Some factors that can lead to poor peak shape in hydrophilic interaction chromatography, and possibilities for their remediation.

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Abstract

Some factors which present difficulties for obtaining good peak shape in hydrophilic interaction chromatography (HILIC) were studied. The effect of injection solvent composition and volume were systematically investigated using a selection of weak and stronger basic compounds on a hybrid bare silica phase. Increasing the mismatch between the injection solvent (range 95 to 0 % ACN, v/v) and the mobile phase (maintained at 95 % ACN v/v) gave increasing deterioration in peak shape. With the 2.1 mm ID columns used, injections in the mobile phase of increasing volume (1-20 µL) gave poorer peak shape, but the magnitude of the effect was considerably smaller than that of solvent mismatch over this range. Some solute structural features such as galloyl (trihydroxy benzene), catechol (benzene diol) and phosphate (in nucleotides) gave serious peak tailing, attributed to interactions with metals in the stationary phase or the chromatographic hardware. These undesirable effects can be moderated by including complexing agents in the mobile phase, by changing the stationary phase chemistry, or by altering the mobile phase pH.

1. Introduction

Hydrophilic interaction liquid chromatography (HILIC) is fast becoming an established tool for the separation of polar and/or ionised compounds. This technique offers an alternative approach for compounds difficult to retain by reversed-phase (RP) methods. Stationary phases in HILIC include bare silica and polar bonded phases. Retention occurs through solute partitioning between a pseudo-immobilised water layer and the bulk mobile phase, as well as by adsorption and ionic interactions. There are important applications of the technique in biomedical and biological science, including in pharmaceutical analysis [1], metabolomic fingerprinting [2,3], anti-doping investigations [4,5] and clinical applications [6]. The mobile phases used in HILIC are typically hydro-organic mixtures of an aprotic solvent such as acetonitrile (>70%ACN, v/v) in the presence of a soluble buffer (e.g. ammonium-formate/acetate/bicarbonate). These buffers are also volatile and thus useful for electrospray ionisation (ESI) mass spectrometry. Buffers are required at sufficient ionic strength to improve peak shape and control retention, particularly for ionogenic compounds [7].

Method development in HILIC can sometimes be more challenging compared with RPLC. For instance, the diverse chemical functionality of many hydrophilic compounds (e.g. the presence of a combination of one or more ionisable amino, carboxyl, diol, phosphate groups) can lead to complex interactions. Certain solute structural features may lead to unwanted, strong secondary interactions with the column and/or with the instrumentation, that can lead to deterioration of chromatographic performance [8]. These effects are clearly of concern in the LC-MS analysis of complex biological matrices where high separation efficiency is required [9]. Some recent articles have outlined approaches for overcoming poor peak shapes in metabolite profiling, notably by using either polymeric zwitterionic columns combined with high pH [10] or by adopting ion-pair chromatography [11]. Another factor is the influence of injection solvent composition on peak efficiency/capacity with different solutes and stationary phases during HILIC method development. In general, injection solvent mismatch should be minimised to obtain optimum chromatographic efficiency. However, problems may occur when poor sample solubility is encountered with a given mobile phase. For instance, highly polar compounds may have limited solubility in ACN-rich (> 90 % v/v) mobile phases. This limitation may be problematic in scaling up HILIC separations for preparative work. Previous studies have studied substituting amounts of methanol [12] and/or isopropyl alcohol [13] in place of acetonitrile while including or excluding some of the water content in the injection solvent. However, no detailed measurements of the effects on column efficiency were made, or on the solution properties of different compounds. In the present study, we systematically investigated the influence of sample diluent from 0-95% ACN using a bare silica stationary phase (BEH HILIC) and a selection of small solutes. The influence of increasing injection volume on

column efficiency was also evaluated using the flow-through needle autosampler design in the instrument used.

A further problem in HILIC is the unexplained poor peak shapes of some compounds that are unrelated to injection effects. For example, a previous investigation showed very poor peak shapes in the analysis of catecholamines by HILIC on a bare silica column [14]. In the present work, we have investigated the possible role of metals in the stationary phase or in the chromatographic hardware. Throughout all of these studies, we exclusively used isocratic elution to minimise the influence of gradient peak compression, which may otherwise complicate the interpretation of results [13].

2. Experimental

All experiments were performed using a 1290 ultra-high pressure liquid chromatograph (UHPLC) (Agilent, Waldbronn, Germany) consisting of a binary pump, autosampler and photodiode array UV detector (0.6 μL flow cell). Chemstation software was used for data handling and instrument control. The columns used were Acquity BEH HILIC (un-bonded) and Amide (bonded) phases (100 x 2.1 mm ID, both having particle size 1.7 μm, pore size 130 Å, surface area 185 m^2/g) from Waters Corp. (Milford, USA). Columns were held at 30 ^oC in the column thermostat. 1 μL injections were made unless otherwise stated. The columns were operated at a flow rate of 0.4 mL/min. Acetonitrile (far UV grade) and formic acid (FA) were purchased from Fisher (Loughborough, UK). Ammonium formate (AF), disodium ethylenediaminetetraacetic acid (EDTA), and ammonium citrate dibasic were purchased from Sigma-Aldrich (Poole, UK). Standards were prepared at a concentration of 20 – 50 mg/L diluted in the exact mobile phase unless stated otherwise. Buffered mobile phases (quoted as $_w^w$pH) were prepared by adjusting the pH of the aqueous portion before the addition of acetonitrile. All test compounds were purchased from Sigma-Aldrich (Poole, UK). Their structures and logD at $_w^w$pH 3 and 9 are indicated in Fig. 1. LogD values were determined as averages from three different prediction software programs: ACD version 12.0 (ACD labs, Toronto, Canada), Marvin (ChemAxon, Budapest, Hungary) and MedChem Designer (Simulation Plus, Lancaster, USA). Column efficiencies were determined using the 5σ method (unless stated otherwise) and asymmetry values reported at 10% peak height ($As_{0.1}$) using the Chemstation software.

