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Ammonia as a preferred additive in chiral and achiral applications of supercritical fluid chromatography for small, drug-like molecules

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ABSTRACT

Supercritical fluid chromatography is routinely utilized by analytical separations groups in the pharmaceutical industry to efficiently handle separations for discovery medicinal chemistry purposes. Purifications are performed on samples ranging from a few milligrams up to hundreds of grams. Basic additives dissolved into the liquid component of the SFC mobile phase are commonly used to improve peak shape and efficiency in achiral and chiral separations. While for purposes of analysis there is minimal consequence to additive introduction in the mobile phase, for preparative separations one needs to consider the potential effect of an additive's presence when concentrated with the desired compound. Following an SFC purification using an additive-containing modifier, the resulting fractions will contain an easily evaporated modifier, and after its evaporation perhaps still significant levels of the less volatile additive. Depending on the aqueous solubility and basicity of the final product, the process of removing basic amine additives can be time-consuming and can result in reduced yields. NMR analysis following preparative isolation and evaporation often reveals the fact of insufficient removal of the chromatographic additive even after aqueous work up steps. In this study, ammonia is evaluated as an alternative additive to strong bases such as diethylamine (DEA) in SFC purification and analysis and to the authors' knowledge no previous publication has been written describing the application of methanolic ammonia as an additive for SFC separations. Dimethylethylamine (DMEA), a more volatile additive than DEA, is also evaluated relative to ammonia for its potential to simplify the isolation process after purification and in terms of chromatographic performance. The loss in concentration of ammonia in methanol modifier over time due to evaporation and effects of that loss are also described. Furthermore, for ammonia the analytical benefit is shown to extend to on-line mass spectrometric detection relative to other basic additives.

1. Introduction

Supercritical fluid chromatography is routinely utilized by analytical groups in discovery pharmaceutical applications to efficiently handle separations for medicinal chemistry. Both chiral and achiral purifications are performed on samples ranging from a few milligrams up to the kilogram range [1–13].

Basic additives dissolved into the liquid component of the SFC mobile phase have been shown to improve peak shape and efficiency in achiral and chiral separations of drug-like molecules [14–25]. It has been proposed that a major influence of basic additives on separations by SFC is due to their masking of silanols to inhibit non-specific interactions with basic solutes [25–29]. In addition, these bases may compete for stationary phase binding sites thus influencing solute-specific retention.

Following SFC purification using an additive-containing modifier solvent or "co-solvent" the resulting fractions will contain both the co-solvent and the additive, which must be removed. Often the primary co-solvent component is readily evaporated, but evaporation and removal of the less volatile additive may not be fast or trivial.

Basic additives such as diethylamine (DEA) and dimethylethylamine (DEA) are popular for use in chiral separation applications [3,6,29,30]. Such additives are highly basic and do not partition completely into the aqueous phase in work ups to isolate fractions from SFC purification. Their presence is often only detected once ^{1}H NMR for structure confirmation is performed. Depending on the aqueous solubility and basicity of an isolated compound, there may be challenges in removing diethylamine or another basic additive that can be costly in terms of time and final yield.

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SFC purification provides an advantage relative to HPLC resulting from the lower boiling point mobile phase required and lower collected solvent volumes [1,6,31–33] from most separations. With an undesired basic chromatographic additive remaining in a dry final product fraction the advantage in rapid evaporation is diminished. Thus it is desirable to achieve a separation in SFC without such an additive. For most basic drug-like solutes it is a significant hindrance and often impossible to achieve a reasonably efficient purification without basic additives. In principle if the basic additive is more volatile than the mobile phase, however, it can be expected to be removed in the solvent evaporation step following purification [34]. We thus consider using ammonia (NH_3) in the SFC co-solvent. Ammonia is basic and exists as a gas at room temperature thus facilitating its rapid removal with the modifier solvent in the post-purification evaporation step.

