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Immobilized artificial membrane liquid chromatography: proposed guidelines for technical optimization of retention measurements

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Abstract

The objectives of this study were to establish guidelines for the proper measurement of capacity factors (log k_{IAMw}) on immobilized artificial membrane (IAM) stationary phases. In this context, some aspects related to the extrapolation of log k_{IAMw} values, the stability and properties of IAM.PC.DD2 stationary phases and the column-to-column variability are discussed. No significant difference was observed when using either acetonitrile or methanol for the linear extrapolation of log k_{IAM} values. However, methanol seems more appropriate when working with ionized compounds. Plotting isocratic capacity factors against the percentage (v/v) of co-solvent instead of the mole fraction leads to more reliable log k_{IAMw} values. Furthermore, our results with a YMC ODS-AQ and an IAM.PC.DD2 HPLC column indicate that only small differences arise between extrapolated capacity factors when using the ${}^{w}_{w}$pH or the ${}^{s}_{w}$pH operational scale and correcting or not the ionic strength for dilution caused by the co-solvent. The use of the ${}^{s}_{w}$pH scale is recommended when working with ionized compounds in order to avoid parabolic relationships during linear extrapolation. The pH-dependent retention of three ionizable drugs on an IAM.PC.DD2 phase showed that secondary interactions with the charged moieties of the chromatographic surface affect the retention of ionized compounds around physiological pH. Finally, it was shown that column ageing occurs also with IAM.PC.DD2 stationary phases and that it depends on the column as well as on the investigated analyte. The intra-batch variability for IAM.PC.DD2 phases was small, whereas a marked and solute-dependent batch-to-batch variability was apparent.

Keywords: Immobilized artificial membranes; Drug–membrane interactions; Mobile phase composition; pH measurements; Stationary phases, LC; Retention factors

1. Introduction

Immobilized artificial membranes (IAMs) mimic the lipid environment of a fluid cell membrane on a solid matrix and are thus of particular interest for the prediction of drug partitioning into biological membranes [1,2]. In fact, they are prepared by linking synthetic phospholipid analogues at monolayer density to silica particles, producing a high-performance liquid chromatography (HPLC) column packing material which allows solute partitioning in an artificial lipid membrane to be measured by fast HPLC [3,4].

In this context, technical aspects relevant to the proper measurement of capacity factors (or retention factor according to IUPAC recommendations) on

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reversed-phase (RP) chromatographic surfaces have also to be considered when determining IAM lipophilicity descriptors (log k_{IAMw}). Here, we discuss some aspects related to the extrapolation of log k_{IAMw} values, the stability and properties of IAM.PC.DD2 stationary phases and the column-to-column variability.

The addition of an organic modifier to the mobile phase is needed for the elution of highly lipophilic compounds. However, extrapolations to 100% aqueous phase are required (log k_{IAMw}) to compare capacity factors independently of the amount and type of organic co-solvent and to avoid fictitious interaction scales due to differences in the elution order occurring at different percentages of co-solvent [5–7]. Methanol and acetonitrile are the most popular co-solvents. Extensive studies have been undertaken to evaluate the influence of organic modifiers on RP-HPLC retention behaviour [8–13], but only a few authors have investigated the extrapolation of log k_{IAM} values from methanol–water and acetonitrile–water eluents [14].

In the present study, capacity factors extrapolated by linear regression to 100% aqueous mobile phase using either methanol or acetonitrile were compared for a congeneric series of five β-blockers. The benefit of using mole fractions instead of the percentages (v/v) of organic modifier [14] was also studied. The addition of methanol or acetonitrile to a buffered aqueous mobile phase changes its pH [15–17]. Actually, three different operational pH scales exist to measure the pH in solvent–water mixtures [16,18], namely the ${}^{w}_{w}$pH, the ${}^{s}_{w}$pH and the ${}^{s}_{s}$pH scales. When one calibrates the electrode system with aqueous buffers and measures the pH of the HPLC aqueous buffer before mixing it with the organic modifier, ${}^{w}_{w}$pH is obtained. However, the pH of the solution changes after dilution of the aqueous buffer with the organic modifier. ${}^{s}_{w}$pH is obtained when the electrode system is calibrated with aqueous buffers but pH measured after adding the organic modifier. Finally, ${}^{s}_{s}$pH is obtained when the electrode system is calibrated with buffers containing the same proportion of co-solvent as the mobile phase, and the pH measured in the actual eluent.

Indeed, it is known that the presence of an organic co-solvent leads to a shift in the apparent pK_a value of ionizable solutes [19,20]. The apparent pK_a of bases decreases with an increasing percentage of organic modifier, whereas it increases for acids. As the retention time of an ionizable compound depends on its ionization state (and thus on its apparent pK_a value) and on the pH of the mobile phase [21–23], the use of the ${}^{s}_{w}$pH or even ${}^{s}_{s}$pH scale instead of ${}^{w}_{w}$pH to adjust the pH of mobile phases may lead to significantly better extrapolations of log k_{IAMw} values, and even to different log k_{IAMw} values for a given solute. The addition of an organic solvent to the aqueous buffer will also decrease its ionic strength, leading to enhanced IAM capacity factors [6].

Here, we studied the influence of the pH operational scale (${}^{w}_{w}$pH, ${}^{s}_{w}$pH, ${}^{s}_{s}$pH) on the log k_w values obtained by linear extrapolation with an RP C_{18} HPLC column and an IAM.PC.DD2 stationary phase using neutral and ionized acidic and basic model compounds. In fact, the quality of the extrapolation may also be influenced by the ionizable phosphate group of the IAM stationary phase. We also corrected the ionic strength for dilution by the organic modifier.

The lipophilicity profile, defined as the variation of the distribution coefficient (log D) as a function of pH, is essential to interpret pharmacokinetic and pharmacodynamic behaviour. The pH-partition diagrams measured in liposomal systems or on immobilized artificial membranes are of particular interest since most drugs are ionizable, and charged compounds interact by electrostatic bonds with the polar head-groups of biological and artificial membranes. Ottiger and Wunderli-Allenspach [14] compared the lipophilicity profiles of five ionizable drugs obtained from partitioning in large unilamellar PhC-liposomes [24] and from retention on an single-chain IAM.PC.DD stationary phase. They revealed systematic differences between the pH-partition diagrams derived from partitioning in the two systems [14] and ascribed the shoulder observed with the IAM.PC.DD phase between pH 5 and 8 to secondary interactions with the silica surface.

For double-chain chromatographic IAM surfaces, steric hindrance due to the second, freely movable fatty acid of the covalently bound phospholipids can be assumed to prevent the charged moieties present on the chromatographic surface from interacting with ionized analytes. To verify this hypothesis, the pH-

dependent retention behaviour of three of the five ionizable drugs studied by Ottiger and Wunderli-Allenspach [14] was investigated on an IAM.PC.DD2 phase in the range of pH 4.5 to 7.0.

Ageing or even premature failure of some IAM HPLC columns was observed, leading to shorter retention times and decreased log k_{IAMw} values [25–27]. To investigate the ageing of IAM.PC.DD2 stationary phases more closely, the capacity factors of six model solutes neutral at pH 7.0 were monitored over time on four different IAM.PC.DD2 surfaces. Simultaneously, column-to-column reproducibility was investigated. Indeed, as the four columns were from two different batches the intra- as well as the inter-batch variability could be assessed.