3. Results and discussion

3.1 Influence of the injection solvent composition on efficiency in HILIC

For this study we used basic compounds $\log D_{pH\ 3}$ values -2.5 to 1.1 as well as the quaternary ammonium salt trimethyphenylammonium chloride (TMPAC). These compounds are well retained (k = 3.8 - 9.5 on BEH HILIC) by a combination of ionic interactions and partitioning processes using 95 % ACN containing pH 3 buffer. Variation of the injection diluent from 0-95% ACN was performed while using this fixed mobile phase composition. The overall concentration of ammonium formate $_{w}^{w}$pH 3 (5 mM) was maintained in each injected solution. Solutions of single solutes were injected separately. Fig. 2 shows that for all solutes, losses in performance were moderate for concentrations of 80-95% ACN in the injection solvent when 1 μL volumes were used. The greatest loss in efficiency over this range was obtained for nortriptyline (from 19,000 to 13,000 plates). Concomitantly, at 80% ACN the asymmetry ($As_{0.1}$) values were also reasonable at 1.0, 1.1, 1.5, 1.2 and 1.2 (full range of data not shown) for nortriptyline, procainamide, adenine, cytosine and TMPAC respectively. However, with further increase in the aqueous concentration in the injection solvent, severe deterioration in column efficiency was observed. Peak splitting occurred for TMPAC and procainamide when using ACN concentrations lower than 60% as the injection solvent (efficiencies not plotted for lower ACN concentrations in Fig. 2). For all other solutes, severe fronting accompanied the dramatic losses in efficiency. These results agree reasonably well with the results of a previous study that used gradient elution [13]. Our results suggest a degree of practical flexibility in the composition of the dissolution solvent i.e. small increases in water content in the injection solvent may be acceptable and necessary (when small injection volumes are used) to accommodate compounds that are poorly soluble in acetonitrile rich mobile phases (e.g. > 90% ACN).

3.2 Effect of increasing injection volume on efficiency in HILIC

Larger injection volumes can be beneficial for obtaining higher signal-to-noise values for trace analysis, as well as for increasing sample loads in preparative chromatography, while limiting problems of solubility. Fig. 3 (a-c) show the influence of increasing injection volume from 1-20 μL on column efficiency when 95, 90 and 80% ACN respectively containing 5 mM buffer pH 3 was used as the injection solvent. As before, the mobile phase was 95% ACN containing 5 mM buffer pH 3. The increase in extra-column variance arising from increasing the injection volume alone can be predicted according to [15,16]:

$$\sigma_v^2(inj) = \frac{V_{Inj}^2}{12} \quad (1)$$

The total variance measured from the chromatogram is the sum of the variance produced by the individual components of the system:

$$\sigma_v^2(tot.) = \sigma_v^2(inj.) + \sigma_v^2(det.) + \sigma_v^2(tubing) + \sigma_v^2(column) \quad (2)$$

Fig. 3a shows plots of the experimentally measured efficiency N against injection volume for the 5 probes, where injections were made in the mobile phase. Fig. 4 shows the predicted % loss in N for each probe against injection volume, (assuming the injection solvent is the same as the mobile phase i.e. 95 % ACN containing 5 mM buffer pH 3), estimating the contribution of the injection volume from equation (1). From equation (1), the increase in variance resulting from the use of a 20 μL injection instead of 1 μL is therefore $(20^2\text{-}1^2)/12 = 33.3\ \mu L^2$, where there is no additional contribution from mismatch of the injection solvent and mobile phase. The influence of the injection process alone can be estimated by subtracting the variance (σ^2) due to the injection from the total variance and expressing as a %. Clearly, the loss in N in Fig. 4 is predicted to be greatest for peaks with low *k* (and also high efficiency) as the variance due to the injection volume will contribute proportionally more to the total variance. Indeed notriptyline and adenine (*k* =4.3 and 3.8 respectively) are predicted to show greater losses than TMPAC and procainamide (*k* = 7.9 and 9.5 respectively). Whereas the experimentally measured loss in efficiency follows these predicted trends, with the decrease in efficiency (indicated by the greater slopes of the plots in Fig. 3a) being highest for adenine and nortriptyline, the actual losses in efficiency are considerably greater than predicted by Fig.4. For example, the actual loss in efficiency for adenine is 41% over the range of injection volumes, whereas the predicted value is 18 %. The actual losses for TMPAC and procainamide are 16 and 20 % respectively whereas the predicted losses are around 6%. Thus, it seems equation (1) underestimates the actual losses in efficiency due to increase in the injection volume. Nevertheless, Fig. 3a demonstrates that practically useful efficiencies can still be obtained with up to 20 μL injections with matched mobile phase and injection solvent, at least for the solutes used here which have moderate to high *k*.

Practically, using 95% ACN as the injection solvent may not be suitable for the dissolution of high concentrations of very polar compounds and it may be necessary to use mismatched injection solvents with higher water concentration. As shown in Fig. 3b, the losses in efficiency using sample volumes greater than 1 μL were considerably worse even if the degree of mismatch between injection solvent and mobile phase was only 5% (90 % ACN). The worst results (Fig. 3c) were using the 80 %

ACN injection solvent, with severe loss in efficiency encountered even when using a 5 μL injection. Nevertheless, acceptable results with this solvent could still be obtained when restricting the injection volume to 1 μL. The relative strength of the injection solvent, together with viscosity differences compared with the mobile phase, are likely to be the causes of the efficiency loss [17–19]. Losses in efficiency due to solute overloading were unlikely to influence the results. The maximum loads used at the highest injection volume (20 μL) were 0.4 μg for nortriptyline, procainamide, adenine, and cytosine and 1.0 μg of TMPAC. In any case, the sample loads are the same as in Fig. 3a, the only variable being the injection solvent composition.

3.4 Problems with metals and possible remediation

Problems resulting from solute-metal interactions have been studied in RP separations [20-23], particularly with regard to the analysis of compounds of biological interest. Some manufacturers supply biocompatible systems that are supposed to be inert to such interactions, using alternative material to steel where possible [24,25]. Elsewhere [26], it has been shown that replacement of stainless steel with polyethylene frits could be beneficial for improving peak shapes of phosphorylated compounds in RP chromatography. This is important since it has also been shown [23] that the corrosion of stainless steel frits in the presence of acidic mobile phases results in the release of metal-oxides such as Fe^{2+}/Fe^{3+}. Nevertheless, little work on the problem of metals and steps for remediation has been published with regard to HILIC separations.

3.4.1 Hydroxybenzoic acids containing vicinal hydroxyl groups

In a previous paper [7] it was shown that 3,4,5-THBA gave very asymmetrical peaks on some columns in both ammonium formate (AF) and formic acid (FA) buffered eluents. It is known [27] that compounds bearing catechol (benzene diol) and galloyl (trihydroxy benzene) groups complex with transition metals in various oxidation states, particularly with iron. As the retention of benzoic acids was poor on bare silica at $_{w}^{w}$ pH 3 we examined the effects of mobile phase additives using a BEH amide column only. A mobile phase containing 80% ACN containing 5 mM AF was used for convenience in the first instance; higher acetonitrile concentrations produced very broad asymmetrical peaks as well as high retention for 3,4,5-THBA. Fig. 5a illustrates the poor peak shape (N = 3300) obtained for 3,4,5-THBA using an 80% ACN AF mobile phase. When a small amount of disodium EDTA (0.1 mM) was included in the mobile phase, a dramatic improvement in peak shape occurred (Fig. 5b, N = 11200). EDTA has one

of the highest known binding constants for iron at physiological pH. Although this was a promising result, EDTA is poorly soluble in > 85% ACN and therefore has limited use in HILIC. Citrate is also a known iron-chelator [28] and was investigated as an alternative additive, due to its higher solubility. Figs. 5c (80 % ACN) and 5d (90 % ACN) show that citrate was indeed sufficiently strong enough to prevent metal complexation of 3,4,5-THBA (N = 11,000 and 12300 respectively). We ensured that 5 mM overall ammonium ions and $_{w}^{w}$pH 3 was maintained in all experiments. The exact sites of metal-solute interactions are as yet unknown, but may result from the column frits, which have a high surface area in contact with the mobile phase.

