analytical chemistry

Article

pubs.acs.org/ac

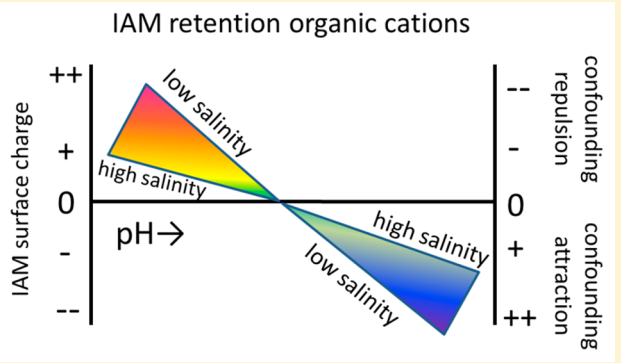
Dealing with Confounding pH-Dependent Surface Charges in Immobilized Artificial Membrane HPLC Columns

Steven T. J. Droge*

Institute for Risk Assessment Sciences, Utrecht University, Yalelaan 104, 3508 TD Utrecht, The Netherlands

Supporting Information

ABSTRACT: The retention capacity factor (k_{IAM}) on immobilized artificial membrane chromatography columns (IAM-HPLC) is widely used as experimental descriptor of lipophilicity. For predominantly ionized compounds, however, unexpected and significant effects of pH, buffers, and salinity on k_{IAM} have been reported. Besides zwitterionic phospholipids, IAM particles contain acidic silanol moieties and positively charged propylamine groups. The electrostatic model and experimental k_{IAM} values presented in this study for organic cations show that the net IAM surface charge is positive below pH 5 and negative above pH 5. The resulting confounding electrostatic repulsion/attraction is strongly influenced by eluent salinity: k_{IAM} values for cations differ by more than 2 orders of magnitude over the tested range of aqueous eluents. In phosphate buffered saline medium the actual lipophilicity of cationic drugs ($K_{PLIPW,cation}$) is overestimated by at least a factor of 2. The $K_{PLIPW,cation}$ can be readily determined by IAM-HPLC in any 10 mM buffered eluent at pH 5. Accounting for, or avoiding, confounding electrostatic effects in IAM-HPLC considerably advances assessments of (phospho)lipophilicity for drug discovery and for environmental risk assessment of organic cations.



The octanol–water partition coefficient (K_{OW}) is commonly applied as a descriptor of lipophilicity in pharmacological and environmental sciences to predict the passive uptake and accumulation of dissolved drugs and organic chemicals into biotic tissues. Although for some compound classes this generalization introduces systematic errors, for many classes of neutral chemicals bulk partitioning into octanol includes comparable molecular interactions as partitioning into storage lipid and phospholipid membranes.[1−3] The majority of pharmaceuticals and vast amounts of industrial chemicals, however, are ionizable compounds[4−6] that are predominantly present as charged species at neutral pH. Octanol is not the ideal surrogate phase to determine lipophilicity for ionic compounds in relation to partitioning to membranes, as octanol lacks critical molecular features of phospholipids such as the zwitterionic headgroups[7] and anisotropic structuring.[8] The need of accurate lipophilic properties for many toxicologically relevant ionizable compounds warrants large data sets on measured sorption affinities of organic ions to phospholipid membranes ($K_{PLIPW,ion}$). Astonishingly, though, recent reviews on membrane affinities for ionic compounds[8−10] could only list $K_{PLIPW,ion}$ for 50 acids and 25 bases from publically available literature. This data scarcity strongly hampers development of accurate structure–activity relationships required for bioaccumulation modeling[10] and validation of molecular simulations.[8]

Immobilized artificial membrane chromatography (IAM-HPLC) has become a commonly used, high throughput screening method in pharmacological research and drug development to determine lipophilicity.[11,12] IAM column particles contain a monolayer coating of biomimetic phospholipids, which should be representative of the anisotropic structuring of phospholipids in cell membranes. For most neutral compounds, column retention capacities (k_{IAM}) obtained by IAM-HPLC indeed correlate well with phospholipid–water sorption coefficients (K_{PLIPW}) obtained with artificial liposomal bilayers, although some outliers were noted.[13] IAM-HPLC data are regularly published for many ionizable pharmaceuticals (e.g., see refs 14–27). The simplicity and consistency of a chromatographic tool seems ideal to create a vast, high quality data set of $K_{PLIPW,ion}$ values.[28] These IAM-retention values for ionizable chemicals, however, were not considered by the aforementioned reviews on $K_{PLIPW,ion}$ values,[8−10] because of concerns raised in several IAM-HPLC studies on confounding charged groups on IAM particles. The charged groups influence the retention capacity factors of strongly ionized compounds.[15,16,18,22,29] If these confounding effects could be adequately quantified or minimized, IAM-HPLC would be a suitable tool to derive experimental lipophilicity descriptors for ionic compounds, adequate input parameters in chemical fate, or kinetic modeling in environmental and pharmacological studies.

Received: October 1, 2015
Accepted: December 6, 2015
Published: December 6, 2015

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960–967

This study aims to unravel the role of the undesirable surface charge interactions for ionizable bases in IAM-HPLC. Previous chromatographic studies with soil organic matter[30] and clay minerals[31] demonstrated that salinity can strongly influence the retention of positively charged compounds to HPLC columns packed with negatively charged sorbents. This study systematically measures how the IAM-HPLC retention of a diverse set of charged amines, anionic tracer compounds, and a neutral reference compound was influenced by salinity over a broad pH range. The second goal is to model the electrostatic processes causing the effects of salinity on retention, in order to quantify the role and properties of the charged surface moieties. The measurements and modeling work in this study should (i) elucidate the significance of the (undesired) charged groups on the overall sorption, for the most widely used IAM column (IAM.PC.DD2), (ii) establish the experimental conditions where these surface interactions can be minimized, or accounted for, and (iii) evaluate whether IAM-HPLC can be used to derive accurate K_{PLIPW} values for organic ions.