Replacing basic additives such as DEA with ammonia can be considered advantageous for the application of preparative SFC if the following conditions are satisfied: (1) its addition to mobile phase co-solvent achieves separation performance enhancement comparable to other non-volatile basic additives, (2) it is effectively eliminated in evaporation, and (3) co-solvents containing ammonia are shown to be sufficiently robust and not require labor-intensive monitoring or replacement. These figures of merit will be examined in this work, using a number of chiral SFC standards and comparing NH_3 to DEA and DMEA in this respect. Seeking to avoid introducing water and any chromatographic effects due to its adsorption to stationary phase silica, methanolic ammonia (non-aqueous) was utilized in this study instead of ammonium hydroxide solution as the mobile phase additive.

2. Materials and methods

2.1.1. Mobile phase components

Solvents methanol (Chromasolv Plus®), 2.0 M methanolic ammonia, diethylamine, and N,N-dimethylethylamine were purchased from Sigma–Aldrich (St. Louis, MO). For all analytical experiments, the liquid CO_2 used was 4.0 Instrument grade (99.99%) from Praxair, Inc. (Danbury, CT). For preparative SFC, the CO_2 supply was Medical grade from 180 L dewars also from Praxair, Inc. A Thar BDS-500 from Waters Corp. (Milford, MA) was used to pump out the liquid CO_2 and pressurize it to >900 psi for delivery to the preparative SFC system.

2.1.2. Standards

Standards used in various experiments were purchased from various vendors: benzoin methyl ether, 2-bromo-1-indanol, benzoin, and 4-benzoyloxy-2-azetidinone were purchased from Acros Organics (Fair Lawn, NJ). Naringenin was purchased from Alexis Biochemicals (Lausen, Switzerland). 3-(2-methylphenoxy)-1,2-propanediol and Hydrobenzoin were purchased from Alfa Aesar (Haysham, Lancashire, UK). Chlormezanone, atropine, thioridazine HCl, fenoterol HBr, sulfinpyrazone and atenolol were purchased from MP Biomedicals (Solon, OH). ketoprofen, metoprolol tartrate and fluoxetine HCl were purchased from Spectrum Chemical Corp. (Gardena, CA). Napropamid, mianserin, proglumide sodium salt, benzyl mandelate, mandelamide, clenbuterol HCl, tropicamide, pindolol, norphenylephrine HCl, hydroxyzine dihydrochloride, warfarin, bendroflumethiazide, sulconazole, disopyramide, diperodon HCl, carvedilol, verapamil HCl, glafenine HCl, trans-stilbene oxide, alprenolol HCl and indapamide were purchased from Sigma–Aldrich.

2.1.3. Analytical instrumentation

For all analytical SFC and SFC/MS experiments, a (Thar) SFC Methods Station with ZQ2000 mass spectrometer from Waters was used to generate analytical data. Preparative separations were performed on a (Thar) SFC 80 from Waters.

2.2. Chiral SFC stability analysis method with various basic additives

For the chiral SFC stability experiment with ammonia mobile phase additive, the basic standard, thioridazine, at 2 mg/mL in methanol was used as the sample. The experiment consisted of repetitive 10 μL injections of this sample followed by isocratic chiral separations of using 80% "A" = CO_2, plus 20% "B" = methanol (20 mM NH_3), as the mobile phase with a total flow rate of 4 mL/min. The stationary phase used was a new, 100 mm × 4.6 mm, 5 μm Chiral Technologies Chiralpak® AD-H column.

2.3. Achiral separations of neutral or basic compounds method

Analytical columns utilized for achiral separations with and without NH_3 additive were ES Industries (West Berlin, NJ) GreenSep™ Ethyl Pyridine and GreenSep™ Silica. The dimension for both columns was 150 mm × 4.6 mm, with 5- μm particle size. The experiment consisted of 10 μL injections of naringenin and thioridazine on each column using 80% CO_2, plus either 20% methanol or 20% methanol (20 mM NH_3), as the mobile phase with a total flow rate of 4 mL/min.

2.4. Chiral gradient and isocratic retention comparison methods

Gradients used in the retention comparison of 30 racemic standards for the three modifiers consisted of a linear ramp from 5 to 60% B from 0 to 4.5 min, followed by a hold at 60% B until 5.5 min., then followed by a return from 60 to 5% B finishing at 6.0 min. For the comparisons, B = methanol (0.2% DEA), methanol (0.2% DMEA) or methanol (20 mM NH_3). The flow rate was 5.0 mL/min. at 40 °C with a fixed outlet pressure of 100 bar. All the above conditions were maintained except for the fixed modifier composition in the isocratic separation experiment applied to each standard. The peak 1 and peak 2 enantiomer retention times from each 10 μL (~2 mg/mL) of all standards were recorded and plotted together for either the gradient or isocratic experiment.