3.4.2 Catecholamines and structurally related amino compounds

In an earlier report [14] poor peak shapes were obtained for catecholamines on bare silica in the HILIC mode, although no rationalisation of this finding was proposed. It was shown subsequently [29] that ZIC-HILIC and amide bonded phases could be used to chromatograph catecholamines with good peak symmetry, retention and column efficiency. Here, some catecholamines, and also some structurally related amino compounds (Fig. 1) that do not bear the catechol functional group, were chosen to ascertain whether catechol-metal interactions were responsible for the poor peak shapes seen on bare silica.

Fig. 6a illustrates that good peak shapes and separation can indeed be obtained for catecholamines and structurally related amines using a BEH amide column with a simple 90% ACN AF mobile phase, similar to the previous findings [29]. In contrast, Fig. 6b shows the same trend of poor peak shape as seen previously [14] except using the un-bonded BEH HILIC phase, with only phenylephrine and halostachine yielding acceptable asymmetry values ($As_{0.1}$ < 1.4). Peak asymmetry values for dopamine (peak 3), epinephrine (peak 4) and norepinephrine (peak 5) were 12.0, 6.5 and 8.1 respectively. Phenylephrine (peak 2) and halostachine (peak 1) do not bear the catechol functional group, which therefore seems to be the source of the detrimental interaction for the other compounds. Fig. 6c indicates much reduced tailing ($As_{0.1}$ < 1.9) of catecholamines on bare silica with the inclusion of citrate in the mobile phase. A difficulty with the use of citrate is its limited solubility in mobile phases containing > 90 % ACN. However, the experiment gives some indication of solute metal interactions. It is unclear why catecholamines exhibit reasonable peak shapes on the equivalent amide phase without the inclusion of metal complexing agents in the mobile phase. It is possible that the catechol group cannot gain access to surface silanol-associated iron when the amide bonding is present. Increasing the mobile phase pH may in some cases, limit metal complexation effects, although alkaline conditions may not be suitable for catecholamine analysis since oxidative degradation is known to occur [30]. As shown by Lindner *et al*.

[8], solute-metal complexation is likely to be different between instruments due to the passivation of steel surfaces over time.

3.4.3 Nucleotides

The chromatography of nucleotides can be challenging when considering both their hydrophilicity (see Fig. 1 logD values) and negative charge, which arises from the presence of one or more phosphate groups. The phosphate functionality of phosphoproteins is known to have an affinity for Fe^{3+} as exploited in iron-metal affinity chromatographic (IMAC) enrichment strategies [31]. By analogy, the same interaction is known to take place with nucleotides [31]. The analysis of nucleotides under typical RPLC conditions can result in severely tailed peaks, which is a result of the metal components contained within the wetted parts of chromatographic and electrospray systems [33–35]. In this study, adenosine (nucleoside), adenosine-monophosphate (AMP), adenosine-diphosphate (ADP) and adenosine-triphosphate (ATP) were chosen as model test compounds to probe the severity of these interactions in HILIC.

Figs. 7 and 8 illustrate the chromatograms and performance data for the nucleotide test mix using the BEH amide column under various conditions. Fig. 7a shows that acceptable peak shapes for adenosine and AMP (peaks 1 and 2) could be obtained using a simple 70% ACN AF $_{w}^{w}$pH 3 mobile phase, whereas ADP and particularly ATP (peaks 3 and 4, $As_{0.1} > 10$) showed considerably worse peak tailing. Fig. 7b shows that the introduction of a small amount of EDTA dramatically reduced the peak tailing of ADP and ATP, most likely due to minimisation of interactions with system metals. Note that these results were obtained on the amide column, which had given acceptable results for the diol compounds in the absence of EDTA, implying that nucleotide-metal interactions may be more serious. Using EDTA in the mobile phase for ascertaining the degree of nucleotide-metal interactions has been demonstrated previously under RPLC conditions [35]. Moreover, co-injection of EDTA [36] in the sample solution has also been shown to be useful for nucleotide analysis. However, when interfacing with ESI-MS for instance, the introduction of non-volatile constituents into the ionisation source should be restricted to avoid contamination. As stated earlier, the low solubility of EDTA in HILIC eluents composed of > 85% ACN further limits its use for substances of lower hydrophilicity, particularly if gradient analysis is required. However, since the release of phosphoproteins in IMAC from bound Fe^{3+} is influenced by pH [31] we investigated the effect of operating in more alkaline conditions. This is possible since the BEH amide column can be operated between $_{w}^{w}$pH 2 - 9 according to the manufacturer. Figs. 7c and Fig. 8 show that the peak shape of ADP and ATP were greatly improved upon raising the mobile phase pH, comparable to

the results with EDTA at low pH. This promising result indicates that very low nucleotide-metal binding occurs under alkaline conditions for ADP and ATP, inferring that the use of ion-pair reagents normally used in nucleotide analysis may be avoidable. Johnsen *et al*. [37] discussed that silica based ZIC-HILIC could not be used to chromatograph nucleotides and observed broad, asymmetrical peaks (there was no discussion of conditions used). The workers chose instead to use a polymeric ZIC-pHILIC column using ammonium carbonate at $_{w}^{w}$pH 8.9 with favourable results. Their work also showed an increase in retention with increasing buffer concentration, indicating a partition based retention mechanism. Finally, Fig. 7d indicates the use of ammonium bicarbonate adjusted to $_{w}^{w}$pH 9 instead of AF buffer. Interestingly, losses in retention were observed, as well as little evidence of strong peak tailing due to metal-phosphate interactions. Further work needs to be done to study the influence of anionic mobile phase component, methods of buffer preparation and their influence on retention effects in HILIC.

4 Conclusions

The effects of some factors that influence column efficiency and peak shape in HILIC were investigated. Increasing the injection volume (sample dissolved in the exact mobile phase) from 1-20 μL with a 2.1 mm ID column gave gradual reduction in efficiency. Approximately 60% of the small injection volume efficiency remained with a 20 μL injection for a solute of $k \sim 4$. A potential drawback with HILIC is that some compounds may not be very soluble in acetonitrile-rich mobile phases, which may be necessary to obtain acceptable retention. Thus we investigated the effect of injection solvents richer in water (lower ACN concentration). Up to 15% ACN mismatch between the composition of the injection solvent (80 % ACN) and mobile phase (95% ACN) gave relatively small losses in efficiency for the smallest injection volume (1 μL in conjunction with a 100 x 2.1 mm ID column) but dramatic losses in efficiency as the injection volume was increased only to a few μL. The detrimental effects of increased injection volume and solvent mismatch were more serious for solutes with smaller retention factors.