Modeling Confounding Electrostatic Interactions in IAM. The IAM retention capacity factors (k_{IAM}) in fully aqueous eluent can be directly converted to a sorption coefficient to the column material by accounting for the medium/phospholipid volume ratio (φ).[32] If retention is only due to the phospholipid coating, measurements for K_{PLIPW} values are obtained by

$$K_{PLIPW=} = \varphi k_{IAM} = 18.9(t_r - t_0)/t_0 \quad (1)$$

where φ is 18.9 for IAM.PC.DD2 columns (1/0.053 in ref 32), t_r is the retention time of the test compound, and t_0 is the elution time of a nonretained tracer. For ionizable compounds, the observed K_{PLIPW} relates to the contributions of both neutral and ionic species according to their dissociation equilibrium in the eluent. As a result of the normal process of column particle manufacturing, a substantial fraction of both basic aminopropyl silica and acidic free silanol groups will reside on the IAM surface, even after end-capping, as summarized in Figure 1.[12,16,19,33]

Based on a silanol pK_a of 6.8 and propylamine pK_a of 10.7,[16] the IAM surface at pH below 5 is dominated by SiOH and NH_3^+ groups, and thus positively charged. Escher et al.[16] argued that at pH 3 their cationic test compounds were repulsed by the -NH_3^+ moieties, resulting in underestimated lipophilicity. With an excess of dissociated silanol moieties to free amine groups, a transition to SiO^- and NH_3^+ around pH 5−7 results in a negatively charged IAM surface at neutral pH.[29] Although the operational range of the IAM.PC.DD2 column is between pH 2.5 and 7.5, most IAM studies have been conducted at physiological pH. As a result, cationic compounds are typically additionally attracted by the negatively charged silanol groups on top of their interactions with phospholipid, resulting in overestimated lipophilicity.

Following the Gouy−Chapman theory, a charged surface on the IAM particles electrostatically attracts oppositely charged ions in order to maintain electroneutrality. This results in the formation of a "diffusive water layer" (DL), or electrical double layer, surrounding the charged IAM-particle surfaces with a different concentration of ions compared to the bulk solution.[34] The ratio between the aqueous concentration in the DL ($C_{aq,DL}$) and the bulk aqueous concentration ($C_{aq,bulk}$) is the Boltzmann factor (B), as conceptualized for IAM-HPLC in Figure 2.

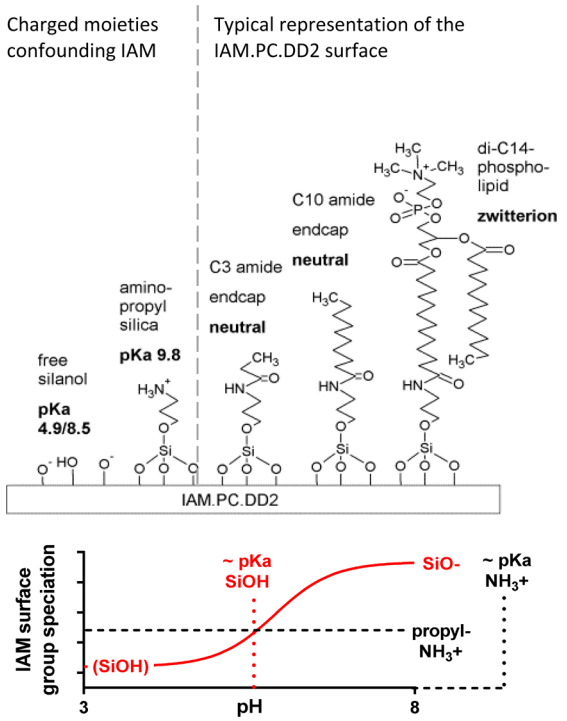


Figure 1. Schematic overview of five different surface groups in IAM-HPLC and speciation profiles for confounding groups between pH 3 and pH 8.

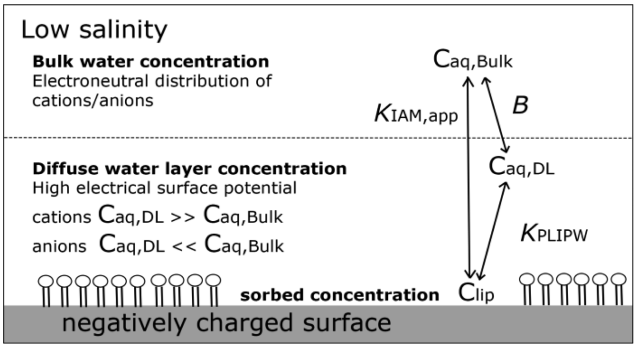


Figure 2. Schematic overview of the nonspecific electrostatic attraction/repulsion (by Boltzmann factor B) of organic ions from the bulk eluent ($C_{aq,bulk}$) to the diffuse water layer surrounding IAM surfaces ($C_{aq,DL}$). The apparent IAM column retention (due to sorption coefficient $K_{IAM,app}$) is based on $C_{aq,bulk}$, while the intrinsic affinity to the phospholipid coating (K_{PLIPW}) relates to $C_{aq,DL}$. Higher salinity lowers the surface potential, hence reducing the factor B. At low pH, the IAM surface is positively charged, reversing the ionic attraction/repulsion. The proportions of phospholipid lengths and diffuse water layer thickness are not realistic.

The intrinsic K_{PLIPW} (or $K_{IAM,intr}$) only relates to the ratio between $C_{aq,DL}$ and the concentration sorbed in the phospholipid coating (C_S). Electrostatic attraction results in an increased apparent retention capacity ($k_{IAM,app}$) because $C_{aq,DL}$ is higher than $C_{aq,bulk}$, and therefore also an increased apparent sorption affinity to the IAM material ($K_{IAM,app}$). Solutes with a similarly charged sign as the surface will be repulsed from the diffusive layer, resulting in a lower $K_{IAM,app}$ than that due to phospholipid binding alone. Log K_{PLIPW} is thus simply the difference between log $K_{IAM,app}$ and log B, if specific sorption to silanol and propylamine groups is neglected:

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960−967

$$\log K_{IAM,app} = \log\left(\frac{C_S}{C_{AQ,bulk}}\right) = \log\left(\frac{B}{C_{AQ,DL}}\frac{C_S}{1}\right)$$
$$= \log(BK_{PLIPW}) = \log K_{PLIPW} + \log B \quad (2)$$