2.5. Removal/analysis of basic additives after SFC purification method

The preparative column used for SFC isolation of fractions of Atenolol for the NMR experiment was a Chiral Technologies Chiralcel® OD-H, 5-micron, of dimension 250 mm × 21.2 mm. This racemic standard was injected repetitively onto a 250 mm × 21.2 mm Chiralcel OD-H column (up to 2.0 mL of 50 mg/mL methanol solution) and fractions of peak 1 collected with each injection using a Thar 80 SFC with 20% modifier (with either 0.2% DEA or 20 mM NH_3) at a total flow rate of 80 g/min. (CO_2 + modifier). Purified methanolic fractions were vacuum evaporated for 1 h on a Buchi (New Castle, DE) R–210 rotary evaporator with 60 °C bath temperature under 100 mTorr vacuum. ^1H NMR data were acquired from a Bruker Daltonics (Billerica, MA) 500 MHz NMR.

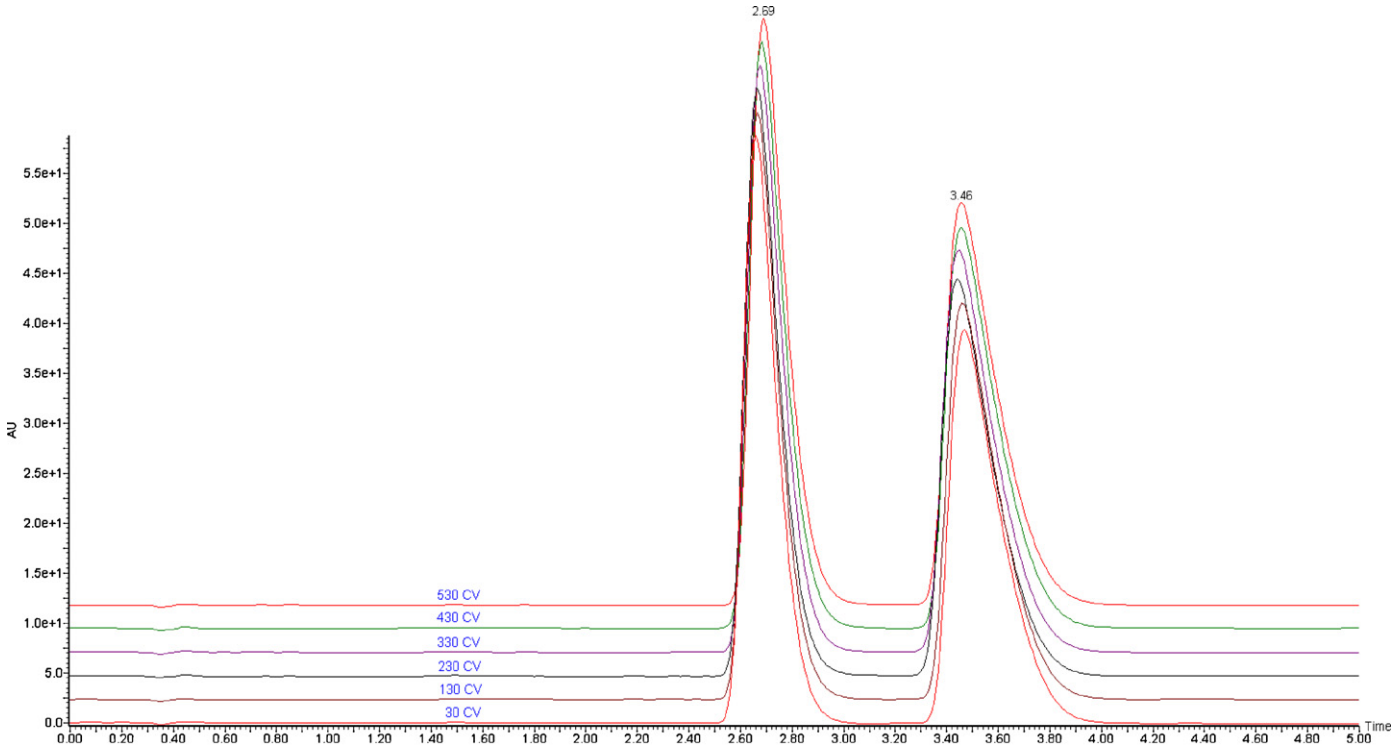

Fig. 1. Chiral separation of basic compound thioridazine on AD-H column after 30 column volume equilibration with 20% modifier (20 mM NH_3 in methanol) in mobile phase (lowermost chromatogram) followed by separations obtained after additional 100 CV modifier, incrementally through 530 CV.

2.6. Analysis of stability of ammonia concentration in SFC modifier methanol

For quantitative spectrophotometric measurement of modifier ammonia concentration, a Sigma Ammonia Assay Kit was utilized to prepare samples. The kit employs an enzymatic reaction in which NADPH is converted to $NADP^+$ stoichiometrically with NH_4^+. With the loss of NADPH in solution, the UV absorbance at 340 nm is thus attenuated linearly based on [NH_4^+]. Ammonia present in this experiment's methanolic samples is converted to ammonium for this test by dilution with excess water (30 μL plus 970 μL H_2O) which also brings the concentrations sampled into the ammonia assay kit's specified linear dynamic range. The kit could thus be used to measure ammonia at levels in the range of interest for this loss determination analysis as follows. Three sample mobile phase bottles were prepared containing 400 mL solutions of ammonia in methanol at concentrations of 10 mM, 20 mM, and 50 mM, from dilutions of a stock solution of 2.0 M ammonia in methanol. The bottles used were 500 mL Schott glass solvent containers with sealing caps which included a 1/8″ hole in the top to allow a somewhat liberal