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14 Influence of metals in the column or instrument on performance in hydrophilic interaction
15 liquid chromatography.
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38 ## Abstract

39 A method is proposed for measuring the relative contribution of extracolumn and column
40 effects to the detrimental interactions which occur between metal-sensitive solutes and the
41 complete HPLC system. The method involves the substitution of a length of narrow bore
42 silica tubing for the column and measuring the extracolumn contribution, which is
43 subtracted from the total bandspreading measured with a column in place to yield the
44 column contribution. The investigation focussed on HILIC separations, which have been
45 relatively little studied compared with similar effects in RPLC. Metal-solute interactions can
46 lead to tailing peaks and reduced sensitivity or even irreversible adsorption of particularly
47 challenging solutes such as mono-, di- and triphosphorylated nucleotides, which show
48 strong interactions between their phosphate groups and metals. A deactivated HILIC
49 column, treated by a vapour deposition procedure gave generally good results when using
50 high pH (pH 9.0) mobile phases, which suppress the effects of metals. The addition of
51 metal complexing agents such as citrate at low millimolar concentration gave further
52 improvements in peak shape at high pH, and even micromolar concentrations of citrate or
53 medronic acid showed good results. These lower concentrations are more favourable for
54 LC-MS. Addition of the higher concentration of citrate gave acceptable results for the
55 nucleotides even at low pH (pH 3.0). With the standard UHPLC instrument used, loss of
56 efficiency due to metal solute interactions was 25 % or less, with most losses due to
57 interactions with the column, although this result will depend on the condition and design
58 of the instrument, which is easily assessed by the proposed procedure.

59

1. Introduction.

Hydrophilic interaction chromatography is now a valuable technique for the separation of polar and ionised compounds, which are poorly retained by RPLC. It has many applications in the analysis of compounds of pharmaceutical, biomedical and clinical relevance; its mechanism is increasingly understood [1-3]. It has long been known that interaction of metals from the column hardware/packing or the HPLC instrumentation with the solute can be seriously detrimental to separations in HPLC. Whereas most of the earlier studies have concerned RP separations [4,5], problems also occur in HILIC [6]. Solutes containing vicinal hydroxyl groups (e.g. trihydroxybenzoic acid or catecholamines) can interact with metals leading to tailing peaks and sometimes irreversible solute adsorption [7]. Particularly strong interactions can take place between metals and compounds with a phosphate functionality such as the nucleotides adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Severely tailed peaks can result both in RP and HILIC separations [6,8-10]. Problems of peak shape and irreversible adsorption in HILIC [6] were found to increase for nucleotides with multiple phosphate groups. The phosphate functionality of phosphoproteins is known to have an affinity for Fe^{3+} as exploited in iron-metal affinity chromatography enrichment strategies [11] .

A number of alternatives to stainless steel used to manufacture column hardware or instrument components have been explored, including titanium, which has been regarded as being more inert compared with stainless steel HPLC systems. However, titanium based systems can still cause problems, such as corrosion when using anhydrous mixtures of acetonitrile and methanol, or with pure short chain alcohols [12]. It was proposed that titanium cations, immobilised in the stationary phase, can form complexes with chelating solutes such as fluoroquinones. In an earlier study, a column with titanium frits showed significant epimerisation and elimination of the S-epimer of the ethylsulphoxide of tipredane, a sensitive indicator of metal interactions in RP separations [5]. These authors also proposed the ratio of the efficiency of 2,7-dihydroxynaphthalene divided by that of 2,3-dihydroxynaphthalene (DERT value) as a routine test for metal activity. The latter can chelate metals but the former cannot; however, these solutes are too hydrophobic to be useful in tests of HILIC systems.

Mobile phase additives have been employed to reduce metal-ion adsorption, some recent studies have also used the nucleotides as test compounds [13]. Ethylenediamine has been employed as additive, but can be strongly retained on the column and can cause

ion suppression effects in LC-MS. Furthermore, slow or incomplete removal of adsorbed EDTA may generate changes in column selectivity and performance with time (see below). The use of low concentrations of medronic acid, which contains two phosphate groups flanking a central carbon atom, was investigated in HILIC mode [13]. Gradient elution separations were used with solvent A 10 mM ammonium acetate pH 9.0 in water and solvent B 10 mM ammonium acetate in ACN. Medronic acid was spiked into each solvent to generate a concentration typically of 5 μM. Unlike EDTA, the additive was found not to produce ion suppression, nor to be difficult to remove from the column or HPLC system. An Agilent Poroshell HILIC -Z column was used. Similar good results were obtained with phosphorylated pesticides or peptides. The authors reported that performance of the separation with the mobile phase additive was inferior at low pH..

The performance of medronic acid and citrate as mobile phase additives has also been studied in the RP-LC analysis of peptides that possess negative charge bearing groups (such as aspartic acid or glutamic acid) [14]. It was found that in gradient analysis using 0.1 % formic acid in water-acetonitrile in conjunction with a Waters CSH C18 column, citric acid at a concentration of 1 ppm or medronic acid at a concentration of 5 μM produced reduction in the US Pharmacopeia tailing factor of as much as 40% for these peptides. Care was taken to prevent irreproducible results in these studies by e.g monitoring performance of sequential injections over a period of in some cases 67 hours. Repeated injections of solute over a short time period may deactivate metal sites by strong adsorption of part of the sample, rendering a more inert surface for subsequent injections.

A further alternative procedure is to create a so-called “bio inert” metal free fluidic pathway by treating stainless steel with a non-interacting material. Waters have introduced a hybrid organic-inorganic surface technology to limit analyte-metal interactions, applied using vapour deposition on column and instrument components. This technology is based on the ethylene bridged siloxane polymer of composition $[O_{1.5}SiCH_2CH_2SiO_{1.5}]_n$, similar to that which the company has used as the base material for the preparation of inert column packings [15]. The performance of the treatment was evaluated in RP separations. The surface was reported to be more hydrophilic than PEEK (an alternative inert coating material), making it less prone to hydrophobic adsorption. Titanium frits gave rise to considerable loss of ATP (used as a test solute) by adsorption. However, adsorption was almost completely prevented on surfaces treated by the vapour deposition method. Frits were stated to account for about half the metal surface area accessible to the analyte in a typical UHPLC system. Using a BEH C18 column in conjunction with a mobile phase of aqueous ammonium acetate (10 mM, pH 6.8) it was demonstrated that ATP was

129 completely adsorbed when using a standard UHPLC system and standard column,
130 partially adsorbed with severe peak tailing on a standard UHPLC system with treated
131 column, and eluted with reasonable peak shape on a treated UHPLC system with treated
132 column. The authors investigated the effect of pH on peak shape using a treated UHPLC
133 system and either a standard or a treated column. It was found that raising of the pH from
134 4.5 to 6.8 to 8.5 of the 20 mM ammonium acetate mobile phase caused improvements in
135 performance for this RP separation. A rationale for this behaviour is that the oxide layer on
136 316 stainless steel has been estimated to have an isoelectric point of ~ 7 [16] . Thus at pH
137 9.0, surface charge is reduced and metal adsorption of phosphorylated peptides/
138 phophorothioated oligonucleotides was reduced. Rather similar results were obtained for
139 all metal complexing solutes studied, indicating that a simple nucleotides test may give
140 results representative of a wide range of compounds.

141 Sandra and co-workers [17] evaluated a new zwitterionic HILIC stationary phase
142 (Poroshell 120 with 2.7 μm particles) which has a PEEK lined column option with PEEK
143 frits for particularly challenging compounds. This is a different approach to vapour
144 deposition procedures. Columns were evaluated on both conventional and "bioinert"
145 systems. Nucleotides were separated by gradient analysis using decreasing
146 concentrations of acetonitrile in 10 mM ammonium acetate pH 9.0. The authors showed
147 poor results for AMP, ADP and ATP on a conventional column format using either a
148 conventional or a bioinert HPLC system but improved results using the PEEK lined
149 column, especially with the bioinert system. Results were very similar for equivalent
150 nucleotides of guanosine, cytidine and uridine. Different mobile phase conditions were not
151 investigated in this study, nor the use of complexing additives. PEEK systems may
152 however be unsuitable for use with certain solvents e.g. tetrahydrofuran. The contribution
153 of the instrument to poor peak shape was not formally assessed.