2. Experimental

2.1. Materials

2.1.1. Chemicals

Acebutolol, acetylsalicylic acid (aspirin), alprenolol, metoprolol, (*S*)-(+)-naproxen, oxprenolol, phenytoin, pindolol, propranolol and warfarin were purchased from Sigma (Buchs, Switzerland). Carazolol was kindly offered by Boehringer Mannheim (Mannheim, Germany) and salicylic acid as well as the small organic solutes acetophenone, aniline, benzylamine, nitrobenzene, phenol, *N*-ethylaniline and 2-chloroaniline were bought in the highest available purity from Fluka (Buchs, Switzerland). Racemates were used when not specified otherwise.

Acetonitrile and methanol of superpure quality for HPLC were purchased from Romil (Cambridge, UK) and all other chemicals were of analytical grade. Deionized water was used throughout.

2.1.2. Equipment

The retention measurements on all IAM.PC.DD2 stationary phases were performed by HPLC using a liquid chromatograph consisting of a pump type LC 414-T Kontron Analytica (Kontron Instruments, Zürich, Switzerland) equipped with an Uvikon 730 S LC Kontron UV Spectrophotometer (Kontron) set at 254 or 220 nm. The chromatograms were recorded using an integrator type 3390 A purchased from Hewlett-Packard (Avondale, PA, USA). The column temperature was maintained at 25±2 °C using a water-bath circulator (Haake, Digitana, Lausanne, Switzerland) and a column jacket, and the eluent mixtures were prepared manually and degassed prior to use. The stainless steel columns were immobilized artificial membrane S12-300 IAM.PC.DD2 stationary phases (100×4.6 mm, 12 μm, 300 Å; Regis Technology, Morton Grove, IL, USA) from two different batches.

The retention measurements on the RP C_{18} HPLC column were performed by HPLC using a liquid chromatograph Waters 2690 Separation module (Waters-Millipore, Milford, MA, USA) equipped with a Waters 2487 Dual Wavelength Absorbance Detector (Waters) set at 210 nm. The chromatograms were analysed using the Millenium^{32} software (Waters) and the column temperature was maintained at 25±1 °C using a column oven type Igloo 560 from CIL Cluzeau Info Labo (Puteaux-la-Défense, France). The RP C_{18} HPLC column used was an YMC ODS-AQ stationary phase (250×4.6 mm, 5 μm, 120 Å; Batch AQ12SO52546FT, Serial No. 80484077; YMC, Wilmington, NC, USA).

Prior to addition of the organic solvent, all aqueous portions of the mobile phases were filtered through 0.45 μm HA Millipore filters (Millipore, Milford, MA, USA). The pH was measured with a Metrohm pH-meter type 654 (Metrohm, Herisau, Switzerland). The glass electrode (Metrohm) was filled with an aqueous 3 *M* KCl electrolyte solution for all pH determinations.

2.2. Calculations

All chromatographic retention data are the mean of at least three determinations and are expressed by the logarithm of the capacity factor, log *k* (or log k_{IAM}), defined as:

$$\log k = \log[(t_R - t_0)/t_0] \quad (1)$$

where t_R and t_0 are the retention times of the solute and of a non-retained compound (citric acid for IAM and 0.01% KBr for RP C_{18} HPLC measurements), respectively.

The capacity factor, k (or k_{IAM}), is linearly related to the equilibrium partition coefficient, K (or K_{IAM}):

$$K = \frac{V_m}{V_s} \cdot k = \frac{k}{\phi} \quad (2)$$

where V_m is the total volume of solvent within the HPLC column; V_s is the volume of the interphase created by the covalently bound, lipidic stationary phase; and $\phi = V_s/V_m$ is the phase ratio, which is constant for a given column.

The volume of the mobile phase equals:

$$V_m = f_r t_0 \quad (3)$$

where f_r is the flow-rate.

The volume of the stationary phase was 0.125 ml. It was calculated by the ligand density on the IAM.PC.DD2 column, which is 70.0:6.7:1.2 (PhC:C_{10}:C_3) mg/g packing material [28]. A 100× 4.6 mm IAM.PC.DD2 column is filled with 1.6 g packing material (Regis Technology, personal communication, 1999). The specific weight of PhC (δ_{PhC}) is 1.01779 g/ml and of C_{10}/C_3 ($\delta_{C10/C3}$) 0.86 g/ml. V_s is the sum of the different ligand volumes, i.e., weight (W) of the ligand linked to the silica divided by its specific weight [2]:

$$V_{s(IAM)} = \frac{W_{PhC}}{\delta_{PhC}} + \frac{W_{C10}}{\delta_{C10}} + \frac{W_{C3}}{\delta_{C3}} \quad (4)$$

2.3. Organic modifiers used to obtain extrapolated log k_{IAMw} values

The isocratic capacity factors of five β-blocking agents (acebutolol, alprenolol, carazolol, metoprolol and propranolol) were measured on an IAM.PC.DD2 stationary phase (Batch P122-19-1, Serial No. 1000038) using methanol or acetonitrile as co-solvent. When methanol was used, the eluents were mixtures of organic modifier and phosphate buffer, pH 7.0 (0.02 M KH_2PO_4 and 0.15 M KCl) containing 10 to 80% methanol according to the lipophilicity of the compounds. The increment of methanol between two mobile phases was set to 10% as normally done in IAM-HPLC [14,25] and at least four mobile phases with different percentages of methanol were used for each compound to obtain its log $k^{7.0}_{IAMw}$ value by linear regression.

When acetonitrile was used, the mobile phases were mixtures of the organic solvent and phosphate buffer, pH 7.0 (0.02 M KH_2PO_4 and 0.15 M KCl) containing 5 to 30% acetonitrile according to the lipophilicity of the β-blockers. The increment of acetonitrile between two mobile phases was set to 5% as normally done using acetonitrile as co-solvent [5–7]. For each drug at least four mobile phases with different percentages of acetonitrile were used to estimate its log $k^{7.0}_{IAMw}$ value.

The capacity factors at 100% aqueous phase were obtained by linear extrapolation by plotting the log $k^{7.0}_{IAM}$ values versus either percentage (v/v) or mole fraction of organic modifier. The log $k^{7.0}_{IAM}$ values were calculated according to Eq. (1) and the mole fractions according to:

$$\chi_{org} = \frac{\chi_{org}}{(\chi_{org} + \chi_{water})} \quad (5)$$

where χ_{org} and χ_{water} are the number of mole of organic solute and water in the mixture. For the two more polar β-blockers, acebutolol and metoprolol, the log $k^{7.0}_{IAMw}$ values were also determined using a 100% aqueous mobile phase.

Flow-rates ranging from 1.4 to 2.0 ml/min according to the solute's lipophilicity were used. Stock solutions (10^{-2} M) of the drugs were prepared in methanol and diluted with the respective eluent prior to injection (20 μl) in order to obtain end-concentrations ranging from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-3}$ M.

