular metabolism or abiotic degradation (e.g., hydrolysis or photolysis), as well as to volatile and semi-volatile chemicals that can be lost extensively from the bioassay system.[25] For these chemicals time-resolved kinetic models are required to predict freely dissolved and total cellular concentrations.

## ASSOCIATED CONTENT

**Supporting Information**. More detailed list of test chemicals, overview of fiber types and concentrations of serum albumin, liposomes, FBS and cell numbers used for SPME experiments, information on chemical analysis, additional figures comparing the determined distribution ratios with octanol-water partitioning of neutral chemicals, BSA-water and liposome-water distribution ratios, kinetics of fiber uptake, fiber sorption isotherms and FBS sorption isotherms, cell-water distribution ratios for individual cell lines and ANOVA test (PDF). Standard operating procedure for the C18-SPME experiments (PDF). Compilation of all distribution ratios (Excel file).

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