154 Estimation of the instrumental contribution to band broadening of these sensitive
155 solutes is important as this contribution is likely to vary from instrument to instrument and
156 even on the same instrument dependent on the history of its use. Few, if any studies have
157 used a quantitative approach to estimate this contribution, especially in HILIC separations.
158 Such an estimation might assist a decision on whether the purchase of a new treated or
159 bioinert HPLC system is justified, or whether the replacement of the column alone with a
160 more inert version would suffice.

161 The aims of the present study can be summarised as:

-to focus on detrimental metal interactions in HILIC as this mechanism may present greater or at least different challenges than RP chromatography. Most previous studies have used RP conditions.

-to measure separately and quantitatively the instrumental contribution to band broadening of these sensitive solutes to determine the relative contribution of the instrument and column.

-to use isocratic elution for evaluation rather than gradient analysis as the latter may show optimistic results due to gradient compression of sample bands (the rear of a peak experiences a stronger eluent than the front of the peak [18]).

-to present quantitative column efficiency data for various mobile phase conditions.

-to investigate the performance of a new deactivated HILIC column.

2. Experimental

Experiments were performed using a 1290 ultra-high performance liquid chromatograph (UHPLC, Agilent, Waldbronn, Germany) comprising a binary pump, autosampler and photodiode array UV detector (0.6 μL flow cell) set at 260 nm with data collection rate 160 Hz. The instrument was fitted with the ultra-low dispersion needle seat and flow-through needle options. A few experiments were conducted on an Agilent 1100 system using a variable wavelength detector (1.0 μL flow cell) and data collection rate 14 Hz. Columns (all 10 cm x 0.21 cm i.d.) were: BEH amide (totally porous, Waters, Milford USA) treated (Premier) and non-treated, particle diameter 1.7 μm, pore diameter 130 Å, surface area 185 m^2/g. For measurement of instrumental contribution to band broadening, the column was replaced with a 10 cm x 50 μm ID PEEKSIL tube of PEEK coated fused silica (Trajan Scientific, Milton Keynes, U.K.). The temperature of the oven was set at 30 ^{o}C. The mobile phase was ACN-water (70:30 v/v), containing various additives, at a flow of 0.40 mL/ min. $_{w}^{w}$ pH was measured before addition of the organic solvent. Solutes were obtained from Sigma-Aldrich (Poole, U.K.). Mobile phase solvents and additives ACN (gradient UV grade), ammonium formate (AF), ammonium acetate (AA) diammonium citrate (AC) formic acid (FA) and ammonia solution (all MS grade) were from Fisher (Loughborough, U.K.).

The HPLC systems were initially washed using the PEEK tubing in place of the column for 10 hours with 0.1 % orthophosphoric acid in 70-30 ACN-water (v/v), 10 hours with 5 mM ammonium formate pH 3.0 in 70-30 ACN-water, and 10 hours with 90-10 ACN-

195 water (v/v). Mobile phase solvents were made up in a single reservoir by weight rather
196 than directly by volume. After each set of results was obtained using a particular mobile
197 phase, the column was washed for 1 hour with 90-10 ACN-water (v/v) and left to stand in
198 this solution for at least 12 hours. The new mobile phase was pumped through the PEEK
199 tubing or column (dependent on the experiment) for 2 hours at 0.4 mL/min. prior to solute
200 introduction [19,20]. Solutes were injected in duplicate or triplicate at 10 minute intervals
201 and further repeat injections were performed after a period of 2 hours of purging the
202 column with mobile phase to check the reproducibility of the results. Under the specified
203 conditions, no evidence was seen for "loading effects" as observed previously for peptides
204 or oligonucleotides in RP separations [14,21]. Injection volume was 1.0 μL for the UHPLC
205 system. t_M was determined by injection of a solution of toluene. All solutes were injected at
206 a concentration of 20 mg/L dissolved in the mobile phase.

207 Log D_{ow} (the ratio at equilibrium of the concentration of charged plus neutral solute
208 in octanol devided by their concentration in water) and solute charge were estimated as
209 the average of the values given from the programs Marvin (ChemAxon, Budapest,
210 Hungary) and I-Lab (ACD, Toronto, Canada).

211

212 **3. Results and Discussion**

213 *3.1 Determination of instrumental contribution to peak broadening-principles of the*
214 *method.*

215 The UHPLC system was first cleaned as outlined above. Passivation with concentrated
216 solutions of strong acids or complexing agents (EDTA) was not carried out because these
217 reagents may only slowly be removed from the systems, which return to their initial state
218 after a period of time, leading to irreproducible results. The UHPLC system used for these
219 experiments had been employed mostly for the analysis of simple pharmaceutical
220 standard solutions; a new injection needle and needle seat were fitted prior to the
221 experiments but the instrument was composed of standard components that were not
222 treated other than as described above.

223 The peak broadening caused by the instrument was measured by substituting the
224 column with a fused silica lined PEEK tube which generated sufficient pressure under the
225 conditions used to ensure a reproducible flow from the pump. The total band spreading
226 can be expressed as the variance in volume units $\sigma^2_{tot,v}$ where

227

228 $$\sigma^2_{tot,v} = \sigma^2_{col,v} + \sigma^2_{extra\ col,v} \qquad [1]$$

229
230 where the subscript col refers to the band spreading of the column itself and the subscript
231 extra col. refers to the band spreading produced by the injector, the connecting tubes and
232 the detector of the 1290 instrument. σ_v can be obtained from σ_t (measurement in time
233 units) from the 5 σ_t peak width measured at 4.4 % of peak height using the relationship:
234
235 $\sigma_v = \sigma_t . F$ [2]
236
237 where F is the volumetric flow rate.
238 Fig.1a shows the plots obtained by injection of 20 ppm solutions of adenosine, AMP, ADP,
239 and ATP into the PEEK tube using a mobile phase of 2.5 mM AF, 1.25 mM dibasic AC
240 adjusted to pH 9.0 with ammonia in 70: 30 ACN-water, v/v. This mobile phase was
241 subsequently found to give the best peak shapes with a column in place. It is clear that the
242 peak profiles are reasonably symmetrical with relatively little tailing of the injection band.
243 Fig. 1b shows similar plots using 5 mM AF adjusted to pH 3.0 with FA in 70:30 ACN-water,
244 v/v. This mobile phase was subsequently found to be the one that generated the worst
245 peak shapes with a column in place. While the peak shape for adenosine was similar to
246 that in Fig 1a, the profiles for AMP, and especially ADP and ATP, showed increased peak
247 broadening and tailing. Thus, the results mirror the findings for detrimental *column*
248 performance (see below). Table 1 gives a quantitative view of the broadening that occurs
249 for the instrument for toluene, adenosine, AMP, ADP and ATP in 4 different mobile
250 phases. For the pH 5 mM AF pH 3.0 buffer as used in Fig. 1b, the values of $\sigma^2_{\text{extra col, v}}$ for
251 these 5 solutes were 2.4, 3.5, 6.0, 130 and 520 μL^2 respectively confirming the increase in
252 peak broadening for solutes with multiple phosphate groups observed visually in Fig. 1b.
253 For the pH 9.0 AF buffer, the results were considerably improved especially for ADP and
254 ATP with results for the 5 solutes of 2.4, 3.5, 7.0, 28 and 40 μL^2 respectively. These
255 results are in line with the hypothesis that the charge on metal ions decreases as mobile
256 phase pH increases [16]. This effect should thus decrease metal interactions with
257 negatively charged solutes as pH increases. However, Table 2 shows that the negative
258 charge on the nucleotides increases somewhat with increasing pH over the range pH 3.0
259 to pH 9.0; for instance the charge on ATP increases from -1.8 to -3.4. This effect should
260 conversely somewhat increase metal solute interactions as pH increases. For the 1.25 mM
261 citrate mobile phase at pH 9.0 in Fig.1a the equivalent values of $\sigma^2_{\text{extra col, v}}$ were 2.5, 3.5,
262 5.8, 15 and 20 μL^2 for the 5 solutes respectively, which are clearly smaller still than with