B is part of the Poisson−Boltzmann equation, and for a monovalent ion solution at 25 °C (see Supporting Information (SI) section S1 for details) B can be approximated as an exponential function of the effective surface potential (Ψ_{eff}):

$$B = e^{(-38.94\Psi_{eff})} \quad (3)$$

Ψ_{eff} is lower than the actual surface potential (Ψ_0) that is related to actual surface charge density, since Ψ_{eff} operates at a certain distance from the surface—about ∼1 D length.[35] The Debye length ($1/\kappa$, in nm) is a function of the ionic strength (I) of the solution, which for a monovalent ion solution at 25 °C can be approximated by $[0.304/(\sqrt{I})]$ (see SI section S1 for details). The difference between Ψ_{eff} and Ψ_0 increases exponentially with the inverse of the Debye length, so eq 3 can be extended to

$$B = e^{(-38.94\Psi_0 e^{-\sqrt{I}/0.304})} \quad (4)$$

Since salinity influences the Debye length, salinity also influences the retention behavior of organic cations on the IAM column. The Debye length in common phosphate buffered saline medium (PBS) is 0.8 nm (0.15 M), 2.5 nm in 10 times diluted PBS and 7.8 nm in 100 times diluted PBS.[34] The influence of pH on the retention of the column is due to the pH-dependent density of dissociated free silanol groups (SiO^-). While the maximum density of unreacted aminopropyl groups ($maxNH_3^+$) is a constant, both groups together form the actual surface potential, Ψ_0 in eq 4. Assuming a single pK_a for silanol groups, the Henderson−Hasselbalch equation can be applied to calculate the fraction (f_{ION}) of silanol groups that is dissociated, and the actual surface potential can be described by

$$\Psi_0 = \Psi_{0,maxNH_3^+} + f_{Ion}\Psi_{0,maxSiO^-}$$
$$= \Psi_{0,maxNH_3^+} + \Psi_{0,maxSiO^-}\frac{1}{1 + 10^{(pK_a(SiOH)-pH)}} \quad (5)$$

Combining eqs 4 and 5 gives a full model equation for B that depends on ionic strength as well as the pH of the IAM eluent, which balances the amount of charged surface sites on the IAM material. Incorporating this full equation for B in eq 2 provides a full model to fit to IAM retention over a series of different eluent types. The intrinsic phospholipid sorption affinity of the ionic species and the relative contributions from confounding charged groups is related to

$$\log K_{IAM,app} = \log K_{PLIPW}$$
$$+ \log[\exp(-38.94(\Psi_{0,maxNH_3^+} + f_{ion}\Psi_{0,maxSiO^-})$$
$$e^{-\sqrt{I}/0.304})] \quad (6)$$

Equation 6 shows that low electrolyte concentrations induce a high surface potential, resulting in strong confounding effects on IAM-HPLC retention, while high electrolyte concentrations reduce ("screen") the electric surface potential, thereby reducing also confounding retention. If the net Ψ_0 is 0 ($maxNH_3^+$ groups balancing the amount of ionized silanol groups), there is no attraction into the DL and $K_{IAM,app} = K_{PLIPW}$. Note that eq 6 neglects the contribution of neutral species to the overall sorption affinity and is only valid for monovalent eluent solutions at ∼25 °C for organic cations.

MATERIALS AND METHODS

Test Compounds. Six different amines were selected as test compounds in a first data set for the pH/salinity range, all with UV absorbing moieties: two primary amines (tryptamine, pK_a = 10.7; naphthylmethylamine, pK_a = 9.1), a secondary amine (metoprolol, pK_a = 9.5), a tertiary amine (3-dimethylamino-propiophenone "dmapio", pK_a = 9.1), and two quaternary ammonium compounds (benzyltrimethylammonium and benzyldimethylhexylammonium). Molecular structures and more detailed information are presented in the SI Table S1. 3-Nitroaniline (pK_a = 2.11) was used as a neutral reference compound, following ref 36. Nitrate (as $NaNO_3$) and bromide (as KBr) were used as anionic tracers; thiourea and pure water, as neutral nonretaining tracers. Eight amines were selected to form a second data set for direct comparisons between IAM-based K_{PLIPW} values and liposomal partitioning values: amlodipine (pK_a = 9.0), fluoxetine (pK_a = 10.1), propranolol (pK_a = 9.5), metoprolol (pK_a = 9.5), atenolol (pK_a = 9.5), 4-phenylbutylamine (pK_a = 10.5), lidocaine (pK_a = 7.9), and procaine (pK_a = 9.0). All compounds had a purity of >97%, and stock solutions were prepared as ∼10 mg in 1 mL of methanol (Biosolve BV, Valkenswaard, The Netherlands).

IAM Column and Eluents. A 10 cm × 4.6 mm IAM.PC.DD2 column (Regis Technologies, Inc., Morton Grove, IL, USA) was used with an IAM.PC.DD2 10/300 guard cartridge in front. The column was always conditioned for >1 h to freshly prepared eluent with an eluent flow rate of 1.0 mL/min, the same flow rate as used throughout all measurements. Measurements were performed at room temperature (23 ± 2 °C). Methanol stock solutions of the test chemicals were diluted 100−200 times in the applied eluent, and 20 μL was injected.

The eluents at every pH were all prepared as a ∼10 mM buffer, with an additional 8.0 g/L NaCl (137 mM) and 0.2 g/L KCl (2.7 mM), corresponding to the commonly used salinity (0.15 M) and composition of PBS solution. The range of pH 3−7.4 was covered with buffers for every 0.5 pH unit increment, with phosphate buffer being used for pH 3.0, pH 6.0, pH 7.0, and pH 7.4, formic acid buffer for pH 3.5 and pH 4.0, and acetic acid buffer for pH 4.5, pH 5.0, and pH 5.5. Before and after use of each eluent, the pH was verified to be within 0.05 pH units of the set value with a Schott BlueLine pH electrode (Schott Instruments GmbH, Mainz, Germany). From every pH buffered eluent at 0.15 M salinity a 10× dilution with pure water was made; the pH was adjusted if required, resulting in a 0.015 M eluent buffered at the same pH. Additionally, the 0.015 M eluent was used for another 10× dilution with pure water to create a 0.0015 M eluent, adjusting the pH if necessary. The three buffered eluents at each pH with 0.15, 0.015, and 0.0015 M were used to determine the retention of all test compounds in the pH/salinity series from high salinity to low salinity, before switching to another pH buffered eluent of 0.15 M salinity. Throughout the pH/salinity series, only fully aqueous eluents were used, in order to avoid any possible effect of solvents on the pH-dependent IAM surface charge development. With the experience in many initial tests that triplicate sampling on the same day resulted in log $K_{IAM,app}$ differences of <0.02, for most measurements in the pH/salinity series only one or two measurements were performed.