Peak shapes of some solutes in HILIC seem to be influenced by solute-metal interactions. Poor peak shapes may occur with compounds containing galloyl, catechol and phosphate groups. The exact sites of these interactions are unknown. It is possible that they arise due to the leaching of metals from the chromatographic hardware (particularly the column frits) which then adsorb onto the silica. Direct interaction of solutes with the frit surface area is also possible. These effects were demonstrated by adding small amounts of EDTA or citrate to the mobile phase, which considerably improved the peak shapes of the test solutes used. For nucleotide phosphates, simply raising the pH using a pH stable hybrid silica amide column, gave much improved peak shape. However, this approach may not be applicable for

all compounds that show metal complexing abilities (e.g. catecholamines) due to their instability at higher pH.

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Figure 1

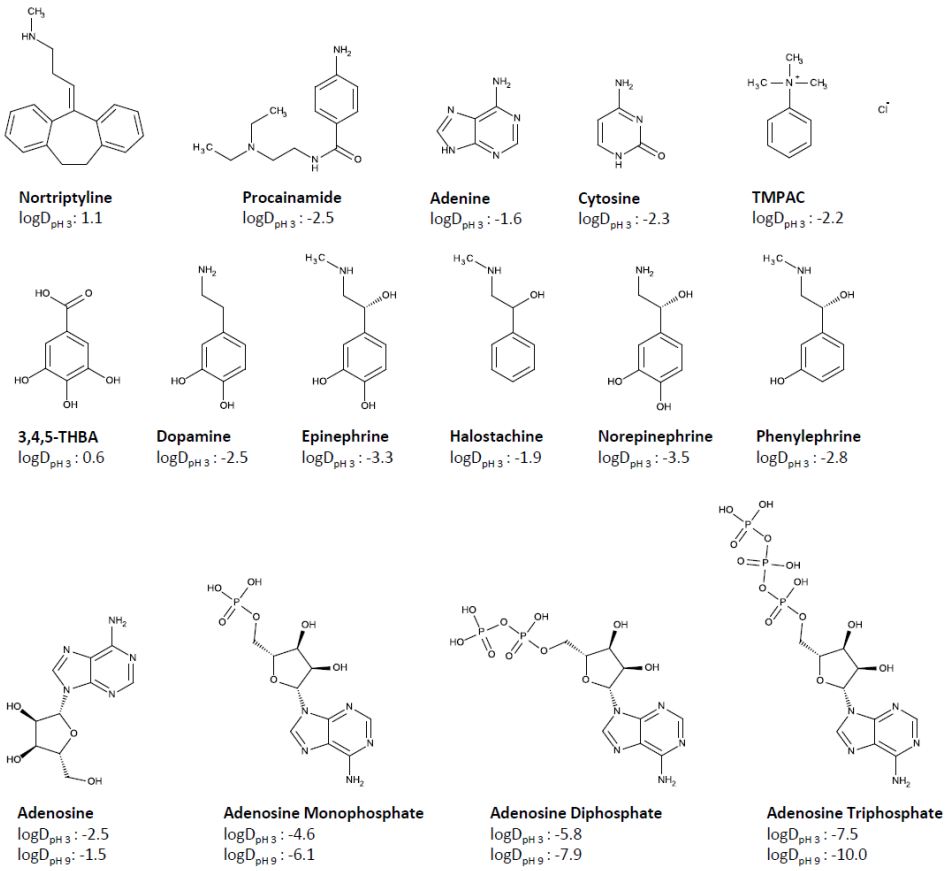


Figure 1

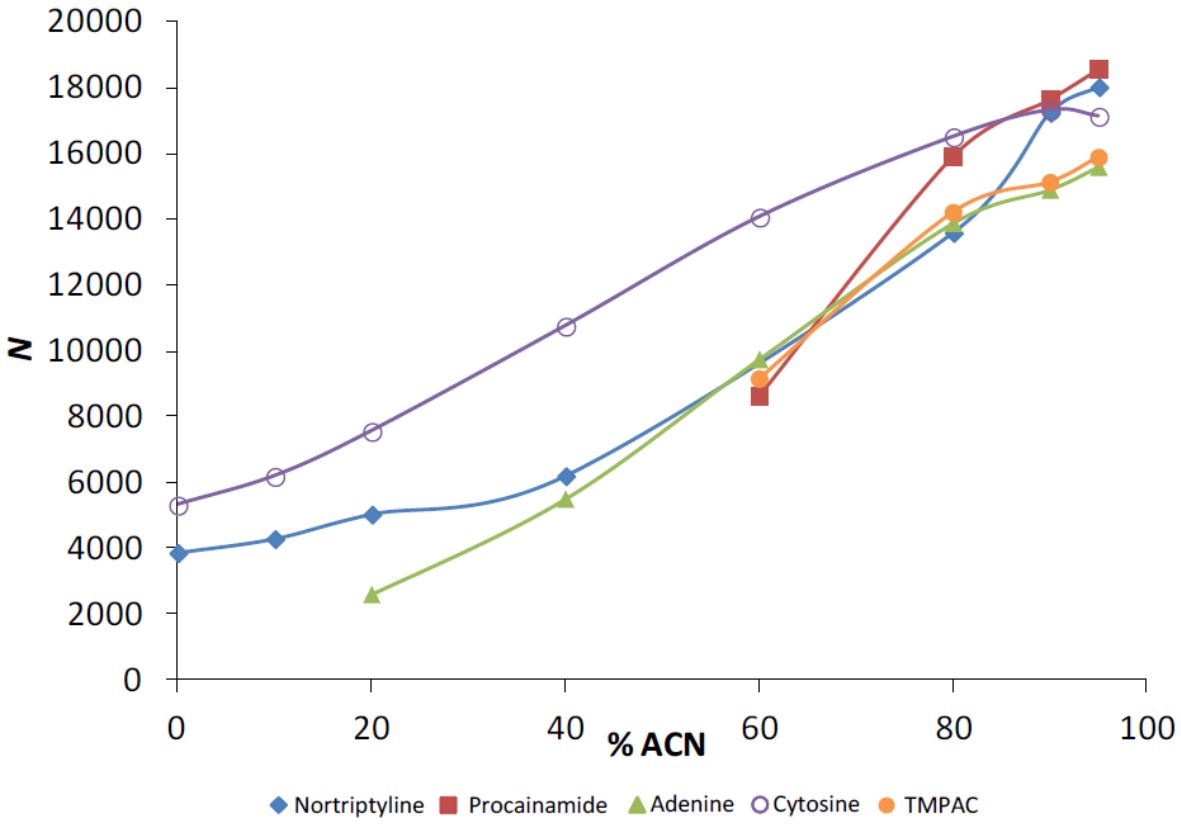

20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
N
0
20
40
% ACN
60
80
100
Nortriptyline
Procainamide
Adenine
Cytosine
TMPAC