263 the same mobile phase but without the complexing agent. Note however that the values
264 for the non-polar compound toluene (2.3- 2.5 μL^2) and for adenosine (no phosphate
265 groups, 3.5 μL^2) were very similar in all mobile phases (Table 1). Of course, instrumental
266 contribution to peak broadening may take place merely due to the system variance of the
267 instrument and connectors rather than adverse adsorption, which is reflected in the small
268 but appreciable $\sigma^2_{extra\ col,\ v}$ value for toluene. Clearly however, the peak variance for
269 toluene is much smaller than that for ADP and ATP in any of the mobile phases in Table 1
270 (see discussion below).
271 Fig. 1c shows the bandspreading produced by the same solutions on an Agilent
272 1100 system, cleaned as for the 1290 instrument; note the x-axis is 10 times the scale of
273 Fig. 1a and 1b. The peaks are much broader on this older instrument which is not
274 optimised for use with small internal diameter columns. The bandspreading for toluene,
275 adenosine, AMP, ADP and ATP was 64, 66, 112, 1665, and 1126 μL^2 respectively using
276 5mM AF buffer pH 3.0 in 70 % aqueous ACN. These values are much higher than those
277 for the 1290 instrument, reflecting particularly for toluene and adenosine, the normal
278 effects of instrumental band broadening. Nevertheless the still higher values for ADP and
279 ATP follow a similar pattern to those on the 1290 instrument, and are indicative of metal
280 interactions. We have previously shown that the effects of instrumental band spreading of
281 conventional instruments with 4.6 mm ID columns can be less severe than those of
282 UHPLC instruments with 2.1 mm columns [22]. Thus the method can also be used to
283 determine the total effects of metals and general band broadening also on conventional
284 HPLC instruments with wider columns. If a suitable column diameter and conditions
285 leading to appropriate *k* values are used, the contribution of metal effects alone to
286 bandspreading can again be estimated on such instruments.
287
288 *3.2 Determination of column contribution to band broadening*.
289 With the column in place, the experimental protocol for changing the mobile phase was
290 strictly followed (see Experimental) in order to achieve reproducible results and counteract
291 any memory effects of use of the previous mobile phase. Some data obtained using a
292 particular mobile phase were repeated on a different day e.g. to determine whether use of
293 higher concentrations of citrate persist on the column, but none was found. This is in
294 agreement with results in a detailed RP study [15] which claimed that multivalent acid
295 washes improved the recovery and peak shape of acid analytes but that the effect was
296 only temporary. This result contrasts with the use of EDTA, which we have found in

297 preliminary studies to persist on the column. Using the injection protocol (see above) there
298 was little evidence of “sample conditioning effects” where peak shape improves on rapid
299 re-injection of the same sample with a given mobile phase, as found for a RP separation of
300 oligonucleotides [21] . These effects are likely to depend also on the separation
301 mechanism, the particular mobile phase and column used, and the solutes. They may be
302 caused by injection of a new sample on the still tailing peak of solute from the previous
303 sample, which temporarily blocks active sites. They are likely to be less significant on a
304 deactivated column as mostly used in the present experiments, which gave much
305 improved results in the nucleotide test of the present study than a standard column of the
306 same stationary phase (see below). Leaving a period of time between each injection (10
307 minutes) also minimises such effects.

308 Fig. 2a shows a chromatogram of the 4 test compounds using a deactivated amide
309 column (Waters Premier BEH amide) and a high pH mobile phase (as Fig. 1a) containing
310 a relatively high concentration of complexing agent (1.25 mM citrate). This column along
311 with some others produced by Waters is based on a bridged ethyl hybrid inorganic/organic
312 substrate which confers high pH stability, in this case over the claimed pH range of 2-12.
313 Columns based on a conventional silica matrix are likely to be much less stable with an
314 upper pH limit of 6-7. The Agilent HILIC Z column studied by others [13,17] also has a high
315 pH limit of 12 and may be based on similar hybrid technology. No detrimental effect of the
316 use of pH 9.0 on the Waters hybrid materials (retention or efficiency changes) were
317 observed in the course of this study. The solutes are eluted in order of increasing numbers
318 of phosphate groups. Table 2 indicates increasing hydrophilicity for adenosine, AMP, ADP,
319 ATP with log D_{ow} values -1.7 to -12.7 along the series at pH 9.0. (Note that large negative
320 log D_{ow} values indicate high hydrophilicity). Ionic interactions, which increases with the
321 number of phosphate groups in the solute, clearly contribute to retention: these
322 interactions increase as the charge on a solute increases at a given pH. For instance, the
323 charge on adenosine, AMP, ADP, ATP is 0, -2.0, -2.4, and -3.4 at pH 9.0. Ionic
324 interactions are however, likely to be suppressed at high pH by the reduced ionisation of
325 metal oxides such as chromium, nickel, cadmium and iron. They are also reduced by the
326 presence of complexing agents such as citrate or medronic acid. These reagents are
327 highly hydrophilic and remain negatively charged at pH 9.0 (see Table 2). Caution must be
328 exercised in use of Table 2 as data are based on the properties of compounds in pure
329 water, rather than 70 % ACN for which data is not available.

330 Having calculated the contribution of the instrument to peak broadening it is now
331 possible to determine the contribution of the column alone. This is done by measuring the

332 total variance σ^2 and subtracting the extracolumn variance. Table 1 shows data for 4
333 different mobile phases using the deactivated column. For ATP using the pH 9.0 high
334 citrate concentration mobile phase, the total variance is ~ 100 μL^2 and the extracolumn
335 variance 20 μL^2 suggesting the column variance is ~ 80 μL^2 . This indicates a true
336 column efficiency using the 5 σ peak width method of 12900 plates (see the penultimate
337 column on the right hand side of Table 1, $N_{true\ col.}$). The uncorrected efficiency is 10,400
338 plates representing a 20 % loss of efficiency due to metal effects (and extra column
339 dispersion). Note this mobile phase gave the best performance for ADP and ATP. For the
340 same mobile phase (1.25 mM citrate) at pH 3.0, the extracolumn variance was larger at 98
341 μL^2 for ATP. However, this value is counterbalanced by a proportionally much higher value
342 of 2400 μL^2 for the total variance for the deactivated column and instrument at pH 3.0,
343 leading to a loss in efficiency of only 3.7 %. These results suggest that the interaction of
344 nucleotides with column metals replicates their interaction with extracolumn metals.
345 Clearly, the proposed method also takes into account peak broadening due to instrumental
346 dead volume (in addition to metal interactions), which is large only at low *k* values. Thus
347 the % loss in efficiency for toluene is ~ 50 % in each mobile phase; however toluene has a
348 retention factor of effectively zero, as it is often used as a void volume marker in HILIC
349 [23]. The effect of instrumental band spreading as measured by toluene (no solute-metal
350 interactions, variance ~2.5 μL^2) is small compared with the peak variance of retained
351 solutes such as ATP (k ~4 in AF/AC pH 9.0) and smaller still in the other mobile phases
352 shown in Table 1, which give greater retention and thus broader chromatographic peaks.
353 Therefore the measurement of peak broadening using ATP is almost entirely due to metal-
354 solute interactions.
355 While it appears that most of the band broadening of metal-interactive solutes takes
356 place due to the column, and in some cases particularly on frits, which have a large
357 surface area [21], the relative contribution of column and instrument will also vary with the
358 condition of the instrument used. However, the proposed method provides a simple way of
359 assessing these contributions. The most detrimental influence of extracolumn interaction
360 with metals would be obtained in the situation where the column generates sharp
361 symmetrical peaks but the instrument itself contributes considerable broadening and tailing
362 to the injected profile. This might be possible for a contaminated or corroded instrument;
363 however the methodology suggested above can easily identify such a situation. This
364 situation may also be less common, in that instrument and column have been shown to