2.4. Correction of pH and ionic strength when extrapolating log k values

The isocratic retention data of aniline, aspirin, benzylamine and phenol on the YMC ODS-AQ HPLC column were measured using mixtures of methanol and buffer (0.02 M KH_2PO_4 and 0.15 M KCl, total ionic strength 0.18 M) containing 10 to 70% methanol. Mobile phases containing 10 to 60% methanol were used to determine the isocratic capacity factors of N-ethylaniline, (S)-(+)-naproxen, oxprenolol, phenytoin and pindolol on the IAM.PC.DD2 stationary phase (Batch P-129-47-2, Serial No. 1000084). In fact, the different hydrophobicity of the two columns did not allow to use the same series of compounds for both stationary phases.

The pH of the mobile phases was adjusted using

either the ${}^{w}_{w}$pH, the ${}^{s}_{w}$pH or the ${}^{s}_{s}$pH scale. In the first case the glass electrode was calibrated using aqueous standard buffer solutions and the pH of the mobile phase was adjusted to 7.0 before mixing buffer and methanol, whereas in case of the ${}^{s}_{w}$pH scale the glass electrode was calibrated as above, but the pH of the mobile phases were adjusted after mixing, in order to obtain ${}^{s}_{w}$pH 7.0. To circumvent the time-consuming preparation of standard buffers in the same solvent composition as the mobile phases (needed for the electrode calibration when working with the ${}^{s}_{s}$pH operational scale) the ${}^{s}_{s}$pH was adjusted using the Eq. (6) and the experimentally measured ${}^{s}_{w}$pH values:

$$ {}^{s}_{s}\mathrm{pH} = {}^{s}_{w}\mathrm{pH} - \delta \quad (6) $$

where the δ terms (including the primary medium effect and the difference of the liquid junction potentials) for the respective methanol–water mixtures were taken from the literature [16,29]. In further experiments the ${}^{w}_{w}$pH or the ${}^{s}_{w}$pH scales were used, but the ionic strength of the mobile phases were corrected for dilution caused by the addition of methanol in order to maintain a constant ionic strength of 0.18 *M*.

The log k_w values were obtained by linear regression plotting the isocratic log *k* values against the percentage (v/v) of methanol in the mobile phases. For a better comparison of results, the log $k_w^{7.0}$ values for all solutes (except for oxprenolol and phenytoin) were also determined with a 100% aqueous phase.

Flow-rates ranging from 1.2 to 2.0 ml/min (according to the solute's lipophilicity) were used and stock solutions (10^{-2} *M*) of all compounds were made in methanol and diluted with the respective eluent prior to injection (20 μl) in order to obtain end-concentrations ranging from $5\cdot10^{-5}$ to $5\cdot10^{-3}$ *M*. The capacity factors were calculated according to Eq. (1).

2.5. pH-dependent partitioning in the IAM.PC.DD2 stationary phase

The pH-dependent partitioning of propranolol, warfarin and salicylic acid was measured on an IAM.PC.DD2 stationary phase (Batch P122-19-1, Serial No. 1000038). The eluents were either SMUBS buffer [24] (ionic strength 0.23 *M*, pH 4.5 to 7.0) or different mixtures of acetonitrile and SMUBS buffer at flow-rates of 1.5 to 2.0 ml/min. Stock solutions (10^{-2} *M*) of the compounds were made in methanol; prior to injection (20 μl) the solutes were diluted with the respective mobile phases to obtain end-concentrations ranging from 10^{-4} to 10^{-3} *M*.

For the elution of the more lipophilic compounds propranolol and warfarin, mixtures of acetonitrile–SMUBS containing up to 30% organic modifier were used. The log k_{IAMw} values at 100% aqueous phase were extrapolated by linear regression plotting the log k_{IAMw} values versus the percentage (v/v) of organic modifier in the eluent mixtures. The IAM partition coefficients, K_{IAMw}, were calculated from the capacity factors, log k_{IAMw}, (Eq. (1)) according to Eq. (2), taking into account the mobile phase volume, V_m (Eq. (3)) and the stationary phase volume, V_s (Eq. (4)).

2.6. Ageing of the IAM.PC.DD2 phases and intra-/inter-batch reproducibility of IAM.PC.DD2 columns

The retention for the six neutral compounds, acetophenone, aniline, nitrobenzene, phenol, *N*-ethylaniline and 2-chloroaniline on four IAM.PC.DD2 stationary phases from two batches was measured using eluents consisting of 0.1 *M* KH_2PO_4 buffer pH 7.0 (flow-rate 1.5 ml/min). Stock solutions (10^{-2} *M*) of the compounds were made in methanol and diluted with eluent prior to injection (20 μl) to reach end-concentrations ranging from 10^{-4} to $5\cdot10^{-4}$ *M*. The capacity factors were calculated according to Eq. (1) and the log $k_{IAMw}^{7.0}$ values were determined with the new columns and then repeatedly during use.

3. Results and discussion

3.1. Organic modifiers used to obtain extrapolated log k_{IAMw} values

To compare IAM capacity factors independently of the amount and type of co-solvent used, extrapola-

tions to 100% aqueous phase (log k_{IAMw}) are required. In the present work, the influence of methanol and acetonitrile on the extrapolated log $k^{7.0}_{IAMw}$ values was studied for a series of five β-blockers. These drugs were chosen because they cover a wide range of lipophilicity (log P_{oct} ranging from 1.95 to 3.73) and are fully protonated at pH 7.0 ($pK_a > 9.0$). In fact, a large influence of the organic modifier on extrapolated log k_{IAMw} values is expected for charged compounds.

Two different extrapolation plots were compared for each co-solvent. The log $k^{7.0}_{IAM}$ values were plotted against both the % (v/v) of organic modifier and its mole fraction (Eq. (5)). The resulting log $k^{7.0}_{IAMw}$ values are reported in Table 1. For acebutolol and metoprolol log $k^{7.0}_{IAMw}$ values could also be determined using a 100% aqueous phase and were higher than the extrapolated capacity factors (Table 1).

The best extrapolated log k_{IAMw} values (see, for example, Fig. 1 for metoprolol) were obtained using methanol and plotting the log $k^{7.0}_{IAM}$ values against the % (v/v) organic modifier. These results contradict those of Ottiger and Wunderli-Allenspach [14], who concluded that better results were obtained plotting the isocratic log $k^{7.0}_{IAM}$ values against the mole fraction of organic modifier in the eluent.

3.2. Correction of pH and ionic strength when extrapolating log k values

The influence of the operational pH scale ($^{w}_{w}$pH, $^{s}_{w}$pH and $^{s}_{s}$pH) used to adjust the eluent pH and of the correction for ionic strength on extrapolated log *k* values was investigated on two different HPLC columns. The extrapolation of capacity factors to 100% aqueous phase was studied on an YMC ODS-AQ C_{18} column and an IAM.PC.DD2 column. The former providing a more hydrophobic environment, the same series of compounds could not be used for both columns.

For the YMC ODS-AQ column, log *k* extrapolation was studied using aniline and phenol (neutral) and benzylamine and aspirin (ionized). The log *k* values extrapolated from methanol using either the $^{w}_{w}$pH, $^{s}_{w}$pH or $^{s}_{s}$pH operational scale are given in Table 2. For all compounds, the log *k* values were also extrapolated using the $^{w}_{w}$pH scale and correcting the ionic strength to maintain a constant value of 0.18 *M* identical to that of a 100% buffer eluent (Table 2). Furthermore, log k_w values were also determined using a 100% aqueous eluent (Table 2).