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960−967

For the amines in the second data set (except metoprolol) that were used in the comparisons between IAM-HPLC and literature reported liposomal partitioning data, only pH 5.0 acetate (10 mM) buffered eluent at 0.15 M was used, with triplicate measurements. For some of the amines, a series of at least four different eluent mixtures with acetonitrile was tested, in order to extrapolate to $K_{IAM,app}$ values in 100% aqueous buffer, using ≤30% acetonitrile in steps of 5%.

HPLC Detection and Analysis. An Agilent 1100 diode-array UV system was set to simultaneous detection of 207, 220, 254, and 278 nm for each compound (all in comparison to 360 nm), in order to confirm peaks of the test compounds for at least one and often at multiple wavelengths. The dip in the UV-absorbance signal at 207 nm after an injection with pure water (Milli-Q, Merck-Millipore) served as the primary nonretained signal tracer to determine the column void volume time (t_0) in all tested eluents. Thiourea, nitrate, and bromide were additionally injected as commonly used tracer signals. Every sampling day, 3-nitroaniline was injected first to guarantee consistency of both the flow rate and retention capacity of the column.

Using fully aqueous eluent, the $K_{IAM,app}$ for each test compound was calculated according to eq 1 using the retention time at the peak apex (t_r). A weighed peak area approach that could account for peak tailing, following,[30] did not result in different $K_{IAM,app}$ values. Retention times were not different for concentrations differing by a factor of 100 for the ionized bases, indicating linear sorption behavior in the IAM column (data not shown). Graphpad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used to fit eq 6 to the data. In the fitting procedure, for each compound the three series with different salinities over the range pH 3–7.4 were simultaneously fit with a shared pK_a (for SiOH, must be >4), a shared $\Psi_{0,maxNH_3^+}$ (in V), a shared $\Psi_{0,maxSiO^-}$ (in V), a shared intrinsic log K_{PLIPW}, and a series specific I (in M) of 0.15, 0.015, and 0.0015.

RESULTS AND DISCUSSION

IAM Retention of Tracers and Neutral Reference Compound. IAM retention of the neutral compound 3-nitroaniline is not influenced by either the range from pH 3.0 to pH 7.4 (all values within 0.15 log units), using various buffers, or a factor of 100 difference in salinity in the tested range, as shown in Figure 3. The average log $K_{IAM,app}$ of 2.1 should represent the phospholipid affinity of 3-nitroaniline, and indeed aligns with the liposomal partitioning value (log K_{MW}) of 2.17 reported for DMPC liposomes at 35 °C.[13,37] The retention of the neutral tracer thiourea (k_{IAM} ~ 0.1 compared to pure water) is also constant at each tested pH and salinity. To simplify our understanding of the effect of surface charge on anionic tracers, we used monovalent anions bromide (SI Figure S4) and nitrate. In neutral pH both anionic tracers elute very fast from the IAM column, bromide even slightly faster than the dip in the UV signal from an injection of pure water. However, Figure 3 demonstrates significant retention of these monovalent anions if the eluent is below pH 5.0, up to almost 9.4 min for nitrate at 0.0015 M (k_{IAM} ~ 4.5). The increased retention of the anionic tracers with decreasing salinity in the lower pH range confirms electrostatic attraction to a positively charged IAM surface, resulting from the presence of residual charged propylamine groups and protonation of the acidic silanol groups.

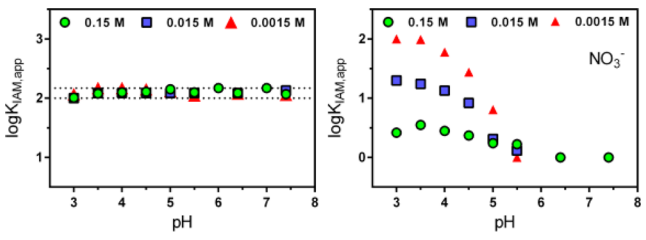


Figure 3. Apparent sorption affinity to the IAM material (log $K_{IAM,app}$ = φk_{IAM}, eq 1) between pH 3 and pH 7.4 at three different salinities, for (left) the neutral reference compound 3-nitroaniline and the anionic tracer nitrate (right). At pH > 5 the signals of nitrate (at lower salinities) eluted faster than the void volume calculated using pure water.

Effect of pH and Salinity on IAM Retention of Charged Bases. For two primary amines (tryptamine ("Tryp") and naphthylmethylamine ("Naph")), the secondary amine metoprolol ("Meto"), the tertiary amine 3-dimethylamino-propiophenone ("Dmapio"), and the quaternary ammonium structure benzyldimethylhexylammonium ("B2m6am"), k_{IAM} values cover as much of the salinity/pH range as feasible, summarized in SI Table S2 and shown in Figure 4. Values for the lowest salinity at pH 7.4 are not included for Naph and B2m6am due to strong sorption (long retention time) with aqueous eluent. Values at lower salinity from pH 3 to pH 5 for B3mam and Dmapio are not used due to elution close to t_0.