Fig 2

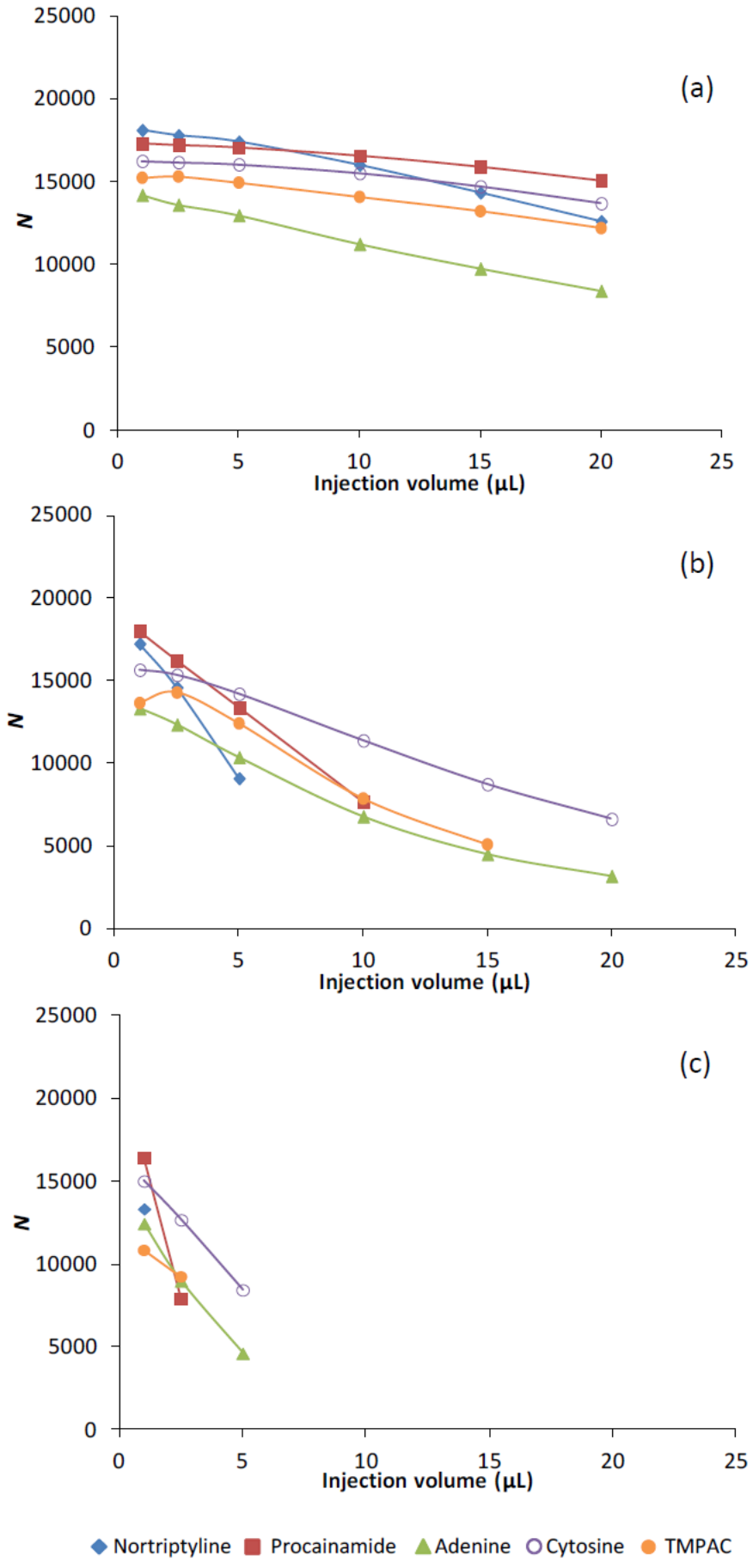

(a)
(b)
(c)
N
Injection volume (µL)
0
5000
10000
15000
20000
25000
0
5
10
15
20
25
Nortriptyline
Procainamide
Adenine
Cytosine
TMPAC

Figure 3

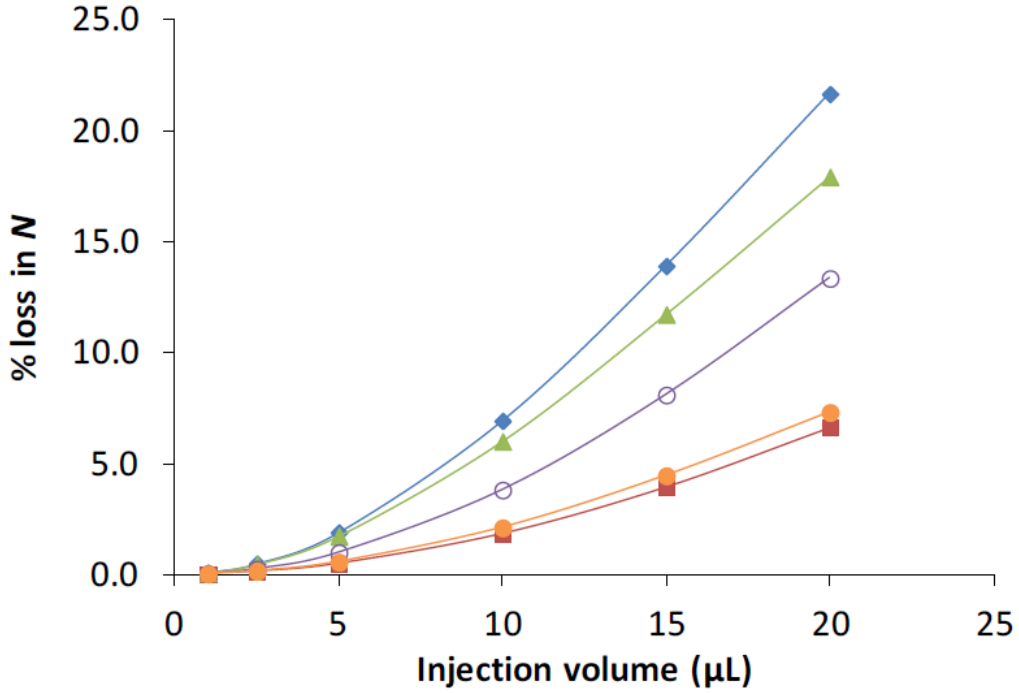

25.0
20.0
15.0
10.0
5.0
0.0
% loss in N
0
5
10
15
20
25
Injection volume (µL)