but realistic rate of vapor escape based on conditions of an analytical SFC/MS system modifier solvent supply bottle. The bottles were kept at room temperature for 28 days and sampled at various time points. The standard calibration curve for this experiment was prepared using solutions at concentrations of 10 mM, 20 mM, 30 mM and 50 mM (prepared from 2.0 M methanolic ammonia) and run each day the assays were performed. One milliliter each of 97:3 water:bottle sample or 97:3 water:standard mixture was prepared on each day of evaluation. 20 μL of each of these ammoniated solutions were added into 180 μL of the assay kit reagent containing alpha-ketoglutaric acid and NADPH, and the resulting solution shaken. Samples thus prepared were analyzed with a Thermo Scientific (Wilmington, DE) Nanodrop ND-1000 spectrophotometer and baseline absorbances were measured for each level. Following this, 2 μL of the enzyme l-glutamate dehydrogenase GDH was added to each sample vial which was shaken to complete the conversion reaction to $NADP^+$ in each case. The resulting final solutions' absorbances were spectrophotometrically measured after at least 2 min of exposure to enzyme and the difference from the baseline measurement was recorded. Five repetitions of each measurement were made to produce adequate precision for data capture. Ammonia concentration values were determined against a given day's calibration curve for each sample measured.

2.7. Method for chromatographic effects of evaporative ammonia loss from mobile phase solvent

For the experiment to highlight the chromatographic effect of ammonia volatility in the mobile phase over a 7-day span, initially 20 mM NH_3 in methanol was used for isocratic separation. Two compounds, thioridazine and fenoterol, were separated on both a 100 mm × 4.6 mm AD-H and a 100 mm × 4.6 mm Silica column with 20% modifier at 4.0 mL/min. Following equilibration at these conditions, data were acquired in triplicate resulting in <1% RSD in retention for either single or enantiomer peaks. Subsequently, 270 mL of same modifier solvent remained in the 1 L solvent supply bottle with a 1/8″ diameter hole open in the top. This bottle was left to rest at room temperature and not used for 7 days, after which the separations were repeated in triplicate after column equilibrations.