The plots so obtained (Fig. 2) show that for the two neutral compounds aniline (Fig. 2A) and phenol (Fig. 2D) the four regression lines are superimposed

Table 1
Extrapolated log $k^{7.0}_{IAMw}$ values (SD < 0.1) of five β-blockers obtained by linear regression[a]

Drug	Log P_{oct}[b]	Methanol			Acetonitrile			Log $k^{7.0}_{IAMw}$ 100% buffer[f]
		Solvent range (%, v/v)[c]	Log $k^{7.0}_{IAMw}$ (%, v/v)[d]	Log $k^{7.0}_{IAMw}$ mole fraction[e]	Solvent range (%, v/v)[c]	Log $k^{7.0}_{IAMw}$ (%, v/v)[d]	Log $k^{7.0}_{IAMw}$ mole fraction[e]	
Acebutolol	2.02	10–40	1.16	1.10	5–30	1.07	0.99	1.33
Alprenolol	3.10	20–50	1.84	1.66	5–30	1.77	1.70	–
Carazolol	3.73	50–80	2.36	1.61	15–30	2.39	2.22	–
Metoprolol	1.95	10–40	0.90	0.83	5–30	0.86	0.81	0.97
Propranolol	2.48	30–80	2.25	1.71	10–30	2.11	1.99	–

The log $k^{7.0}_{IAM}$ values were plotted against (a) the % (v/v) of organic modifier in the co-solvent–phosphate buffer, pH 7.0 mixtures, and (b) the mole fraction of organic modifier.

[a] Done with four to six isocratic capacity factors.

[b] Taken from Ref. [35].

[c] Range of co-solvent used for the linear extrapolation, expressed in % (v/v) of co-solvent in the eluent.

[d] Extrapolated values obtained using the % (v/v) of co-solvent in the eluent. Determination coefficient $r^2 = 0.994–0.999$.

[e] Extrapolated values obtained using the mole fraction of co-solvent in the eluent. Determination coefficient $r^2 = 0.967–0.997$.

[f] Log $k^{7.0}_{IAMw}$ values measured directly, using a 100% aqueous mobile phase.

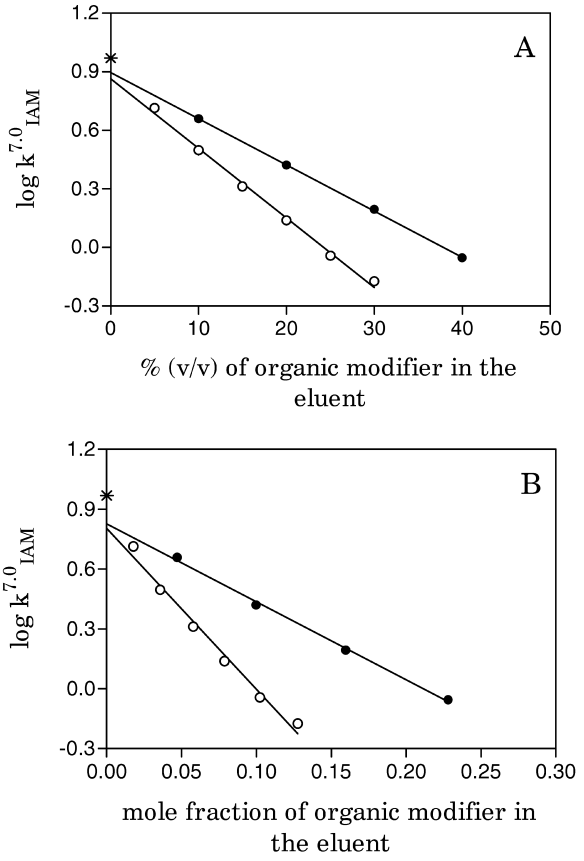

Fig. 1. Linear extrapolation of the log $k^{7.0}_{IAMw}$ values of metoprolol using either methanol (●) or acetonitrile (○) as co-solvent. The log $k^{7.0}_{IAMw}$ value was also measured using a 100% aqueous mobile phase (*). The isocratic log $k^{7.0}_{IAM}$ values were plotted against (A) the % (v/v) of organic modifier in the eluent and (B) against the mole fraction of co-solvent in the mobile phase.

and the extrapolated log k_w values very similar. In contrast, differences appear at high percentages of methanol for the negatively charged aspirin (Fig. 2B). However, the extrapolated log k_w values are similar independently of the operational pH scale used. For the positively charged benzylamine (Fig. 2C), the ${}^{s}_{w}$pH and ${}^{s}_{s}$pH operational scales yield similar (and straight) regression lines. In contrast, the ${}^{w}_{w}$pH scale leads to a parabolic relationship due to the increase of pH and to the lowering of the apparent pK_a with the addition of methanol [15,17,19,20].

The curvature in the relationship between isocratic capacity factors and methanol percentage causes the log k_w value obtained with the ${}^{w}_{w}$pH scale to be significantly smaller. However, using the ${}^{w}_{w}$pH scale but correcting the ionic strength for dilution due to methanol leads to a less pronounced curvature and a better log k_w value.

Overall, it can be seen that all extrapolated values are significantly smaller than the log k_w values measured using a 100% aqueous phase, the differences being particularly important for the ionized compounds.

Superimposed regression lines were found for all compounds when the ${}^{s}_{w}$pH or the ${}^{s}_{s}$pH scale were used to adjust the pH of the mobile phases. The latter is not surprising since the δ values (Eq. (6)) used in this study are similar for all methanol–water mixtures, and the two scales are linearly related [16,29].

The linear extrapolation of log k_{IAM} values (IAM.PC.DD2 stationary phase) was investigated using five model compounds. Under the experimental conditions used, *N*-ethylaniline and phenytoin

Table 2
Extrapolated log k_w values (SD<0.1) obtained by plotting the isocratic log k values obtained on an RP-C_{18} YMC ODS-AQ HPLC column against the percentage (v/v) of methanol in the mobile phases and using either the ${}^{w}_{w}$pH, ${}^{s}_{w}$pH or ${}^{s}_{s}$pH scale to adjust the pH of the eluent

Compound	Log k_w 100% buffer[a]	Log k_w ${}^{w}_{w}$pH	Log k_w ${}^{s}_{w}$pH	Log k_w ${}^{s}_{s}$pH	Log k_w ${}^{w}_{w}$pH, 0.18 *M*[b]
Aniline	1.37	1.15	1.15	1.15	1.16
Aspirin	1.19	0.77	0.76	0.75	0.75
Benzylamine	1.03	0.53	0.68	0.66	0.63
Phenol	1.55	1.43	1.43	1.42	1.44

[a] Log k_w values measured using a 100% aqueous mobile phase (phosphate buffer, pH 7.0).
[b] Extrapolated log k_w values obtained using the ${}^{w}_{w}$pH operational scale to adjust the pH of the eluents, but correcting the ionic strength in all mobile phases to 0.18 *M*.