Fig 4

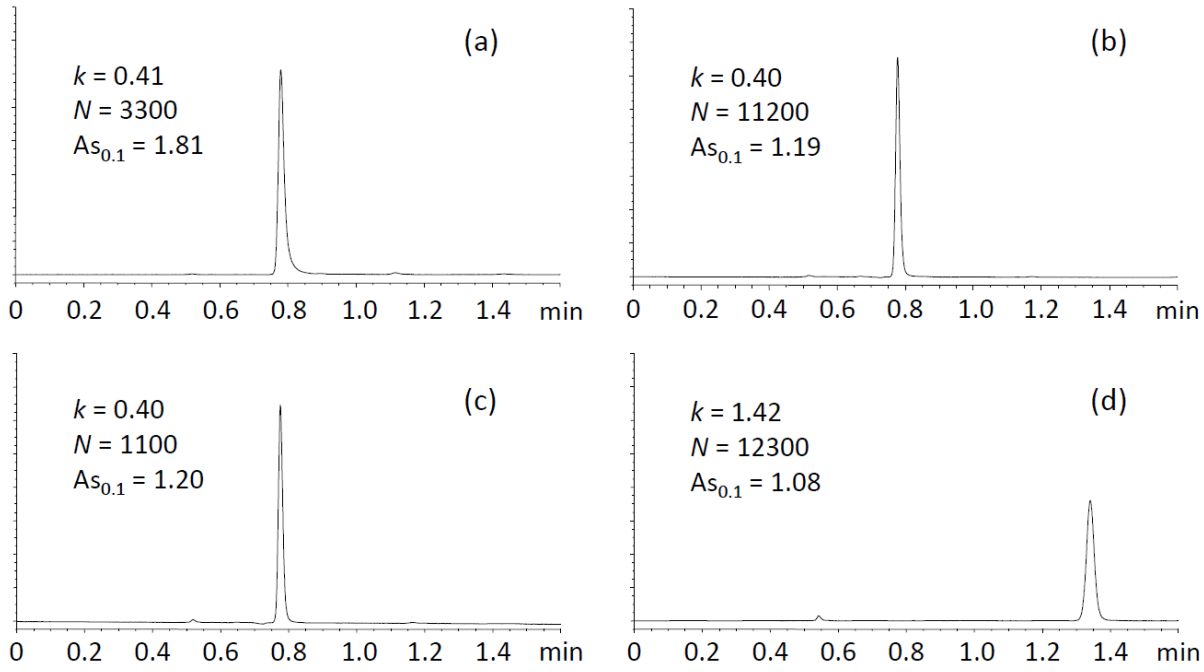

(a)
k = 0.41
N = 3300
As0.1 = 1.81
(b)
k = 0.40
N = 11200
As0.1 = 1.19
(c)
k = 0.40
N = 1100
As0.1 = 1.20
(d)
k = 1.42
N = 12300
As0.1 = 1.08
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 min