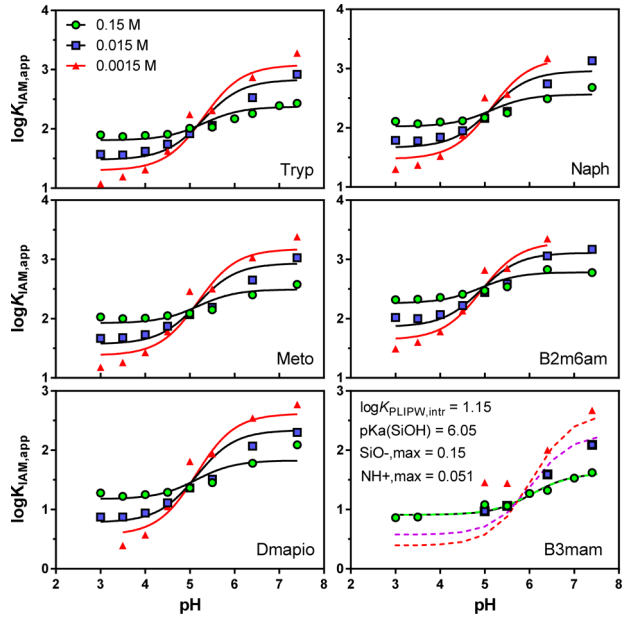


Figure 4. Apparent sorption affinity to the IAM material between pH 3 and pH 7.4 at three different salinities (green dots at 0.15 M, purple squares at 0.015 M, and red triangles at 0.0015 M, including buffer ions and NaCl/KCl), for six cationic amines. Solid lines are fits of the shared data on all salinities for each amine with eq 6. The solid line for benzyltrimethylammonium (B3mam) only fits the 0.15 M data set, and the fitted parameters are extended to the curves fitted to data obtained at the two lower salinies. The broken lines for the two lower salinity data sets for B3mam are curves fitted with the IAM parameters obtained with the highest salinity.

The retention time, and thus the apparent sorption coefficient to the IAM material, increases significantly for all amines with higher pH. The average difference in log $K_{IAM,app}$ values for the six organic cations at highest tested salinity between pH 3.0 and pH 7.4 is 0.63 ± 0.15 (s.d.) log units. At

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960–967

Table 1. Fitting Parameters (Equation 6) per Compound for Intrinsic Phospholipid Sorption Coefficient log $K_{PLIPW,intr}$ (L/kg), Maximal Contribution to the IAM Surface Potential for Residual Propylamines ($\Psi_{maxNH_3^+}$) and Dissociating Silanol Groups (Ψ_{maxSiO^-}), and Standard Deviation of the Residuals (sy.x)

amine	pK_a	log $K_{PLIPW,intr}$ (±s.e.)	$\Psi_{maxNH_3^+}$ (±s.e.)	Ψ_{maxSiO^-} (±s.e.)	pK_a (SiOH) (±s.e.)	shared Df, sy.x[c]
tryptamine	10.7	2.05 (0.07)	0.050 (0.007)	−0.12 (0.007)	5.30 (0.11)	22, 0.143
naphthylmethylamine	9.1	2.28 (0.08)	0.054 (0.008)	−0.11 (0.008)	5.14 (0.12)	19, 0.146
metoprolol	9.5	2.17 (0.09)	0.054 (0.009)	−0.12 (0.008)	5.18 (0.12)	20, 0.158
dmapio	9.1	1.46 (0.09)	0.060 (0.010)	−0.14 (0.009)	5.14 (0.12)	19, 0.172
benzyldimethylhexylammonium	[a]	2.55 (0.07)	0.061 (0.007)	−0.11 (0.006)	4.92 (0.11)	19, 0.125
benzyltrimethylammonium	[a,b]	∼1.15	∼0.051	−0.15 (0.017)	6.05 (0.27)	4, 0.079
average IAM.PC.DD2 properties			0.055	−0.125	5.29	

[a]Permanently charged. [b]Parameters obtained with only the highest salinity data set, which resulted in wide standard errors for log$K_{PLIPW,intr}$ and $\Psi_{0,maxNH_3^+}$. [c]sy.x = $\sqrt{(SS/Df)}$.

the lowest salinity, the maximum differences in k_{IAM} values between pH 3.0 and pH 7.4 for Tryp, Meto, and Dmapio are more than a factor of 100 (2.26 ± 0.10 (s.d.) log units). A similar trend in increasing log $K_{IAM,app}$ at higher pH occurs for the permanently charged B2m6am compared to the ionizable amines, which at pH 7.4 are all >98% charged. There is no apparent difference in how salinity and pH influence the retention behavior of organic cations for different amine types, molecular structure (polar moieties present or not), and level of intrinsic phospholipid sorption affinity (e.g., comparing the analogues B3mam and B2m6am). Since the increasing log $K_{IAM,app}$ is even more strongly related to salinity than to pH, the higher affinity of amines at increased eluent pH must be due to the confounding conditions at the IAM surface that affect the retention of cationic amine species, rather than a contribution of neutral species for the ionizable amines. For an ionizable amine 98.0% present as ionic species at pH 7.4, the sorption affinity of neutral species would need to be 50 times higher than that of the charged species to contribute a factor of 2 (0.3 log units) to the log $K_{IAM,app}$. From the small set of bases for which liposomal partitioning data are available at various pH, it appears that most cationic species sorb less than 1.5 log units (factor of <30) weaker to membranes than their corresponding neutral species (depending on various chemical characteristics[10]), so it seems unlikely that neutral amine species influenced our results at the highest test pH. Furthermore, the increasing trend in log $K_{IAM,app}$ with pH is never proportional to the fraction of neutral species, so at eluent distinctly lower than pH 7.4 the influence of neutral species was negligible.

At pH 4.5 and lower, log $K_{IAM,app}$ values of all amines decrease with lower salinity, corresponding to the inversed trend for anionic tracers, again demonstrating that IAM particles are then positively charged. At pH 5, the difference in log $K_{IAM,app}$ values between 0.15 and 0.015 M is minimal, while at even lower salinity of 0.0015 the log $K_{IAM,app}$ values of the amines slightly increase, indicating a net neutral to slightly negative surface charge of the IAM particles. For the 0.15 and 0.015 M data this corresponds to the anionic tracers, while the lowest salinity data for the anionic tracers still indicate a slight positive surface charge. At pH 5.5 and higher, all data for the amines show a higher retention with lower salinity, demonstrating that the IAM surfaces are negatively charged in those eluent conditions. Close to an eluent of pH 5, the amount of dissociated silanol groups appears to balance the residual amines on the IAM surface, and any electrostatic effects due to the confounding surface charges is rendered to insignificant levels at eluent salinities between 0.015 and 0.15 M.