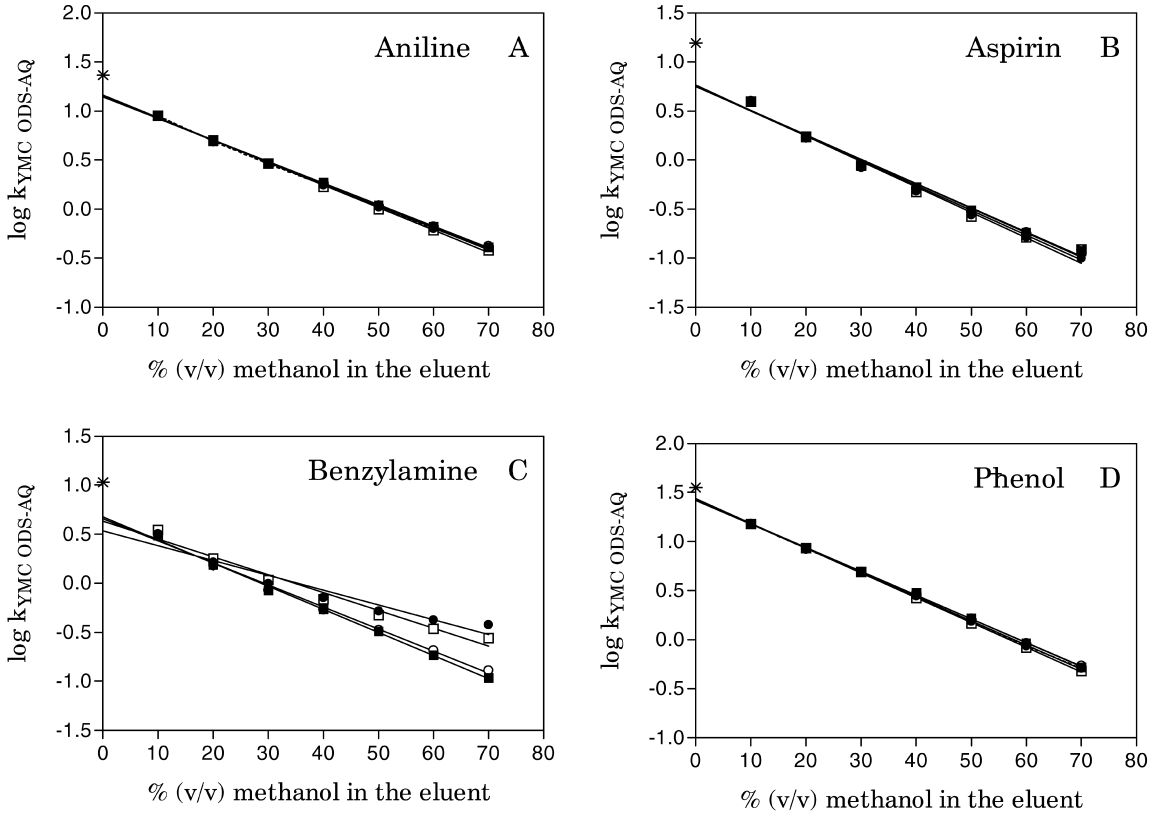

Fig. 2. Log k values extrapolated on an YMC ODS-AQ reversed-phase C_{18} HPLC column, plotting the isocratic log k values against the percentage (v/v) of methanol in the mobile phases and using either the ${}^{w}_{w}$pH (●), ${}^{s}_{w}$pH (■) or ${}^{s}_{s}$pH (○) operational scale to adjust the pH of the eluents. In a further experiment the ${}^{w}_{w}$pH scale was used, but the ionic strength of the eluents was corrected for dilution caused by the addition of methanol to the buffer (□). (*) Log k_w values measured directly, using a 100% aqueous mobile phase.

were mostly neutral, whereas the basic drugs pindolol and oxprenolol were in cationic form and (*S*)-(+)-naproxen in anionic form.

The log k_{IAM} values were extrapolated by linear regression, the pH of the eluents being adjusted using either the ${}^{w}_{w}$pH or the ${}^{s}_{w}$pH operational scale. In two further series of experiments the same scales were used to adjust the pH but the ionic strength of the eluents was corrected for dilution caused by the addition of methanol. For the more polar solutes *N*-ethylaniline, (*S*)-(+)-naproxen and pindolol, the log k_{IAMw} values were also measured using a 100% aqueous mobile phase and these values together with the extrapolated log k_{IAMw} values are listed in Table 3.

For the two mostly neutral compounds *N*-ethylaniline (Fig. 3A) and phenytoin (Fig. 3D), the four extrapolation lines are superimposed as already observed for the YMC ODS-AQ column, but this is not the case for the negatively charged (*S*)-(+)-naproxen (Fig. 3B) or for the positively charged oxprenolol (Fig. 3C) and pindolol (Fig. 3E). However, the regression lines converge towards similar log k_{IAMw} values (Table 3).

The plots obtained for the protonated drugs on the IAM.PC.DD2 stationary phase (Fig. 3C and Fig. 3E) show a less pronounced parabolic shape compared to the behaviour of benzylamine on the YMC ODS-AQ column (Fig. 2C).

Comparing Fig. 3B to Fig. 3C and E shows that the relative positions of the linear regressions obtained using either the ${}^{w}_{w}$pH or the ${}^{s}_{w}$pH scale are interchanged. Indeed, for the two basic compounds the log k_{IAM} values measured in the ${}^{s}_{w}$pH scale lie

Table 3
Extrapolated log k_{IAMw} values (SD<0.1) obtained by plotting the isocratic capacity factors obtained on an IAM.PC.DD2 stationary phase against the percentage (v/v) of organic modifier in the mobile phases and using either the ${}^{w}_{w}$pH or the ${}^{s}_{w}$pH scale to adjust the pH of the eluent

Compound	Log k_{IAMw} 100% buffer[a]	Log k_{IAMw} ${}^{w}_{w}$pH	Log k_{IAMw} ${}^{s}_{w}$pH	Log k_{IAMw}[b] ${}^{w}_{w}$pH, 0.18 *M*	Log k_{IAMw}[b] ${}^{s}_{w}$pH, 0.18 *M*
N-Ethylaniline	1.04	1.02	1.01	1.03	1.00
Phenytoin	–	1.84	1.88	1.87	1.94
Pindolol	1.50	1.39	1.31	1.43	1.34
Oxprenolol	–	1.54	1.46	1.57	1.48
(*S*)-(+)-Naproxen	1.22	1.11	1.11	1.08	1.16

[a] Log k_{IAMw} values measured using a 100% aqueous mobile phase (phosphate buffer, pH 7.0).
[b] Extrapolated log k_{IAMw} values obtained using either the ${}^{w}_{w}$pH or the ${}^{s}_{w}$pH operational scale to adjust the pH of the mobile phases, but correcting the ionic strength in all eluents to 0.18 *M*.

below the capacity factors determined using the ${}^{w}_{w}$pH scale. For the acidic (*S*)-(+)-naproxen in contrast, the log k_{IAM} values obtained in the ${}^{s}_{w}$pH scale lie above those measured using the ${}^{w}_{w}$pH scale.

The log k_{IAMw} values of the more polar compounds *N*-ethylaniline, (*S*)-(+)-naproxen and pindolol were also measured using a 100% aqueous phase. For *N*-ethylaniline (which is present in neutral form) the extrapolated log k_{IAMw} values are similar to those measured directly. In contrast, the extrapolated values of (*S*)-(+)-naproxen and pindolol (which are present in charged form), lie slightly below those determined using a 100% aqueous phase. However, these differences are much smaller than those observed using the YMC ODS-AQ column, indicating that the chromatographic behaviour of the IAM.PC.DD2 stationary phase is the same when using 100% aqueous mobile phases or eluents containing an organic modifier.