365 suffer from similar effects i.e. metal interactions would tend to lead to a broad extracolumn
366 injection profile and also strong adsorptive column interactions.
367 Clearly, it would be possible to substitute different solutes for the nucleotides which
368 were of particular interest, allowing the determination of the instrumental and column
369 contributions for those particular solutes. Of course, a reasonable value of k for such
370 solutes is required to minimise the normal effects of instrumental dead volume on column
371 efficiency.
372 Finally, the use of the 5-sigma method to characterise losses in column efficiency is
373 debatable in that it assumes peaks are Gaussian which is clearly not the case. However,
374 we found the measurements reproducible and straightforward to make. More sophisticated
375 methods have been proposed, but these also may not be accessible to the average
376 practitioner [24].
377
378 *3.3 Effect of mobile phase pH and complexing additives on column performance*.
379 3.3.1 Column efficiency effects.
380 Fig. 3a shows column efficiencies (uncorrected for instrumental band spreading) in a
381 variety of mobile phases at pH 3.0. The performance of the conventional BEH amide
382 column is poor using a simple 5 mM AF buffer, with very low efficiency even for AMP. Fig.
383 2b shows a chromatogram of the deactivated column with the test mixture in this mobile
384 phase, which shows some improvement, however, the peak of AMP is still noticeably
385 tailing, while ADP and ATP were irreversibly adsorbed even on this deactivated column at
386 pH 3.0 in the absence of complexing agents. Addition of 10 μM citrate to the mobile phase
387 at pH 3.0 hardly improved the efficiency with the deactivated column, although there is a
388 slight improvement for AMP and the marginal appearance of a peak for ADP (Fig. 3a). A
389 larger concentration of citrate additive (1.25 mM) produced a marked increase in the
390 efficiency for AMP and ADP, and the appearance of a peak for ATP (Fig. 3a and 3b). Thus
391 at pH 3.0, it seems that relatively high concentrations of metal complexing additives are
392 necessary to mediate metal-solute interactions even when using the deactivated column.
393 Using pH 9.0 conditions the conventional BEH amide column continued to give poor
394 performance. However, the deactivated column produced marked improvement in
395 efficiency over that for the same column at pH 3.0 (Fig. 4a). Performance of the
396 deactivated column was clearly better than that of the standard column at pH 9.0, giving
397 measurable efficiency also for ADP and ATP. Addition of micromolar concentrations of
398 citrate (10 μM) or medronic acid, however produced marked increases in efficiency on the
399 deactivated column compared with the simple AF buffer, resulting in these mobile phases

400 being suitable for use in LC-MS. While performance with medronic acid and citrate was
401 similar at this concentration, we noted longer stabilisation times (several hours) required
402 to produce consistent retention times with the former additive; this result may be
403 connected with the low concentration used compared with some other studies [2]. Fig. 4a
404 shows that further small improvements in efficiency were revealed with 1.25 mM citrate on
405 calculation of the 5 sigma plate counts, although these are hardly apparent from visual
406 comparison of the chromatograms Fig. 2c and Fig. 2a.
407 These results show that addition of complexing agents at pH 9.0 produces further
408 increases in efficiency, despite metal-solute interactions being reduced at this higher pH.
409 Metal-solute interactions appear to be still significant even this high pH. Fig. 4a shows that
410 use of 1.25 mM citrate produces acceptable effiency even for the conventional BEH
411 column, although this mobile phase may not be as suitable for LC-MS applications. The
412 difference is again hardly apparent by mere inspection of chromatograms 2a and 2d.
413 The contribution of extracolumn adsorption to peak shape remains low at pH 9.0,
414 with a maximum loss of efficiency due to the instrument of 20-25 % for the retained peaks
415 of ADP and ATP when using the 1.25 mM citrate buffer. The consistent loss in efficiency of
416 about 25 % for adenosine in all 4 mobile phases in Table 1 is due to the normal
417 bandspreading effect of the instrument on a peak of low k (~0.7) and not due to adsorptive
418 metal interactions. Peaks were consistently symmetrical for adenosine with USP tailing
419 factors <1.25 in all cases.
420 Finally, the advantage of the use of high pH in conjunction with pH-stable columns
421 is confirmed in Fig. 2e. Column efficiency using 5 mM AA at pH 6.5 was for adenosine,
422 AMP, ADP and ATP 9580, 4500, 220 and 430 theoretical plates, compared with at pH 9
423 (Table 1) 9390, 7650, 2420 and 887 respectively. Irreversible adsorption of ADP and ATP
424 at pH 6.5 is clearly shown by the small peak areas for these solutes, which is not shown at
425 pH 9 (Fig. 4b).
426
427 3.3.2 Peak area and "recovery".
428 The plots in Fig. 3a and 3b have a similar appearance showing that the effects of metal
429 interactions on peak shape or detection sensitivity (peak area) are similar at pH 3.0.
430 Clearly ADP and ATP are the most challenging solutes that are only detected at pH 3.0
431 when using 1.25 mM citrate as additive (Fig. 3b). The equivalent graphs at pH 9.0 show
432 greater differences between the peak area plots (Fig. 4b) and the efficiency plots (Fig. 4a).
433 Thus the conventional BEH amide column at pH 9.0 with 5mM AF gave reasonable peak
434 area for AMP, ADP and ATP but very low efficiencies. This result can be explained by

435 reversible strong adsorption of some solutes, giving extensive peak tailing but
436 nevertheless (eventual) release of much of the solute. Accordingly, the peak area plots at
437 pH 9.0 are rather similar to each other, whereas the efficiency plots are not and
438 demonstrate the added benefit of using complexing additives, even at pH 9.0. Clearly the
439 use of peak area alone is insufficient and inadequate to monitor metal interaction effects.
440 It is interesting to note that the (acceptable) performance in terms of peak area and
441 efficiency on the deactivated column are roughly similar for pH 3.0 with1.25 mM citrate and
442 pH 9.0 with ammonium formate (no citrate). This shows that use of low pH is still practical
443 e.g. to improve selectivity, although this higher concentration of complexing agent may be
444 detrimental to MS use. However, no detrimental effects of low mM concentrations of citrate
445 were noted in the present LC-UV study. It seems however, that complexing reagents may
446 still be required to obtain the best results, even at pH 9.0.
447

448 **4. Conclusions**

449 A method is proposed for separate estimation of the contribution of extracolumn and
450 column interactions to peak shape with metal sensitive solutes. The method involves
451 measuring the instrumental bandspreading by substitution of the column with a short piece
452 of 50 μm PEEK coated fused silica tubing (to generate some back pressure in the system)
453 and measuring the peak widths at 4.4 % of peak height (5σ method). The extracolumn
454 effects can then be subtracted from the total bandspreading of instrument and column to
455 leave the column effects. The contribution of normal instrumental dead volume
456 bandspreading, which does not involve metal interactions, can be minimised if desired by
457 avoiding solutes or mobile phase conditions (e.g. in HILIC, higher % water concentrations)
458 that generate low *k*.
459 A deactivated amide HILIC column gave generally good peak shape for the
460 searching nucleotide test compounds used at pH 9.0; results were considerably improved
461 compared with a standard column of the same stationary phase. Results on the
462 deactivated column were much improved over those at low pH (3.0) or at intermediate pH
463 (6.5). Micromolar concentrations of metal complexing agents (citrate or medronic acid)
464 added to the pH 9.0 mobile phase gave further improvements in peak shape. The best
465 results at pH 9.0 were obtained with low mM concentrations of citrate, which also allowed
466 reasonable analysis of the nucleotides even at pH 3.0. Isocratic elution was used
467 throughout as a more demanding test; gradient elution can give peak focussing effects that
468 partially conceal the influence of metal interactions

Lack of any loss in peak area due to metal-solute interaction cannot be taken as meaning these detrimental effects are absent. Deterioration in column efficiency is a much more sensitive indicator of metal effects. Absence of peak area loss may be explained by the presence of strong but reversible interactions.