2.8. Method for comparison of SFC/MS sensitivity between methanol with NH_3, DEA, and DMEA

Naringenin at 2 mg/mL was separated on a 150 mm × 4.6 mm, 5-micron Chiralpak® AD-H column with methanol plus either of three additives, NH_3 (20 mM), DMEA (0.2%), and DEA (0.2%) for evaluation of mobile phase effects on positive electrospray ionization sensitivity in SFC/MS. The mobile phase was split into the mass spectrometer source from the 100 bar controlled pressure

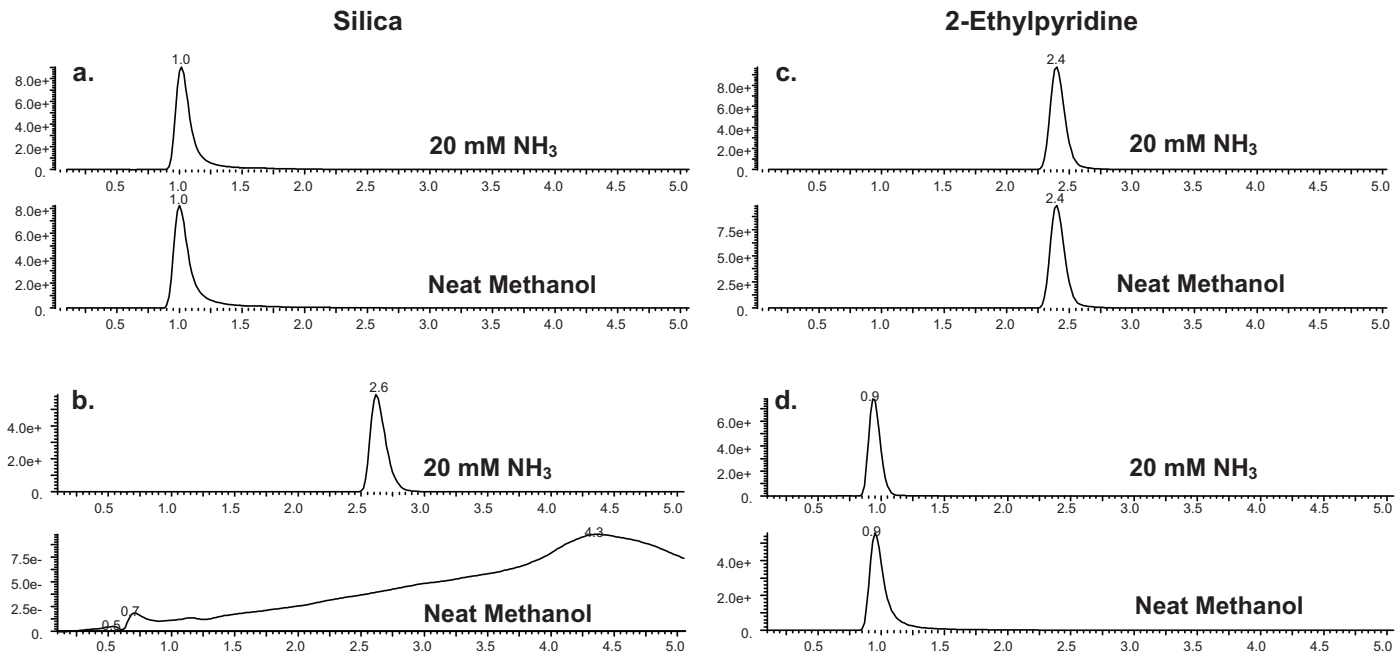

Fig. 2. Chromatograms displaying SFC separation using 20% methanol modifier with and without ammonia additive on (a) silica column with neutral compound naringenin, (b) silica column with basic compound thioridazine, (c) 2-ethypyridine column naringenin and (d) 2-ethylpyridine column with thioridazine.

region just upstream from the automated backpressure regulator. ESI+ mass spectra shown were obtained from on-line analysis of detected peaks.

3. Experiments and results

3.1. Chiral SFC stability analysis with various basic additives

In this experiment a gradient typical for chiral screening was applied to the above standards with the co-solvent methanol modified with either 0.2% DEA (19 mM), 0.2% DMEA (19 mM) and NH_3 (20 mM) diluted from 2.0 M methanolic ammonia stock.