Overall, our results show that with both columns the use of either the ${}^{w}_{w}$pH, ${}^{s}_{w}$pH or ${}^{s}_{s}$pH operational scale has no influence on the extrapolated log k_w values of neutral solutes, and neither has correction for ionic strength. With the RP C_{18} column, however, it seems that for ionized compounds with a pK_a value in a ''critical'' range (e.g., benzylamine), the use of the ${}^{s}_{w}$pH or ${}^{s}_{s}$pH scale leads to significantly higher extrapolated log k_w values and to more linear plots.

3.3. pH-dependent partitioning in the IAM.PC.DD2 stationary phase

The pH-dependent partition behaviour of three ionizable compounds (propranolol, warfarin and salicylic acid) was studied in the pH range 4.5 to 7.0 in order to examine the hypothesis that the steric bulk of the second, freely movable acyl chains of the IAM.PC.DD2 phase can prevent the silanols and non end-capped amino moieties of the stationary phase from interacting with analytes. For these drugs, lipophilicity profiles had previously been determined at 37 °C for partitioning in large unilamellar PhC-liposomes and for retention on a single-chain IAM.PC.DD stationary phase [14]. Based on their results the authors had concluded that secondary interactions between ionized silanol groups and charged compounds occur in the pH range from 5 to 8.

For the more lipophilic compounds propranolol and warfarin, acetonitrile–buffer mixtures were used to extrapolate the log k_{IAMw} values by linear regression from at least four points. Good linear correlations with coefficients of $r^2 = 0.995–0.999$ were obtained.

The pH-partition diagrams derived from liposomal partitioning and IAM retention are shown in Fig. 4. For all three drugs, the pH-partition diagram obtained for the IAM.PC.DD2 stationary phase lies above the other two, suggesting a lower interfacial barrier to solute transport into the IAM hydrocarbon region due to the lower immobilized phospholipid density [2] and an increase of hydrophobic interactions between the solutes and the bounded phase due to the higher hydrocarbon density of the IAM.PC.DD2 phase [28].

The pH-partition diagram obtained for the liposomal partitioning of propranolol (Fig. 4A)

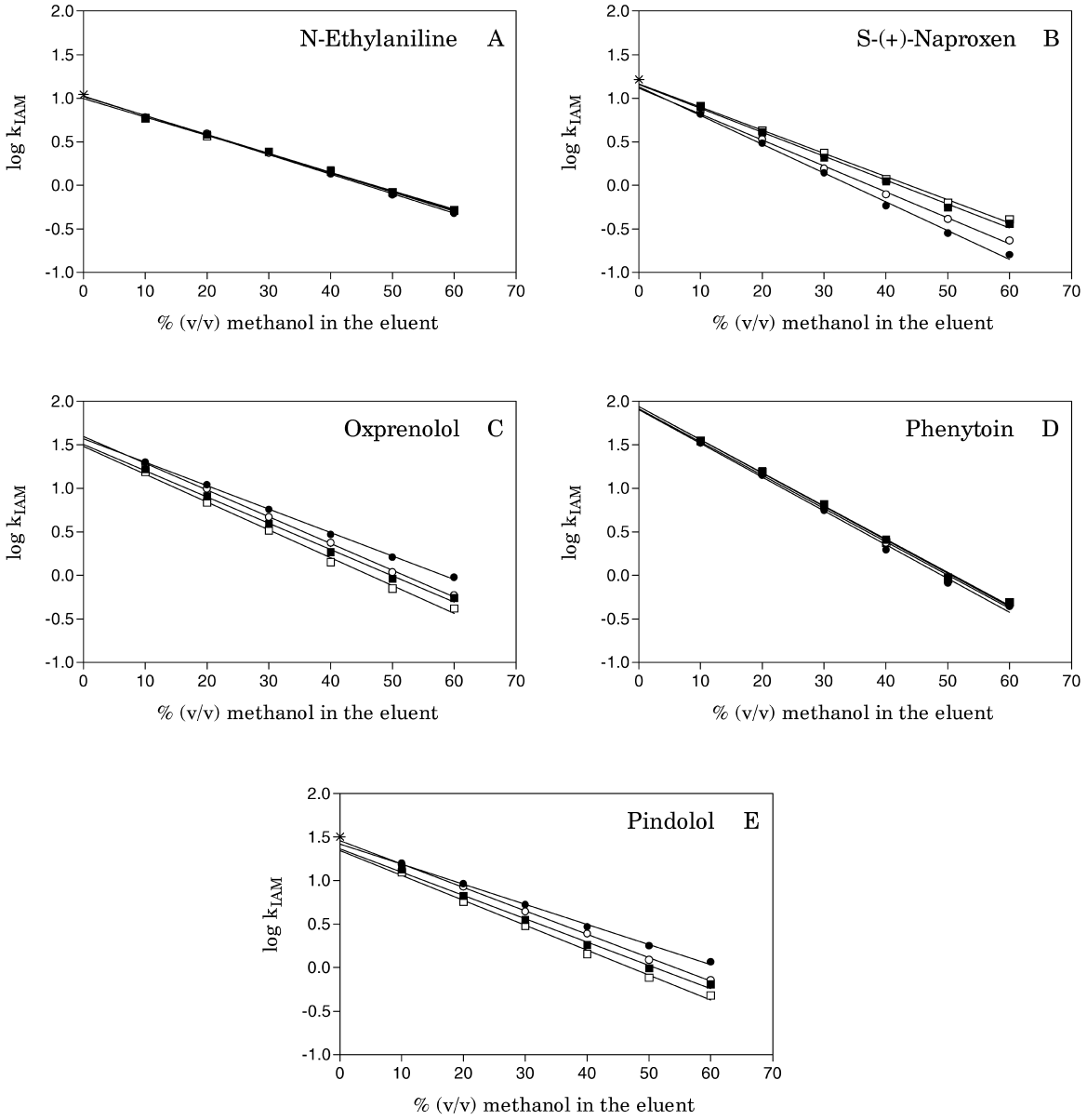

Fig. 3. Extrapolated log k_{IAMw} values obtained on an IAM.PC.DD2 stationary phase by plotting the isocratic log k_{IAM} against the percentage (v/v) of methanol and using either the ${}^{w}_{w}$pH (●) or the ${}^{s}_{w}$pH (■) scale to adjust the pH of the eluents. Additionally, the ionic strength was corrected throughout to 0.18 *M* using either the ${}^{w}_{w}$pH (○) or the ${}^{s}_{w}$pH (□) scale. When possible, the log $k^{7.0}_{IAMw}$ value was measured directly, using a 100% aqueous eluent (*).

shows a sigmoidal shape with an inflection point around the compound's pK_a (9.24 [14]). In fact, a plateau is reached below pH 8. For the IAM.PC.DD phase, in contrast, no plateau is observed. The log K_{IAMw} values increase slightly between pH 3 and 8, suggesting silanophilic interactions [14]. For the double-chain IAM.PC.DD2 phase, log k_{IAMw} values increase even more steeply between pH 6 and 7, due