Fig 5

Figure 6

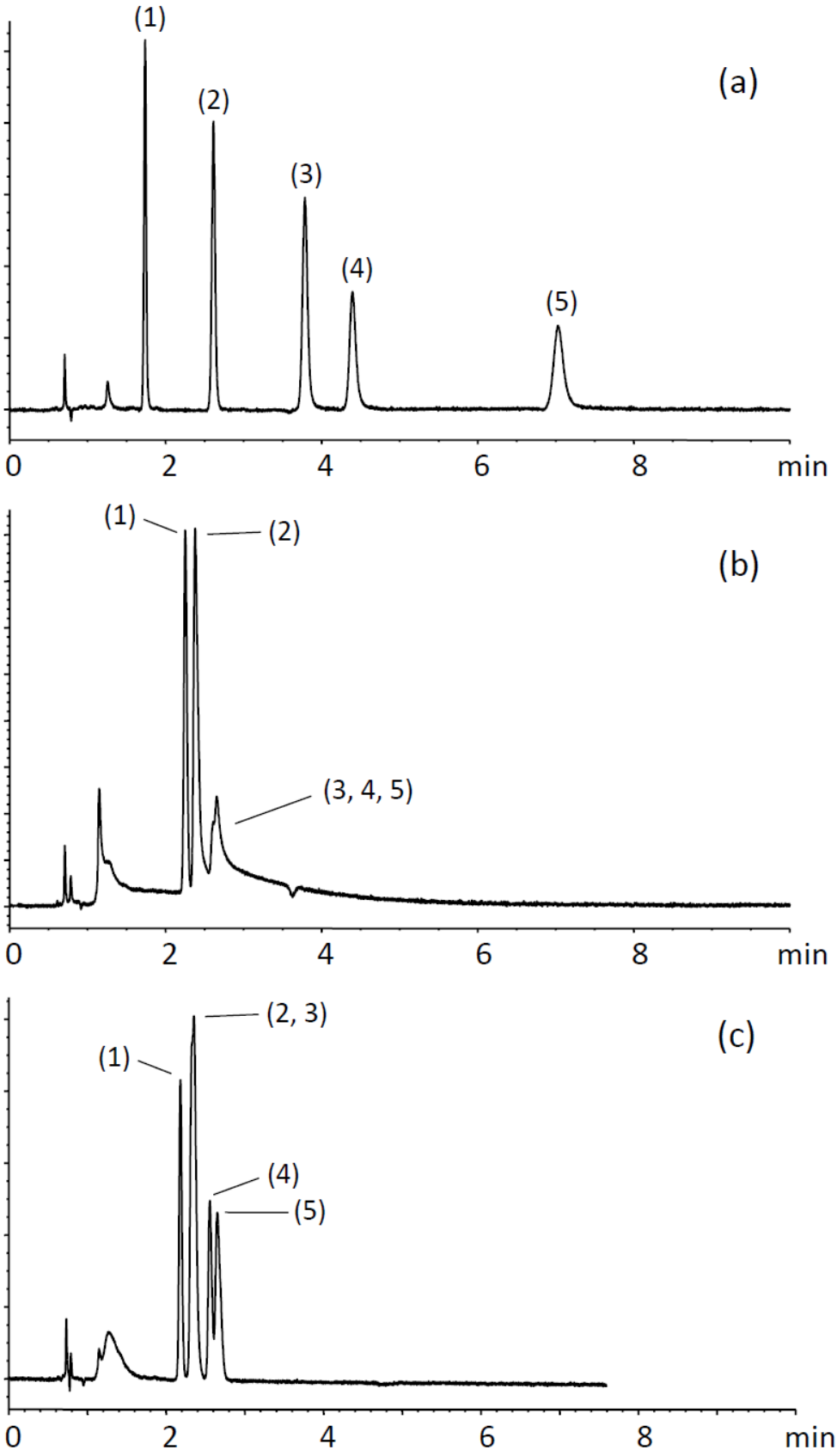


Figure 6

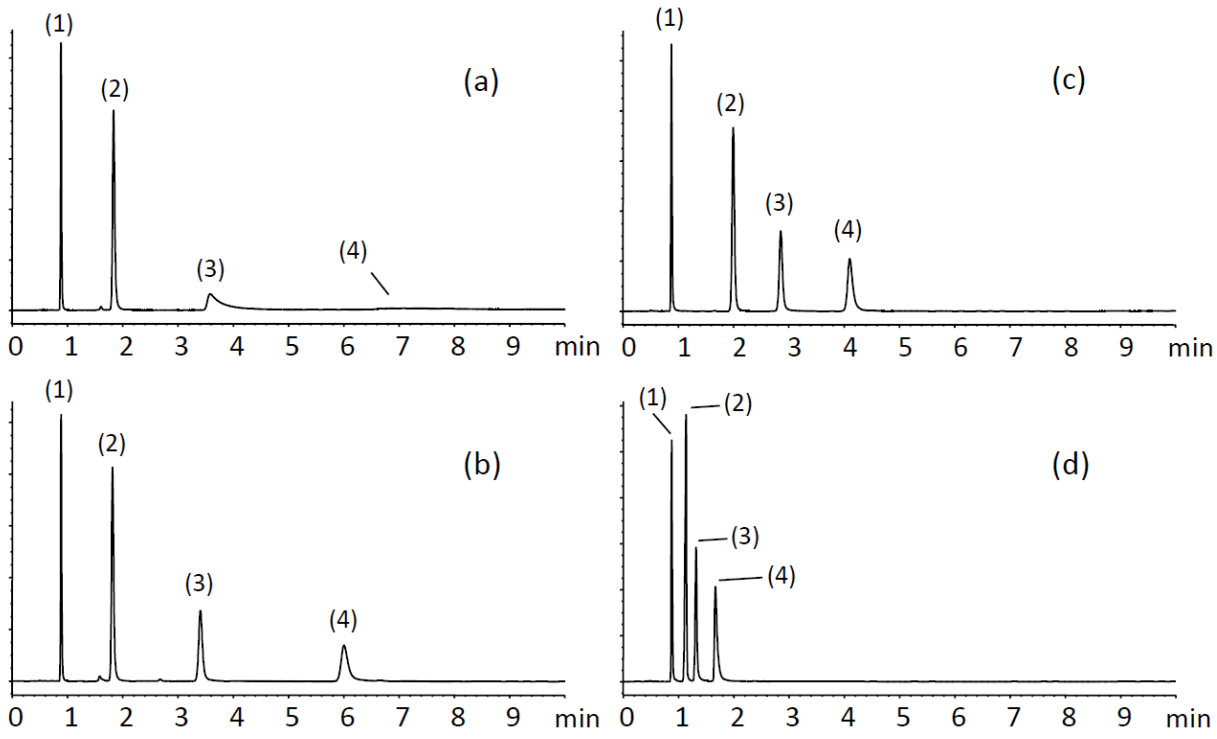


Fig 7

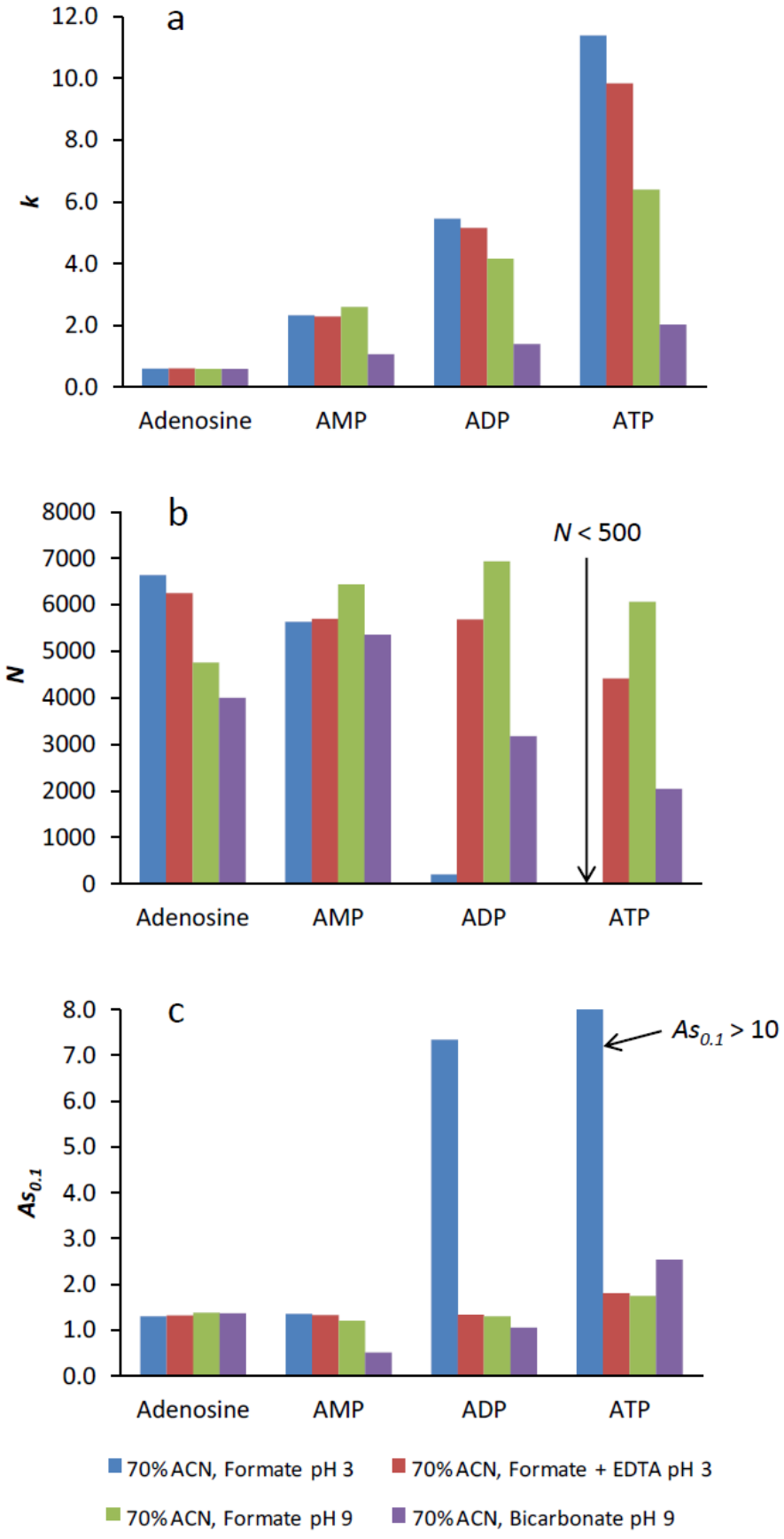

a
12.0
10.0
8.0
6.0
4.0
2.0
0.0
k
Adenosine
AMP
ADP
ATP
b
8000
7000
6000
5000
4000
3000
2000
1000
0
N
N < 500
Adenosine
AMP
ADP
ATP
c
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
As0.1
As0.1 > 10
Adenosine
AMP
ADP
ATP
70% ACN, Formate pH 3
70% ACN, Formate + EDTA pH 3
70% ACN, Formate pH 9
70% ACN, Bicarbonate pH 9

Figure 8

Legend to Figures

Fig. 1 Structures and predicted logD values at $_{w}^{w}$pH 3 and $_{w}^{w}$pH 9 for the probe solutes.