With the instrument used (a standard UHPLC system) extracolumn metal interactions were found to mirror column interactions; column interactions were found to cause the most deleterious effects on efficiency. However, this result depends on the condition of the instrument, which can be easily assessed by the proposed method. Furthermore, whereas the chosen nucleotides may be representative of solutes that give strong metal interactions, substitution of any chosen solutes can be used to probe the extent of these interactions in a particular separation.

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Author contribution

David McCalley: conception, investigation and experimental work, technical aspects, data analysis, writing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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503

504 **6. Legend to Figures**

505 Fig. 1. Injection profiles on Agilent 1290 of 1 μL solutions of 20 ppm adenosine (blue),
506 AMP (red), ADP (green), ATP (pink) dissolved in mobile phase. Flow rate 0.4mL/min.
507 Detection UV at 260 nm. Oven temperature 30 ° C. Mobile phase a) 2.5 mM AF, 1.25 mM
508 dibasic AC adjusted to pH 9.0 with ammonia in 70: 30 ACN-water, v/v; b) 5 mM AF
509 adjusted to pH 3.0 with FA in 70: 30 ACN-water, v/v; c) injection profiles of 5 μL solutions
510 of the same solutes on Agilent 1100, mobile phase as for 1 b). Note the x-axis scale is 10
511 x greater in c) than in a) and b).

512 Fig. 2 Chromatograms on Premier amide column of 1 = adenosine; 2 = AMP; 3 = ADP; 4=
513 ATP using mobile phases 70:30 ACN-water containing: a) 5 mM AF +1.25 mM citrate pH
514 9.0; b) 5 mM AF pH 3.0:. c) 5mM AF + 10 μM citrate pH 9.0; d) as in a) with BEH amide
515 column; e) 5 mM AA pH 6.5 with Premier amide column. Other conditions as in Fig. 1.

516 Fig. 3. a) Column efficiency (5 sigma method) for adenosine (orange); AMP (grey); ADP
517 (yellow); AP (blue) using 70:30 ACN-water (v/v) containing various buffers at pH 3.0.
518 Column BEH amide standard or Premier BEH amide as indicated. b) Peak area for the
519 same columns and mobile phase conditions as in Fig. 3 a). Other conditions as Fig.1.

520 Fig. 4. a) Column efficiency data (5 sigma method) for same solutes (see Fig. 3) using
521 70:30 ACN-water (v/v) containing various buffers/additives at pH 9.0. Columns BEH
522 standard or Premier BEH amide as indicated. b) Peak area for the same columns and
523 mobile phase conditions as in Fig. 4 a). Other conditions as Fig.1.

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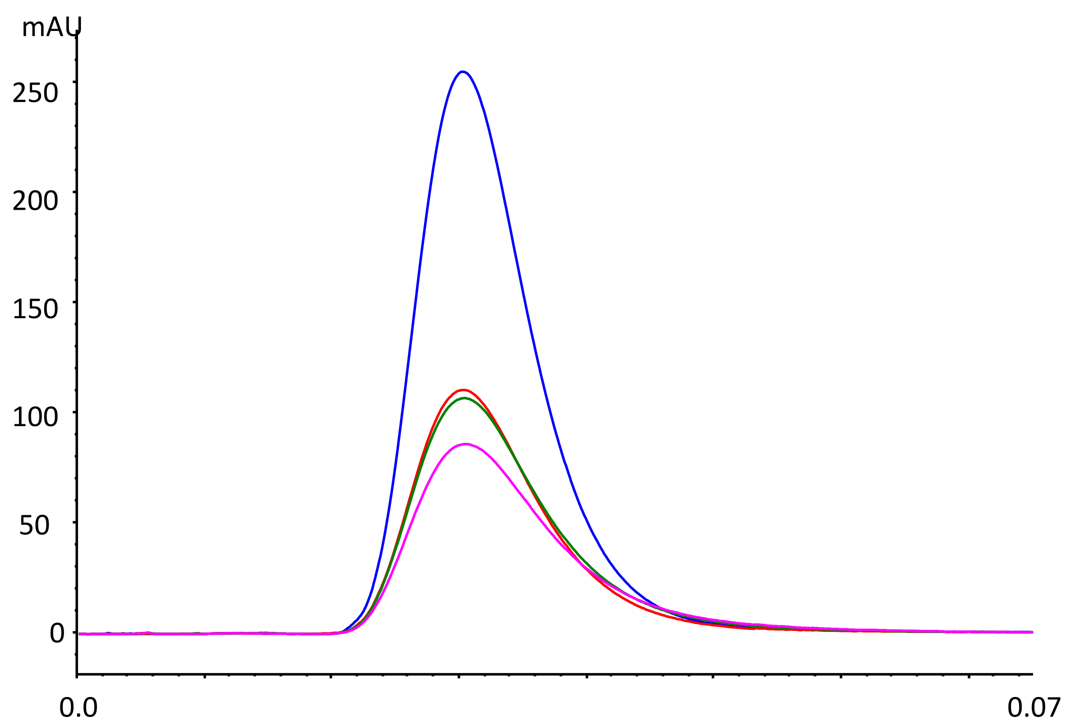

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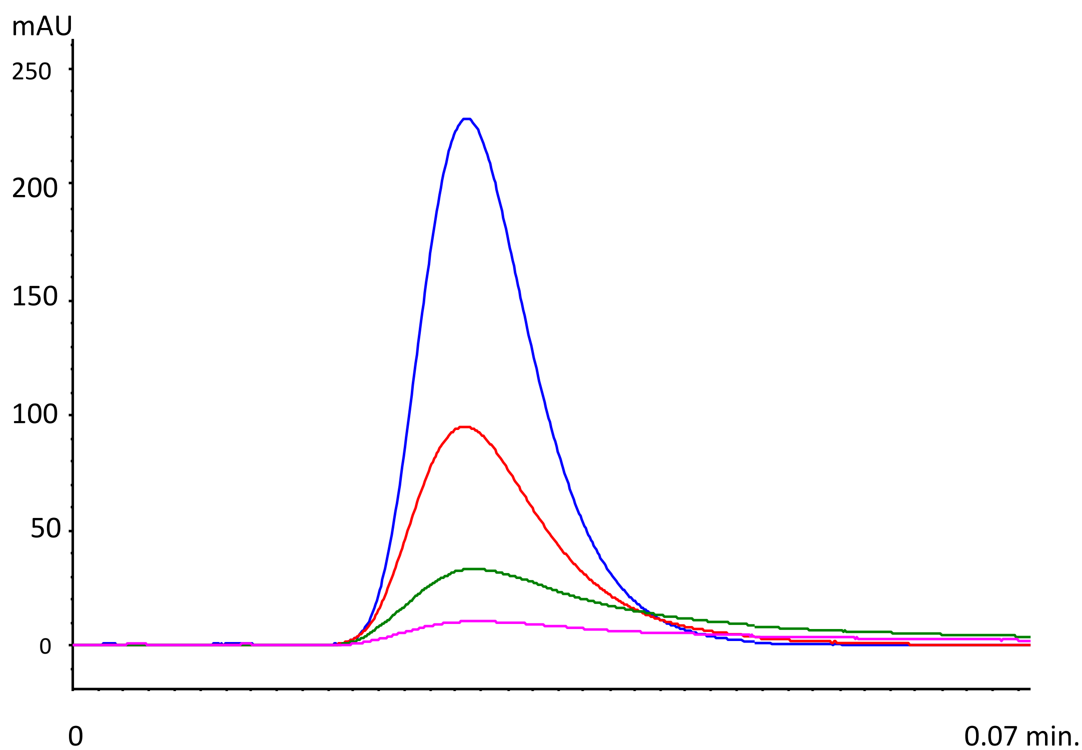