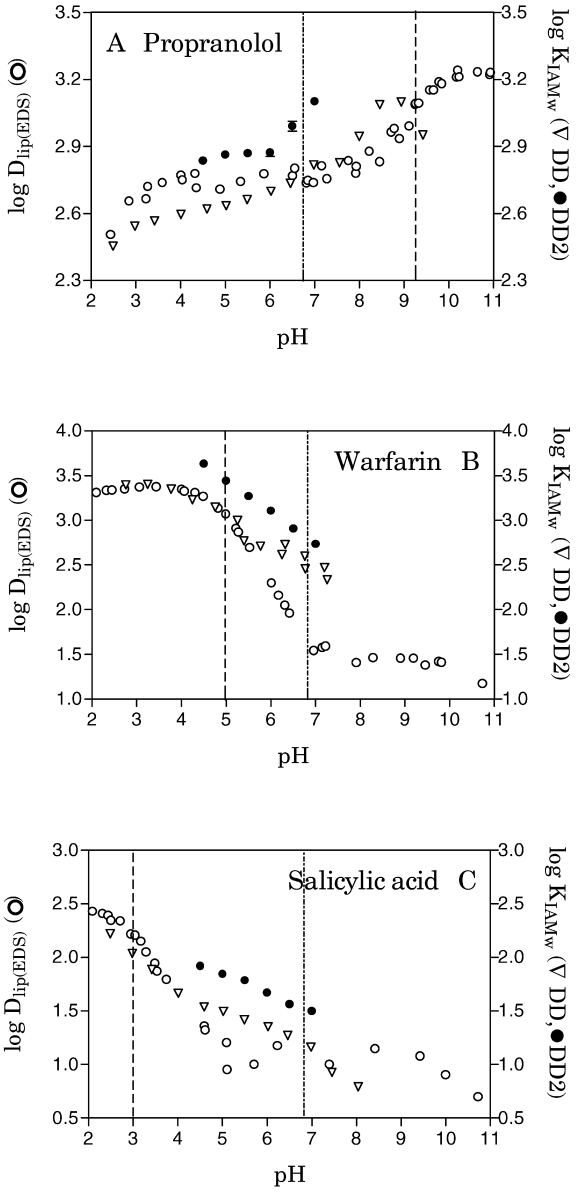

Fig. 4. Comparison between our results and those obtained by the group of Professor Wunderli (Department of Applied BioSciences, Federal Institute of Technology, Zürich, Switzerland). pH-partition diagrams derived from retention on IAMs (log K_{IAMw}: ●, ▽) and from partitioning in PhC-liposomes [log $D_{lip(EDS)}$: ○], measured by equilibrium dialysis. Our data are the log K_{IAMw} measured on the IAM.PC.DD2 phase (●).

to attractive electrostatic interactions between the cationic drug and the silanol groups becoming negatively charged (pK_a~6.8 [30]).

For the acidic warfarin and salicylic acid (Fig. 4B and C, respectively) a plateau is observed in the pH-partition diagram derived from liposomal partitioning above pH 7.0, where the drug is almost fully ionized (pK_a 5.0, and 3.0, respectively [14]). The pH-diagrams obtained for the IAM retention on both stationary phases show a decreasing trend. Beside the changing ionization state of the drug, repulsive interactions between the anionic drug and the negatively charged silanol groups and/or attractive interactions with residual non end-capped amino moieties may be involved [31].

Such secondary interactions with charged moieties in the stationary phase have already been described. Amato et al. [32] studied the retention of amines on an IAM.PC.MG stationary phase and observed smaller capacity factors for totally ionized amines at pH 5.5 than at pH 7.0. For neutral solutes, in contrast, retention was the same at both pH values. Demare et al. [27] determined the capacity factors of some ionizable compounds at pH 5.4 and pH 7.0 and also observed higher capacity factors at pH 5.4 than at pH 7.0 for fully deprotonated acids.

3.4. Ageing of the IAM.PC.DD2 phases and intra-/inter-batch reproducibility of IAM.PC.DD2 columns

Column ageing was investigated with four IAM.PC.DD2 stationary phases from two different batches using six neutral compounds, namely acetophenone, aniline, nitrobenzene, phenol, *N*-ethylaniline and 2-chloroaniline, all being neutral at pH 7.0. In fact, these solutes were chosen because their log $k^{7.0}_{IAMw}$ values cover a wide range.

For all compounds under study, a continuous decrease of capacity factors over time was observed indicating a deterioration of the columns. This is illustrated in Fig. 5 for an IAM.PC.DD2 stationary phase, with the capacity factors plotted against the multiples of column volumes. The column volume is defined as:

$$\text{column volume} = \pi r^2 L \quad (7)$$

where L is the column length; and r the radius. For the IAM.PC.DD2 stationary phases used the column volume equals 1.6 ml.

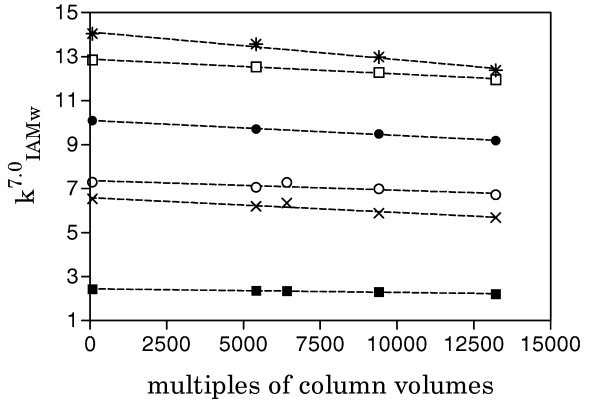

Fig. 5. Capacity factors ($k^{7.0}_{\mathrm{IAMw}}$) of acetophenone (○), aniline (■), nitrobenzene (●), phenol (×), *N*-ethylaniline (□) and 2-chloroaniline (★) determined on the IAM.PC.DD2 stationary phase of the first batch plotted as a function of multiples of column volumes.

For the single column of the first batch (Fig. 5) the percent decrease in capacity factors at 13 000 column volumes compared to 80 column volumes ranked between 8 and 13%, depending on solute. In fact, the extent of the decrease of the IAM capacity factors upon column failure was not identical for all solutes. Compared to the above column, the three stationary phases from the second batch showed a more pronounced ageing. Thus, the percent decrease after 13 400 column volumes compared to 80 column volumes ranked between 16 and 19%.

These results agree with those of other authors who found that column deterioration led to decreased IAM capacity factors of neutral as well as charged acidic and basic compounds, and that the percentage of decrease was not identical for all solutes [25,27]. The above results clearly indicate the need to correct for column ageing when log k_{IAMw} values have to be compared over time. We recommend to use several compounds with varying physicochemical properties (lipophilicity, charge) to follow up column ageing and to normalize the capacity factors of the analytes with respect to the capacity factors of the control compounds.

The column-to-column variability was studied using four IAM.PC.DD2 stationary phases from two batches and the same six neutral compounds. Log $k^{7.0}_{\mathrm{IAMw}}$ values were measured on the new columns, each conditioned using 80 column volumes of mobile phase.