Fig. 2 The effect of decreasing acetonitrile (increasing water content) in the injection solvent on column efficienty ($N_{5\sigma}$) using BEH HILIC for a selection of basic solutes. Column dimensions 100 x 2.1 mm, 1.7 µm particles. Adenine, cytosine, nortriptyline and procainamide were at 20 mg/L whereas TMPAC was at 50 mg/L. Injection volume 1 µL. Conditions: Mobile phase 95% ACN containing 5 mM overall ammonium formate $_{w}^{w}$pH 3.

Fig. 3 Experimentally measured influence of increasing injection volume from 1-20 µL using (a) 95% ACN, (b) 90% ACN and (c) 80% ACN on column efficiency ($N_{5\sigma}$). Experimental conditions and mobile phase as Fig. 2.

Fig. 4 Predicted percentage losses in efficiency ($N_{5\sigma}$) with increasing injection volume from 1-20 µL based on equation (1). Solute annotation same as Fig. 3.

Fig. 5 Retention, peak shape and efficiency ($N_{moments}$) of 3,4,5-THBA on BEH amide using (a) 80% ACN, 5 mM overall ammonium formate $_{w}^{w}$pH 3 (b) 80% ACN, 5 mM overall ammonium formate $_{w}^{w}$pH 3, 0.1 mM EDTA (c) 80% ACN, 2.5 mM overall ammonium formate, 1.25 mM overall ammonium citrate dibasic adjusted to $_{w}^{w}$pH 3 with formic acid (Total 5 mM ammonium cations) (d) 90% ACN, 2.5 mM overall ammonium formate, 1.25 mM overall ammonium citrate dibasic adjusted to $_{w}^{w}$pH 3 with formic acid (Total 5 mM ammonium cations). Detection UV at 270 nm (80 Hz).

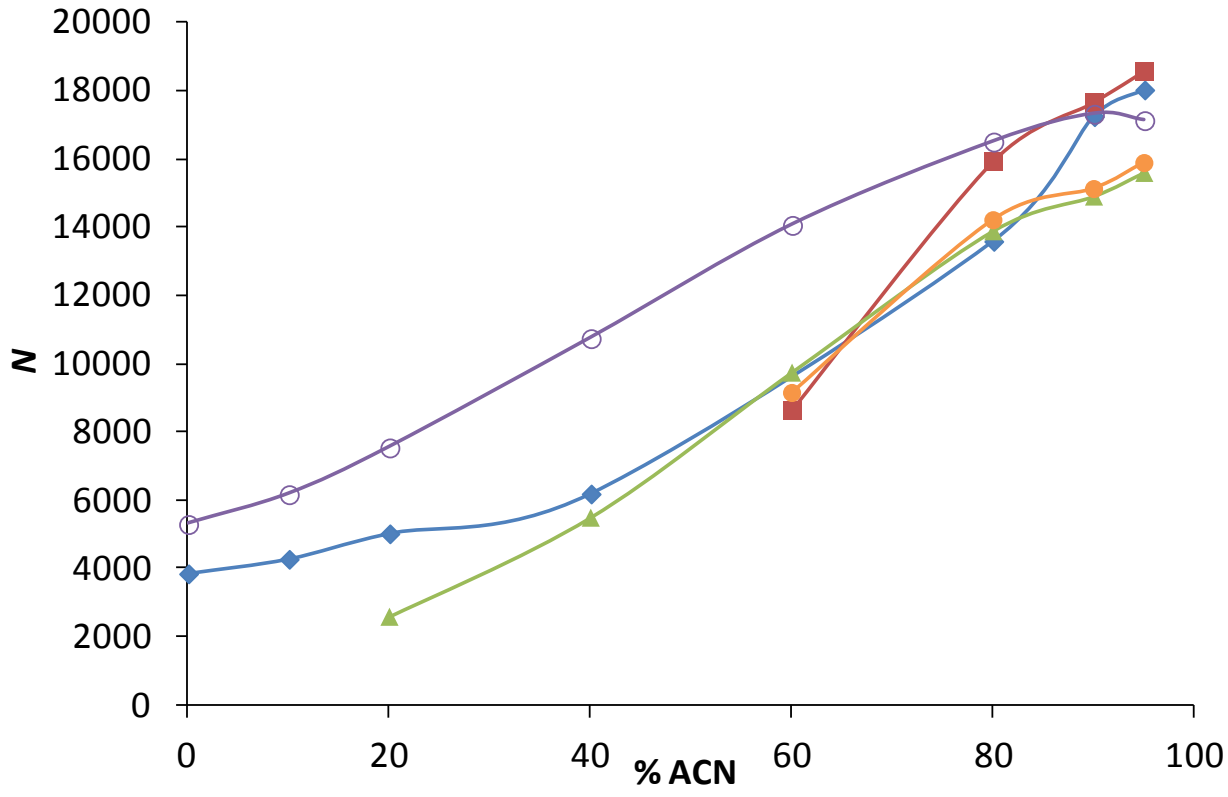
Fig. 6 Separation of catecholamines and structurally related basic compounds on (a) BEH Amide, 90% ACN, 5 mM overall ammonium formate pH 3 (b) BEH HILIC, 90% ACN, 5 mM overall ammonium formate pH 3 (c) BEH HILIC, 90% ACN, 2.5 mM overall ammonium formate, 1.25 mM overall

ammonium citrate dibasic, adjusted to pH 3 with formic acid (5 mM overall ammonium cations). Elution order: (1) Halostachine (2) Phenylephrine (3) Dopamine (4) Epinephrine (5) Norepinephrine. Other conditions: λ = 215 nm (80 Hz).

Fig. 7 Separation of nucleotides on BEH Amide (a) 70% ACN, 5 mM ammonium formate pH 3 (b) 70% ACN, 5 mM ammonium formate, 0.1 mM EDTA, pH 3 (c) 70% ACN, 5 mM ammonium formate pH 9 (d) 70% ACN, 5 mM ammonium bicarbonate pH 9. Elution order: (1) Adenosine (2) Adenosine Monophosphate (3) Adenosine Diphosphate (4) Adenosine Triphosphate. Other conditions: λ = 260 nm (80 Hz).

Fig. 8 Retention factor (a), column efficiency (b) and asymmetry factor (c) measurements for nucleotides on a BEH amide column using 70% ACN containing various buffer components. Column efficiency was calculated using the method of statistical moments.

Fig 2


20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
N
0
20
40
60
80
100
% ACN
Nortriptyline
Procainamide
Adenine
Cytosine
TMPAC