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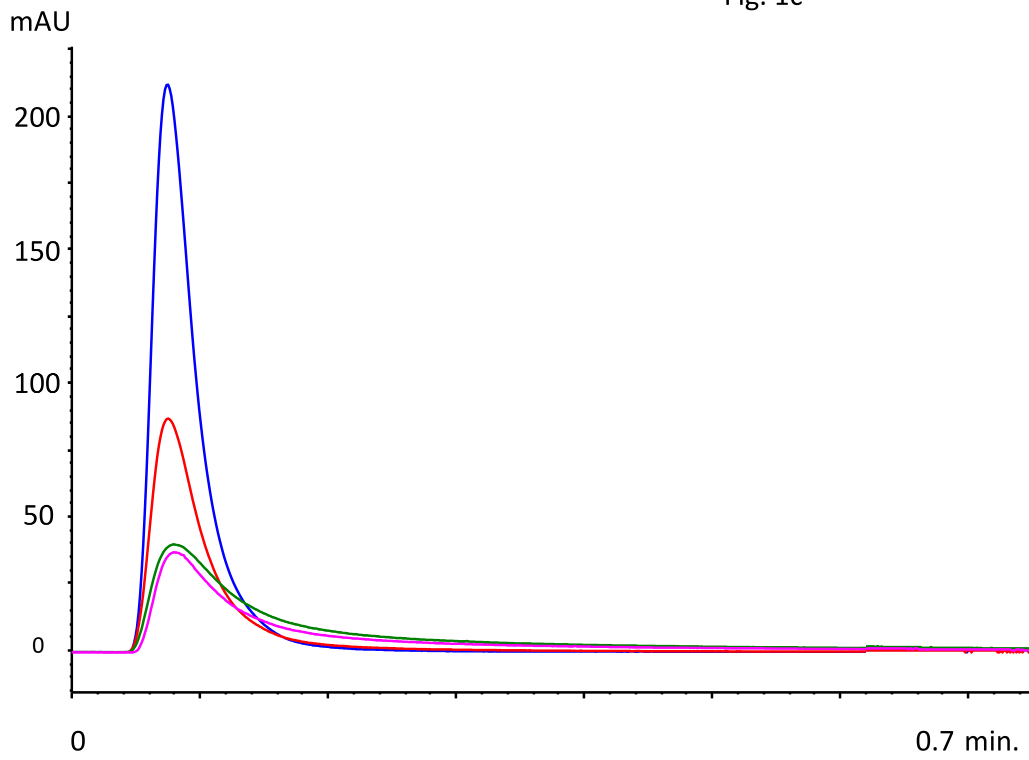

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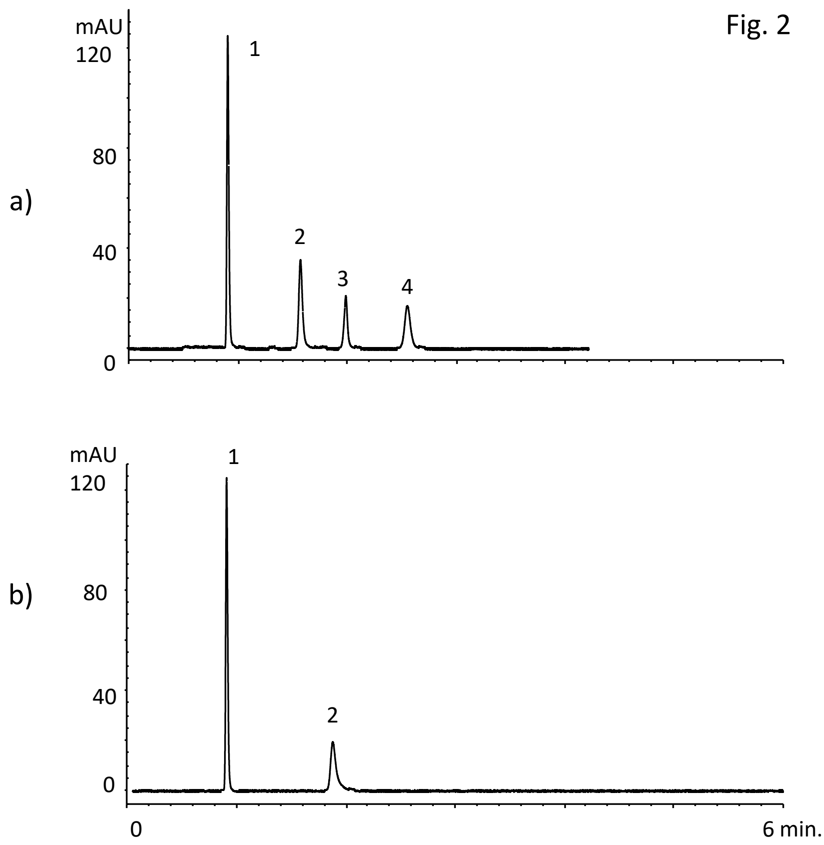

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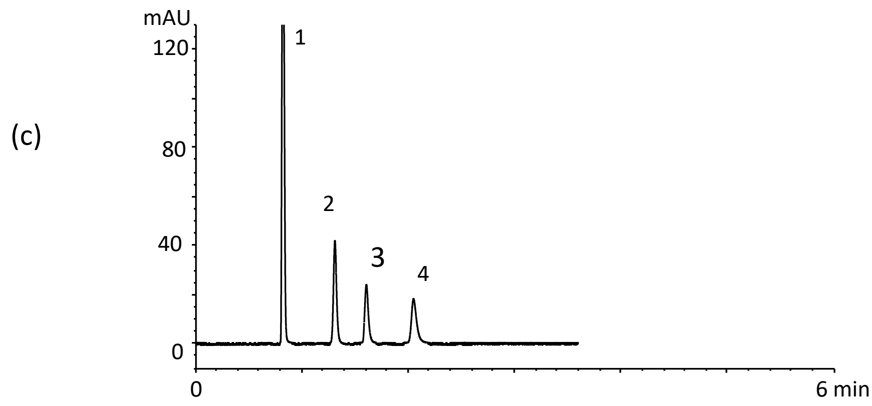

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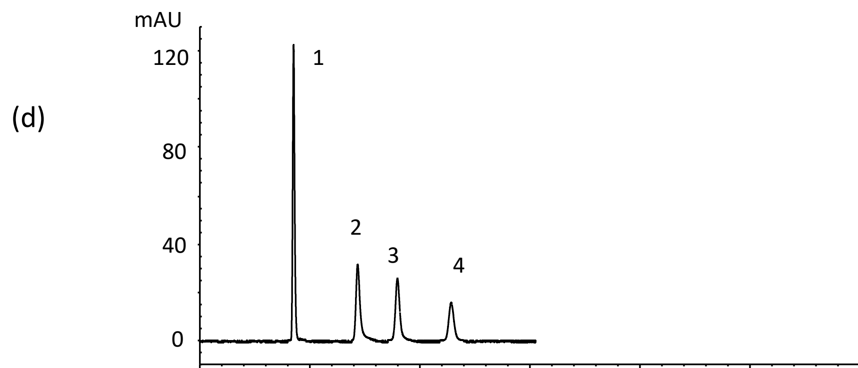