The histograms represented in Fig. 6 show that for aniline (B), phenol (D) and 2-chloroaniline (F) the log $k^{7.0}_{\mathrm{IAMw}}$ values measured with the column from the first batch are significantly higher compared to those obtained with the three stationary phases from the

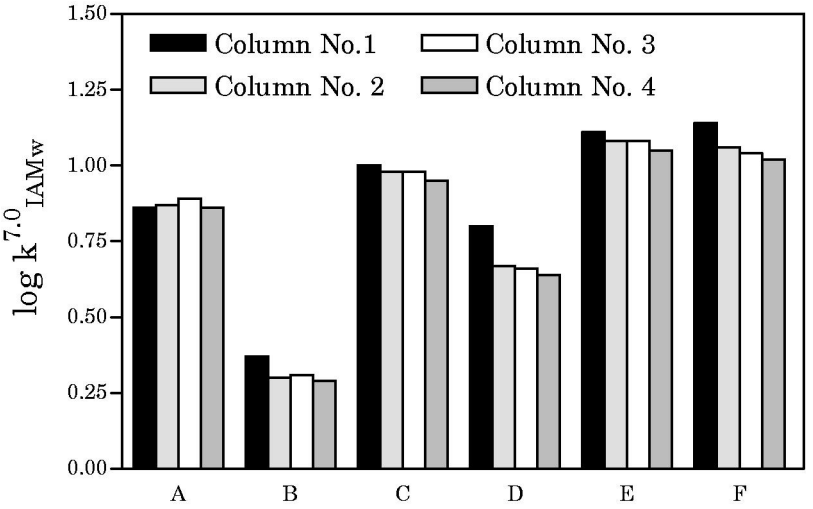

Fig. 6. The intra- and inter-batch variability of IAM.PC.DD2 stationary phases from two batches assessed by the capacity factors of six neutral model compounds, namely (A) acetophenone, (B) aniline, (C) nitrobenzene, (D) phenol, (E) *N*-ethylaniline and (F) 2-chloroaniline. Column No. 1 was from Batch P122-19-1 and the other stationary phases (Nos. 2–4) from Batch P129-47-2.

second batch. Furthermore, on the column from the first batch a slightly higher capacity factor was determined for 2-chloroaniline (F) compared to *N*-ethylaniline (E), in contrast to the ranking seen the stationary phases of the second batch. Additionally, very similar capacity factors were observed for all solutes when comparing the three columns from the second batch. The intra-batch variability thus appears negligible for IAM.PC.DD2 stationary phases.

As mentioned above, differences between the two batches exist not only for new columns, but also with column ageing. Several factors may explain the observed differences, as for example the purity of the silica matrix [33], the density of propylamines and phospholipids linked to the silica sub-surface, and the extent of end-capping [34].

Our results indicate that combining IAM capacity factors determined on stationary phases from different batches in a single analysis should be avoided when column-to-column reproducibility is not assessed. However, some authors have proposed to normalize capacity factors measured on HPLC columns from different batches by dividing them by the capacity factor of a standard solute. Such a normalization can indeed decrease the variation, but it will not be sufficient given that column-to-column variability also depends strongly on the analyte as shown here.

4. Conclusions

With the objective to establish guidelines for the proper measurement of IAM capacity factors, we investigated some methodological aspects related to the extrapolation of log k_{IAMw} values, the stability and properties of IAM.PC.DD2 stationary phases and the column-to-column variability.

The use of either acetonitrile or methanol as organic modifier for the extrapolation of log $k^{7.0}_{IAM}$ values was studied using a series of five β-blockers. Our results indicate that plotting the log $k^{7.0}_{IAM}$ values against the % (v/v) of the organic modifier in order to extrapolate log $k^{7.0}_{IAMw}$ values by linear regression gives more reliable values than the use of the mole fraction of co-solvent. No significant difference was observed between the log $k^{7.0}_{IAMw}$ values extrapolated from either methanol or acetonitrile. However, methanol seems more appropriate when log $k^{7.0}_{IAMw}$ values for charged compounds have to be estimated, since its solvent properties are closer to those of water. Furthermore, when acetonitrile is used, its percentage should not be more than 30% since this would disrupt the water structure [10].

The influence of using the ${}^{w}_{w}$pH, ${}^{s}_{w}$pH or ${}^{s}_{s}$pH operational scale to adjust the pH of mobile phases containing a co-solvent was investigated on a RP C_{18} HPLC column and an IAM.PC.DD2 stationary phase. Furthermore, the effect of correcting the ionic strength for dilution caused by the co-solvent was studied. Our results indicate that with the IAM.PC.DD2 stationary phase, only small differences between the extrapolated log k_{IAMw} values exist when using the ${}^{w}_{w}$pH scale or the ${}^{s}_{w}$pH scale and correcting or not the ionic strength. However, the use of the ${}^{s}_{w}$pH scale seems more appropriate when isocratic capacity factors are compared. With the YMC ODS-AQ column and ionized analytes, the use of the ${}^{s}_{w}$pH scale to adjust the pH is judicious in order to avoid a parabolic relationship between the isocratic log k values and the % (v/v) of organic modifier. Further investigations with more compounds are needed to confirm our results.

The analysis of pH-dependent retention on an IAM.PC.DD2 stationary phase indicates that secondary interactions between analytes and charged moieties of the stationary phase occur not only on the single-chain IAM.PC.DD column but also on the double-chain IAM.PC.DD2 surface. The second, freely movable fatty acids of the phospholipids immobilized on the IAM.PC.DD2 column do not appear to prevent ionized analytes from interacting with silanol and/or amino groups. This presents a real problem since IAMs are commonly used to mimic biological membranes.

Finally, our results show that column ageing occurs also for IAM.PC.DD2 stationary phases and that it depends not only on the column studied but also on the investigated compounds. When log k_{IAMw} values have to be compared over time, correction for column ageing is recommended. But as the capacity factors decrease differently for the various analytes, several compounds must be used for the correction.

The column-to-column variability was studied using four IAM.PC.DD2 stationary phases from two

batches. The intra-batch variability was small, whereas a marked and solute-dependent batch-to-batch variability was seen. Normalization for column-to-column variability using a single compound is not sufficient.

5. Nomenclature

Log D	Logarithm of the distribution coefficient
${}^{w}_{w}$pH	Electrode calibration and pH measured in water
${}^{s}_{w}$pH	Electrode calibration in water and pH measured in organic solvent–water mixtures
${}^{s}_{s}$pH	Electrode calibration and pH measured in organic solvent–water mixtures
IAM	Immobilized artificial membrane
IAM.PC.DD2	Immobilized artificial membrane prepared using a diacylated phosphatidylcholine analogoue and end-capped with C_3- and C_{10}-anhydrides
Log k	Logarithm of the capacity factor
Log k_w	Logarithm of the capacity factor obtained by extrapolation of isocratic capacity factors to a 100% aqueous mobile phase
Log k_{IAMw}	Logarithm of the IAM capacity factor obtained by extrapolation of isocratic capacity factors to a 100% aqueous mobile phase or measured directly using this eluent
Log K_{IAMw}	Partition coefficient in IAM-HPLC with a 100% aqueous eluent
PhC	Phosphatidylcholine
lip	PhC-liposomes
EDS	Equilibrium dialysis system

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