Fitting the Electrostatic Model to IAM Data. Equation 6 is fitted to all data in the pH series at three different salinities for four strongly ionized amines and the permanently charged B2m6am. Fitted parameters are listed in Table 1. The fitting becomes more successful when data at lower salinity are included, as indicated by the relatively high uncertainty margins for each fitting parameter for B3mam, where only 0.15 M data are used. Besides a larger sample size, data at lower salinity show more pronounced differences in log $K_{IAM,app}$ between the lowest and highest tested pH. As shown in Figure 4, for the lowest tested salinity the measured log $K_{IAM,app}$ values are always below the fitted curves at pH 3−4 and always above the fitted curves at pH 7.4. Also the lowest salinity tested at pH 5.0 is always above the curve. Buffering strength at the lowest salinity may have been relatively weak as the buffer was co-diluted from 10 mM in the initial 0.15 mM eluent to 0.1 mM, but was also verified to be constant within 0.1 pH units before and after a testing series. It is not clear if deviations between observations and model predictions are due to experimental errors for some data series (e.g., inappropriate conditioning time upon changing certain eluents). More likely, they are due to theoretical shortcomings (e.g., fitting a single pK_a to silanol groups, neglecting effects of IAM-particle porosity and surface charges extending from the base of the phospholipid monolayer beyond zwitterionic headgroups to the aqueous layer at the surface of the phospholipids). Still, regarding the crude assumptions used to construct the model in eq 6, deviations between model fits and observed data trends are surprisingly small. Residuals for the 0.15 and 0.015 M salinity data are below 0.1 log units, and overall standard deviations of the residuals (sy.x) are 0.15 log units if data on three different salinities were shared in the fitting procedure. The fitted average pK_a values for silanol groups in the range of 4.9−5.3 are lower than the 6.3−6.8 mentioned in other studies,[15,16] or even 7.6 observed with Li^+-retention data.[29] A second pK_a of 3.1 has been reported in lipid-capped silicagel.[15] The single pK_a value fitted in this study may thus represent an overall average for the various silanol groups on the IAM surface, and the existence of silanol groups with a pK_a below 5 may explain the consistent underestimation of our electrostatic IAM model for the observed log $K_{IAM,app}$ at pH 5.0 for the amines.

Rather than evaluating the maximum difference in log $K_{IAM,app}$ values for cations over the whole pH range, it makes more sense to compare differences between observed log

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960−967

$K_{IAM,app}$ values under certain conditions with the fitted intrinsic log K_{PLIPW} values. Using the log $K_{PLIPW,intr}$ values from Table 1, a physiologically saline buffer (0.15 M) between pH 3.0 and pH 5.0 results in a maximum underestimation of only <0.15 log units, while a physiologically saline buffer at pH 7.4 results in an overestimation of 0.4−0.6 log units. This latter difference corresponds to the average difference of 0.45−0.93 log units for the k_{IAM} values of strongly protonated bases (pK_a > 9) between "saline" buffered eluents of pH 5.0 and pH 7.4 determined by Vrakas et al.[22] (0.45 log units for neostigmine, a permanently charged quaternary ammonium compound). As long as the neutral base species can be neglected, this suggests that, on average, all reported k_{IAM} values for strongly protonated bases (e.g., pK_a > 9) determined in PBS may be converted to approximate log$K_{PLIPW,cation}$ values, by reducing the corresponding log$K_{IAM,app}$ with ∼0.5 log units. According to the electrostatic model of eq 6 and average input IAM surface parameters of Table 1, the difference between PBS pH 7.4 and saline (0.15 M) buffer pH 5.0 is 0.39 log units (see Vrakas et al. data sets H and I in SI Table S3). SI Table S4 lists the IAM capacity factors determined for 61 predominantly ionized bases taken from 10 separate studies using the same IAM.PC.DD2 column as tested in this study, but tested under different eluent conditions (SI Table S3), and SI Table S5 presents the converted log$K_{PLIPW,intr.}$ values using this approach. SI Table S5 shows that for many amines a considerable residual difference in extrapolated $K_{PLIPW,intr}$ remains if values from different studies are available, e.g., 0.56 log units for 10 k_{IAM} values on propranolol. This could relate to underestimation of the contribution of neutral base species at pH ≥ 7 (e.g., diltiazem, SI Table S5) and/or interlab differences, e.g., when using an extrapolated series of solvent mixtures for hydrophobic sorbates (SI Table S3). Taking only measurements at sufficiently low pH and/or accepting an uncertainty of ∼0.5 log units for the IAM-HPLC-based K_{PLIPW} values would strongly enlarge the currently available K_{PLIPW} data set for organic cations.

IAM-Retention-Based $K_{PLIPW,intr}$ vs Liposomal K_{MW}. Retention capacity factors (k_{IAM}) under "ideal" IAM eluent composition (pH 5.0, salinity = 0.15 M) are determined for eight strongly protonated basic drugs and one simple amine structure (pK_a range 7.9−10.5) of the second data set. Sorption of fluoxetine and amlodipine to the IAM column was recorded in four different eluent mixtures of acetonitrile (30−15%) with pH 5.0 buffer (see SI Figure S3), and was extrapolated linearly to fully aqueous eluent. Triplicate measurements of log $k_{IAM,app}$ for amlodipine at each eluent mixture are within 0.01 units, resulting in an extrapolated (r^2 = 0.9946) log $k_{IAM,app}$ in aqueous eluent of 4.15 ± 0.04. For propranolol, the full range of 30% to 0% solvent shows a linear trend (SI Figure S3). Fractions of acetonitrile above 30% underestimate the extrapolated $k_{IAM,app}$ in fully aqueous eluent by at least 0.5 log units (SI Figure S3). The resulting K_{PLIPW} for fluoxetine and amlodipine are about 0.5 log units above the values that were reported in another study at pH 5.0 with the same type of IAM column,[22] which were obtained including 35% acetonitrile. Triplicate log k_{IAM} values determined in aqueous eluent are all within 0.01 log units. Our K_{PLIPW} value for propranolol and other less lipophilic amines are within 0.2 log units of those reported in ref 22, indicating good interlaboratory agreement under ideal IAM conditions for basic solutes. Vrakas et al.[22] also determined k_{IAM} for the metabolite norfluoxetine at pH 5.0, allowing for a comparison between IAM-based K_{PLIPW} values and liposomal partitioning data for nine protonated amines in total. Sorption coefficients to dispersed liposomes (K_{MW}) have been summarized elsewhere.[8] The liposomal partitioning data included tests with different pure phospholipids (POPC, DOPC, and DMPC) and egg yolk phospholipid mixtures, in the temperature range 25−37 °C, measured in different pH solutions by equilibrium dialysis or potentiometric titration, and then fitted to the Henderson−Hasselbalch equation. Whereas four liposomal K_{PLIPW} values for protonated propranolol range within 0.45 log units, two reported K_{PLIPW} for protonated atenolol differed by a full log unit. Regarding an uncertainty range of half a log unit for the liposomal partitioning data, Figure 5 shows that the IAM-based $K_{PLIPW,intr}$ values for these protonated bases show a good approximation of the K_{PLIPW} in phospholipid bilayers (0−0.5 log units higher), when tested under negligible confounding surface charge effects of the IAM-HPLC column.