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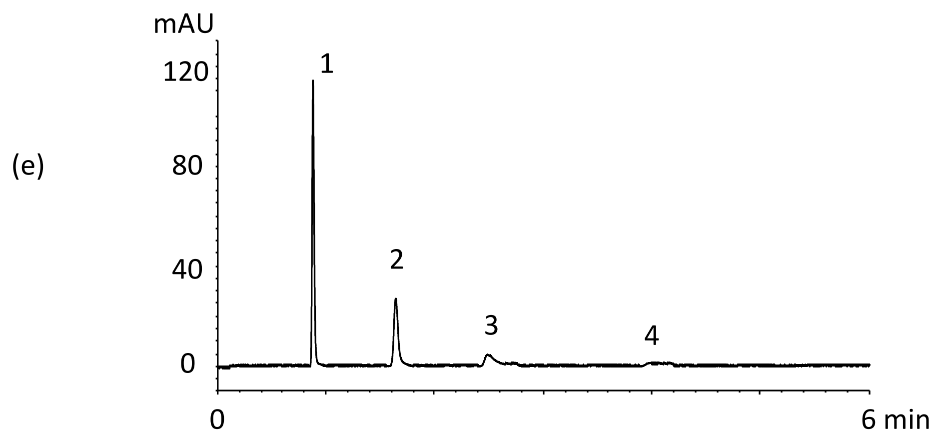