Chromatographic reproducibility is extremely important, especially for isocratic preparative separations utilizing repetitive injections in a time-dependent fashion. This motivates our choice of ammonia introduced with pure methanol instead of through aqueous ammonia solution for an SFC mobile phase additive. It is expected that the higher degree of stationary phase stability will be achieved with non-aqueous containing mobile phase modifier, although aqueous addition to SFC mobile phases has been reported for various applications [24,35–38]. Of course the severities of effects due to aqueous adsorption depend heavily on the experiment – the bonded phase if any, modifier composition in the mobile phase and the type of solutes introduced. Using methanolic ammonia stock, NH_3 concentrations can also be varied as necessary for any purpose without significantly changing water content in the mobile phase and consequently, the level of adsorption to the stationary phase. An experiment was performed to demonstrate performance stability for chiral separation of a basic molecule when using a mobile phase of methanol with NH_3 additive diluted from methanolic ammonia stock, in this case to 20 mM.

In Fig. 1, the first chromatogram (lowest in the stack) was acquired following equilibration with 30 column volumes (CV = 1.2 mL) of mobile phase modifier. Stacked above for illustration are chromatograms recorded after each additional 100 CV modifier passed through the column successively up to 530 CV. The initial six chromatograms following the 30 CV equilibration resulted in average retention times of 2.658 ± 0.012 min. for peak 1 maxima, and 3.475 ± 0.012 min. for peak 2 maxima. After more than 500 CV the average retention time for peak 1 was 2.682 ± 0.009 min., and 3.454 ± 0.010 min. for peak 2. This represents less than a 1% shift from either initial value. The peak width at 10% height did not increase by more than 3.5% for either peak 1 or peak 2. This demonstrated that chromatographic performance using methanol with 20 mM NH_3 additive was consistent and should be robust for purposes of chiral screening, QC analysis and purification in small molecule discovery applications.

3.2. Achiral separations of neutral or basic compounds

As mentioned in the introduction there are a number of possible influences that a base additive may have in the course of a separation. The explanation that basic additives disrupt silanol interactions in SFC with basic solutes is widely accepted [29]. Without the presence of water in the SFC mobile phase such interactions are not ionic as with reversed phase, but can nonetheless still be strong. To illustrate this effect comparison of an achiral SFC separation with basic and neutral compounds was performed. Here the separations obtained on silica were compared to those from a 2-ethylpyridine bonded phase on the same silica. One would expect the chromatographic behavior of basic compounds to be less affected in the case of the 2-ethylpyridine stationary phase as the ligand itself should provide shielding against active silanols. The addition of a basic additive should then improve the separation for a basic solute on silica. In Fig. 2 the addition of ammonia to the mobile phase modifier on a silica column was explored with basic and neutral compounds. In Fig. 2a, a negligible difference is observable on the silica column between separations with and without ammonia for the neutral compound naringenin, whereas in Fig. 2b for the basic compound thioridazine, no elution at all was possible at this condition without basic additive, i.e. ammonia. In the case of the 2-ethylpyridine column there was again no effect on the elution profile of naringenin as shown in Fig. 2c, and although increased tailing is evident for thioridazine, in Fig. 2d the effect was far less dramatic compared to the performance on silica. This