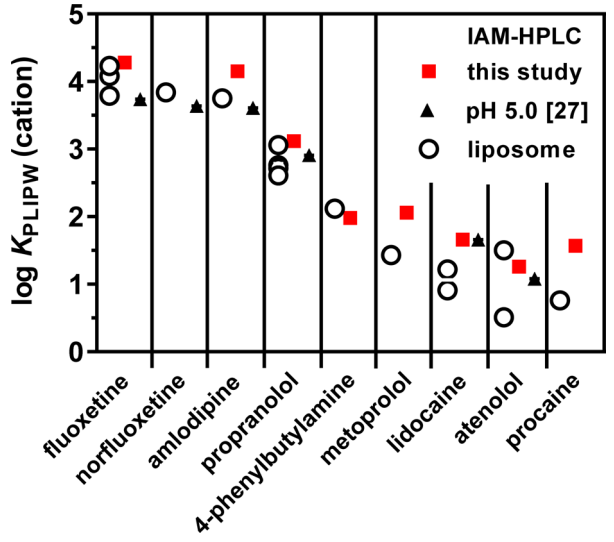


Figure 5. Sorption coefficients to phospholipid membranes for protonated amines obtained with IAM-HPLC (squares, this study; triangles, ref 21) and in liposomal partitioning studies (circles, reviewed in ref 8). All HPLC-based values are retention capacity factors obtained on IAM.PC.DD2 columns at pH 5.0 multiplied by the column's solvent/sorbent ratio of 18.9.[14] Amines are ordered from high to low average liposomal K_{PLIPW} values.

IAM-HPLC as Phospholipophilicity Descriptors for Cations. When the IAM eluent conditions are such that the organic cation is not influenced by confounding high surface potentials (pH 5.0 at salinity >10 mM, or at 150 mM for pH 3.0−5.0), the IAM-HPLC-based K_{PLIPW} values are comparable with liposomal partitioning data on nine structurally very different amines (Figure 5). Under these conditions, IAM-HPLC generates a direct experimental descriptor of phospholipophilicity for organic cations. At pH 5.0 the measured k_{IAM} for bases with pK_a > 7 can be directly converted to a $K_{PLIPW,cation}$ by multiplication with the solvent/sorbent phase ratio of 18.9. Note that in this way the IAM-HPLC method does not directly generate K_{PLIPW} values for the neutral base species. For bases with a pK_a < 6 these can be determined with IAM-HPLC at high salinity at pH 7.4 (which should suppress contributions from the minor fractions of cationic species, including electrostatic attraction). IAM-HPLC does not seem suitable to determine the neutral base K_{PLIPW} for bases with a pK_a > 6; this would require liposomal studies or calculations using K_{OW}

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960−967

regressions or polyparameter linear free energy relationships.[1] For bases with pK_a values between 5 and 7, $K_{IAM,app}$ should be determined at pH 3.0 with high (0.15 M) ionic strength of the eluent, and ∼0.15 log units should be added to generate the $K_{PLIPW,cation}$ value, to account for the electrostatic repulsion under these conditions. For bases with pK_a < 5, the exact determination of logK_{PLIPW} for cationic species may be irrelevant for most pharmaceutical and environmental chemical fate models. As discussed earlier, literature data on k_{IAM} obtained at neutral pH for basic compounds with pK_a > 7 need to be carefully handled.

Vrakas et al.[22] found retention differences between pH 5 and pH 7.4 for organic cations comparable to our measurements and model predictions, suggesting these rules of thumb on confounding effects are rather generic for IAM.PC.DD2 columns.

For liposomes constructed only with zwitterionic phospholipids, the sorption of charged compounds to liposomes is not significantly influenced by electrostatic attraction or repulsion,[7] unless (i) the membrane loading of the charged compound becomes more than 5 mol % of the phospholipid phase,[38] or, analogously, (ii) the liposome contains more than 5 mol % (an)ionic phospholipid structures.[38,39] The inclusion of 10 mol % anionic phospholipid into zwitterionic liposomes yields a factor of 4 higher (apparent) sorption affinities of organic cations in physiologically saline medium, due to resulting electrostatic attraction.[39] While this study aimed to separate out confounding electrostatic effects in the IAM-HPLC method, this realistic electrostatic attraction at membranes in living cells may thus be used as an additional factor to the IAM-based phospholipophilicity descriptor $K_{PLIPW,intr}$.

The current study only demonstrated the effect of eluent composition on the IAM retention, and resulting comparison with liposomal partitioning data, for monovalent organic cations. The behavior of organic anions, zwitterions, and multivalent ions in IAM-HPLC could be evaluated in a comparable series of eluent compositions. Before accepting these other classes of ionogenic chemicals as part of the chemical domain for which IAM-HPLC functions as a predictor of phospholipophilicity, substantial K_{IAM} data obtained under appropriate and consistent eluent conditions should be verified to align with liposomal partitioning data.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b03708.

Details on electrostatic modeling approach, details on test chemicals, raw data retention capacity factors, and graphs with linear extrapolation to fully aqueous eluent using various solvent mixtures, and literature review on k_{IAM} for 61 basic compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: steven.droge@gmail.com. Tel.: +31-30-2535217. Fax: +31-30-2535077.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was funded by Unilever, Safety & Environmental Assurance Centre (SEAC), Colworth Science Park, U.K. G. Hodges, S. Gutsell, J. Roberts, A. Teixeira (all SEAC) and J. Hermens (Utrecht University) provided many useful comments and fruitful discussions on this work. I acknowledge S. Mahawat Khan for performing the pilot salinity experiments with IAM-HPLC that initiated this study.