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Fig. 3a

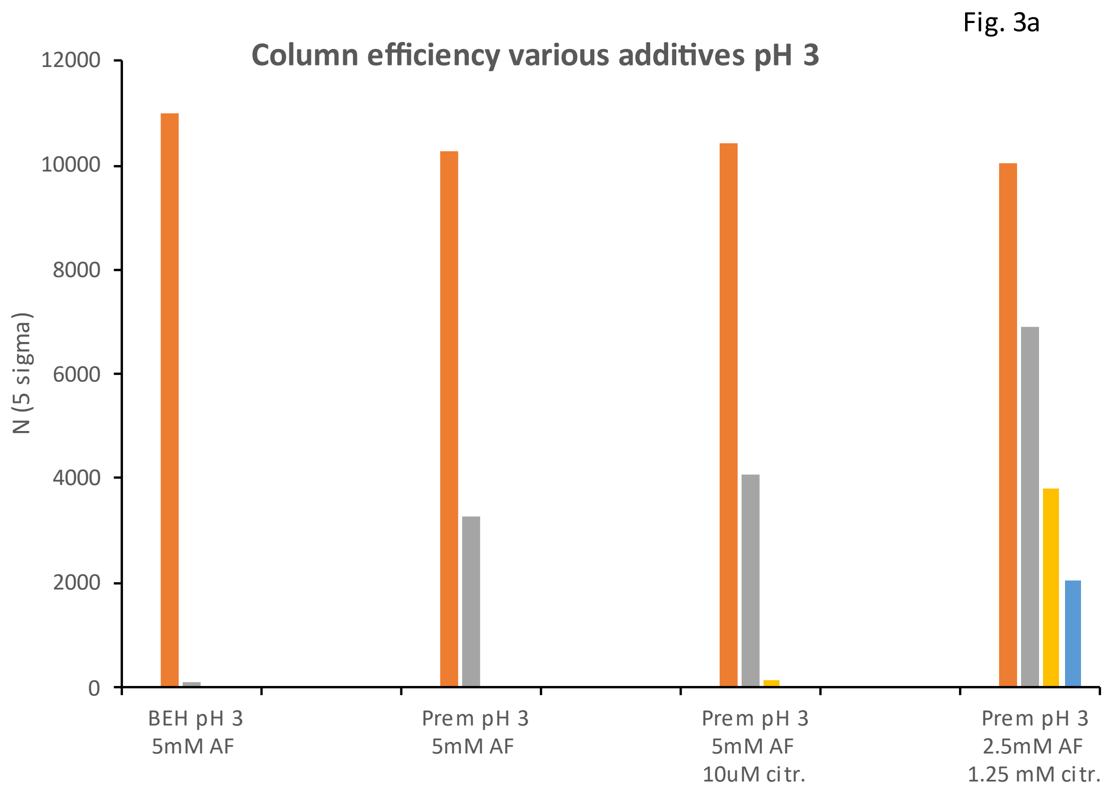

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Fig. 3b

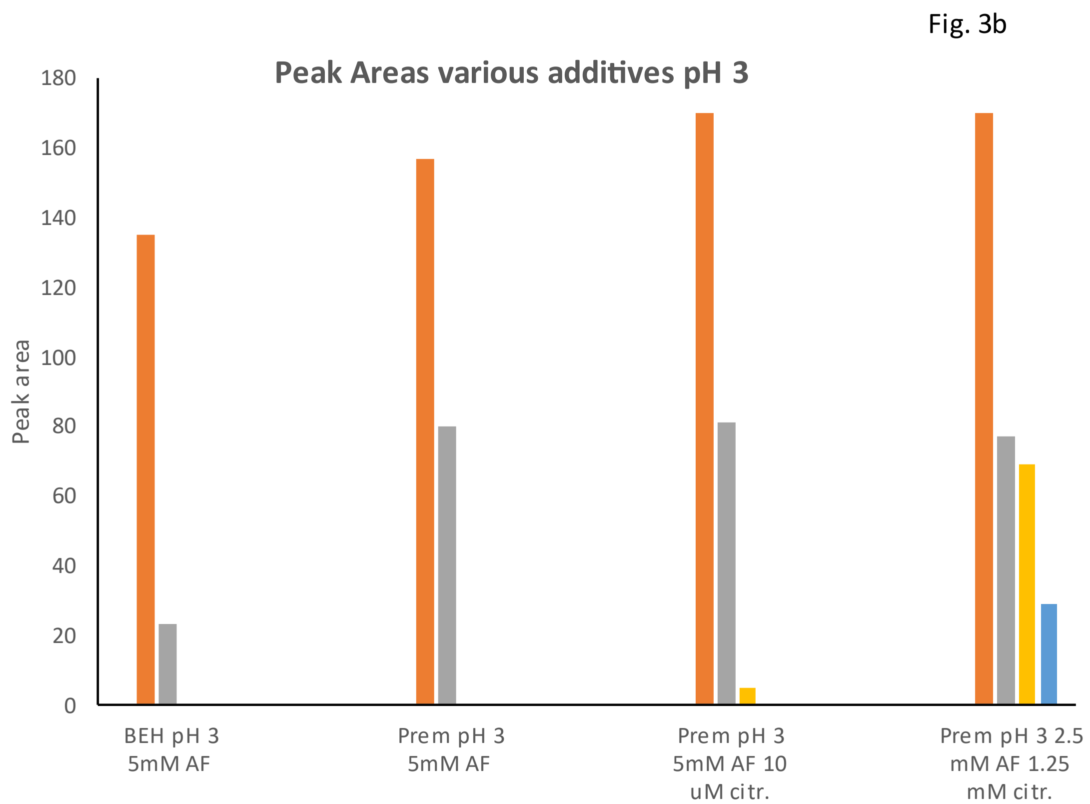

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Fig. 4a

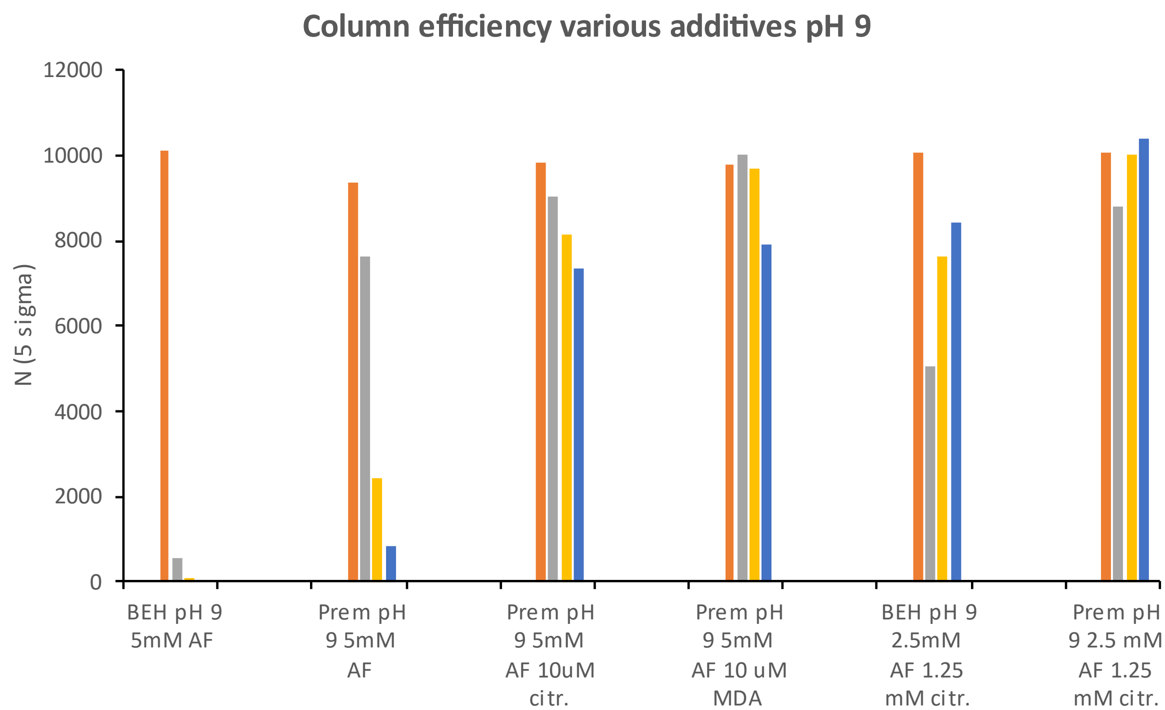

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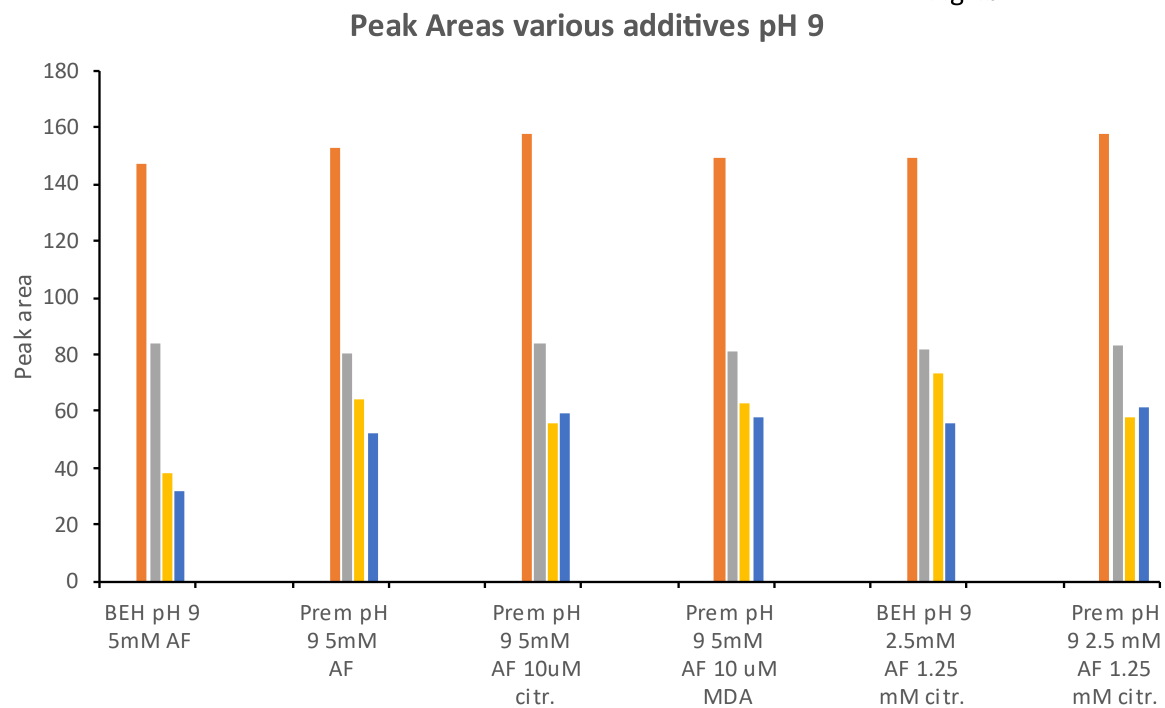
Fig 4b



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	Extracolumn bandspreading (PEEKSIL tube)		Column + extracolumn bandspreading; experimental and true efficiencies						
	Sigma (μL)	Sigma^2 (μL^2)	t_R (min.)	N (exptl)	Sigma tot. (μL)	Sigma^2 tot (μL^2)	Sigma^2 col (μL^2)	N (true col.)	% N loss
5mM AF pH3									
Toluene	1.55	2.4	0.53	9980	2.13	4.5	2.1	21400	53
AD	1.88	3.5	0.90	10300	3.57	13	9.2	14200	28
AMP	2.44	6.0	1.88	3360	13.0	170	160	3470	3.2
ADP	11.5	130	no peak						
ATP	22.9	520	no peak						
2.5mM AF pH3+1.25mM citrate pH 3									
Toluene	1.52	2.3	0.53	10500	2.08	4.4	2.0	22300	53
AD	1.88	3.5	0.89	10100	3.53	13	8.9	14100	29
AMP	2.2	4.8	1.75	6930	8.43	71	66	7430	6.8
ADP	4.54	21	3.20	3820	20.7	430	410	4010	4.7
ATP	9.88	98	5.51	2040	48.9	2400	2300	2110	3.7
5mM AF pH 9									
Toluene	1.56	2.4	0.53	9780	2.13	4.6	2.1	21100	54
AD	1.87	3.5	0.90	9390	3.71	14	10	12600	25
AMP	2.65	7.0	2.27	7650	10.4	110	100	8100	5.9
ADP	5.25	28	3.47	2420	28.4	810	780	2470	2.1
ATP	6.33	40	5.68	887	75.8	5700	5700	905	2.0
2.5mM AF+1.25mM citrate pH 9									
Toluene	1.57	2.5	0.53	9090	2.11	4.5	2.0	22200	56
AD	1.88	3.5	0.89	10100	3.56	13	9.2	14000	28
AMP	2.41	5.8	1.58	8820	6.71	45	39	10200	14
ADP	3.93	15	1.98	10000	7.89	62	47	13400	25
ATP	4.45	20	2.55	10400	10.0	100	81	12900	20

Table 1 instrumental and column bandspreading for solutes using either 10 cm Peeksil tube or Premier Amide Column. Various buffers in 70% ACN

	pH 3		pH 7		pH 9	
Solute	**Charge**	**log D**	**Charge**	**log D**	**Charge**	**log D**
Adenosine	0.9	-2.4	0.01	-1.7	0	-1.7
AMP	-0.1	-5.2	-1.9	-6.7	-2	-7.6
ADP	-0.8	-6.8	-2.4	-8.8	-2.4	-9.9
ATP	-1.8	-9	-3.4	-11.5	-3.4	-12.7
citric acid	-0.5	-1.8	-3	-8.1	-3	-9.6
medronic acid	-1.8	-5.9	-2.3	-8.1	-2.3	-9.4

623 **Table 2 charge and log D values for solutes/additives at various pH.**