■ REFERENCES

(1) Endo, S.; Escher, B. I.; Goss, K.-U. *Environ. Sci. Technol.* **2011**, *45*, 5912−5921.
(2) Geisler, A.; Endo, S.; Goss, K.-U. *Environ. Int.* **2011**, *37*, 1253−1258.
(3) Geisler, A.; Endo, S.; Goss, K.-U. *Environ. Sci. Technol.* **2012**, *46*, 9519−9524.
(4) Manallack, D. T. *Perspect. Med. Chem.* **2007**, *1*, 25−38.
(5) Franco, A.; Ferranti, A.; Davidsen, C.; Trapp, S. *Int. J. Life Cycle Assess.* **2010**, *15*, 321−325.
(6) Rayne, S.; Forest, K. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* **2010**, *45*, 1550−1594.
(7) Austin, R. P.; Davis, A. M.; Manners, C. N. *J. Pharm. Sci.* **1995**, *84*, 1180−1183.
(8) Bittermann, K.; Spycher, S.; Endo, S.; Pohler, L.; Huniar, U.; Goss, K.-U.; Klamt, A. *J. Phys. Chem. B* **2014**, *118*, 14833−14842.
(9) Neuwoehner, J.; Fenner, K.; Escher, B. I. *Environ. Sci. Technol.* **2009**, *43*, 6830−6837.
(10) Armitage, J. M.; Arnot, J. A.; Wania, F.; Mackay, D. *Environ. Toxicol. Chem.* **2013**, *32*, 115−128.
(11) Henchoz, Y.; Bard, B.; Guillarme, D.; Carrupt, P.-A.; Veuthey, J.-L.; Martel, S. *Anal. Bioanal. Chem.* **2009**, *394*, 707−729.
(12) Rhee, D.; Markovich, R.; Chae, W. G.; Qiu, X.; Pidgeon, C. *Anal. Chim. Acta* **1994**, *297*, 377−386.
(13) Endo, S.; Escher, B. I.; Goss, K.-U. *Environ. Sci. Technol.* **2011**, *45*, 5912−5921.
(14) Ong, S.; Liu, H.; Pidgeon, C. *J. Chromatogr A* **1996**, *728*, 113−128.
(15) Ottiger, C.; Wunderli-Allenspach, H. *Pharm. Res.* **1999**, *16*, 643−650.
(16) Escher, B. I.; Schwarzenbach, R. P.; Westall, J. C. *Environ. Sci. Technol.* **2000**, *34*, 3962−3968.
(17) Kangas, H.; Kotiaho, T.; Salminen, T.; Kostiainen, R. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1501−1505.
(18) Taillardat-Bertschinger, A.; Martinet, C. A. M.; Carrupt, P. A.; Reist, M.; Caron, G.; Fruttero, R.; Testa, B. *Pharm. Res.* **2002**, *19*, 729−737.
(19) Yen, T. E.; Agatonovic-Kustrin, S.; Evans, A. M.; Nation, R. L.; Ryand, J. *J. Pharm. Biomed. Anal.* **2005**, *38*, 472−478.
(20) Barbato, F.; di Martino, G.; Grumetto, L.; La Rotonda, M. I. *Eur. J. Pharm. Sci.* **2005**, *25*, 379−386.
(21) Chan, E. C. Y.; Tan, W. L.; Ho, P. C.; Fang, L. J. *J. Chromatogr A* **2005**, *1072*, 159−168.
(22) Vrakas, D.; Giaginis, C.; Tsantili-Kakoulidou, A. *J. Chromatogr A* **2008**, *1187*, 67−78.
(23) Sprunger, L.; Blake-Taylor, B. H.; Wairegi, A.; Acree, W. E., Jr; Abraham, M. H. *J. Chromatogr A* **2007**, *1160*, 235−245.
(24) Kotecha, J.; Shah, S.; Rathod, I.; Subbaiah, G. *Int. J. Pharm.* **2008**, *360*, 96−106.
(25) Li, J.; Sun, J.; Cui, S.; He, Z. *J. Chromatogr A* **2006**, *1132*, 174−182.
(26) Liu, X.; Hefesha, H.; Scriba, G.; Fahr, A. *Helv. Chim. Acta* **2008**, *91*, 1505−1512.
(27) Grumetto, L.; Carpentiero, C.; Barbato, F. *Eur. J. Pharm. Sci.* **2012**, *45*, 685−92.
(28) Ledbetter, M. R.; Gutsell, S.; Hodges, G.; Madden, J. C.; O'Connor, S.; Cronin, M. T. D. *Environ. Toxicol. Chem.* **2011**, *30*, 2701−2708.

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960−967

(29) Lázaro, E.; Ràfols, C.; Rosés, M. *J. Chromatogr A* **2008**, *1182*, 233−236.
(30) Droge, S. T. J.; Goss, K. U. *Environ. Sci. Technol.* **2012**, *46*, 5894−5901.
(31) Droge, S. T. J.; Goss, K. U. *Environ. Sci. Technol.* **2013**, *47*, 14224−14232.
(32) Ong, S.; Pidgeon, D. *Anal. Chem.* **1995**, *67*, 2119−2128.
(33) Ong, S.; Liu, H.; Qiu, X.; Bhat, G.; Pidgeon, C. *Anal. Chem.* **1995**, *67*, 755−762.
(34) Israelachvili, J. N. *Intermolecular & Surface Forces*; Academic Press: London, 1992.
(35) Bohinc, K.; Kralj-Iglič, V.; Iglič, A. *Electrochim. Acta* **2001**, *46*, 3033−3040.
(36) Ledbetter, M. R.; Gutsell, S.; Hodges, G.; Madden, J. C.; Rowe, P. H.; Cronin, M. T. D. *J. Chem. Eng. Data* **2012**, *57*, 3696−3700.
(37) Vaes, W. H. J.; Ramos, E. U.; Hamwijk, C.; van Holsteijn, I.; Blaauboer, B. J.; Seinen, W.; Verhaar, H. J.; Hermens, J. L. M. *Chem. Res. Toxicol.* **1997**, *10*, 1067−1072.
(38) Escher, B. I.; Schwarzenbach, R. P.; Westall, J. C. *Environ. Sci. Technol.* **2000**, *34*, 3954−3961.
(39) Elsayed, M. M. A.; Vierl, U.; Cevc, G. *Pharm. Res.* **2009**, *26* (6), 1332−43.

DOI: 10.1021/acs.analchem.5b03708
Anal. Chem. 2016, 88, 960−967