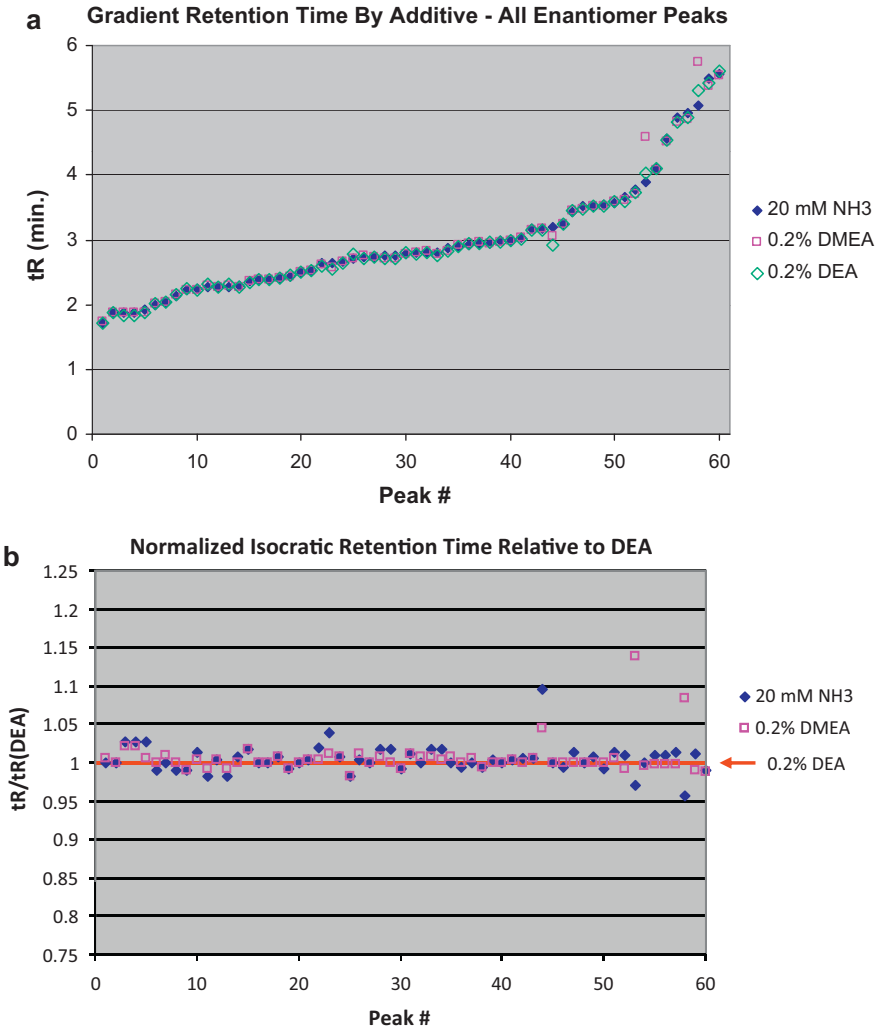

Fig. 3. (a) Consolidation of all peak retention times (two for each chiral standard) from lowest to highest in order for all 30 standards plotted for three different additives. Outlier data points are 53 and 58, corresponding to enantiomers of Sulfinpyrazone. (b) Comparison of all peaks' isocratic retention times associated with additives (♦) NH_3 and (□) DMEA relative to that for DEA (normalized to 1) in order by screening gradient retention time.

is consistent with the idea that 2-ethylpyridine is acting similarly to a basic additive to reduce basic interactions with silanols.

3.3. Retention comparison for chiral separations with basic additives: NH_3, DEA, and DMEA

Chiral SFC is currently heavily utilized for preparative purification in the pharmaceutical industry due to its favorable inherent properties. SFC routinely produces chiral separations faster, and often with higher selectivity than HPLC methods. Preparative fractions collected can then be quickly evaporated to isolate enantiomers from a mixture. In this process, the gradient screening of unknown racemates on several chiral phases is a key first step in preparative method development. Mobile phase modifiers such as methanol generally include basic additives such as diethylamine (DEA) or dimethylethylamine (DMEA) which will usually enhance performance for basic compounds. Separation performance for basic compounds on silica-based chiral columns using basic additives is improved analogously to the achiral performance enhancement described above with bare silica. For a set of 30 diverse chiral standards from the list above, the gradient method previously described was applied using methanol with either 0.2% DEA (19 mM), 0.2% DMEA (19 mM) or NH_3 (20 mM) as the additive. Counting peak 1 and peak 2 enantiomer retention times from each 10 μL (~2 mg/mL) racemate injection as unique data points, the retention times for 60 enantiomers from the set of 30 racemic standards were plotted in Fig. 3a to determine variations in gradient retention resulting from the use of different additives.

Based on this set of standard compounds, SFC gradient retention is almost independent of the choice of basic additive. For only one compound, Sulfinpyrazone, did one of the additive's gradient retention differ by more than 5% relative to the average of the other two. As the next step in scale up method development, isocratic methods derived from these gradient separations would thus be nearly all the same.

Following the gradient screen, for each compound an isocratic method, anywhere between 3% and 35% B as required, was used and each of 60 enantiomer peaks' retention times was tabulated. Besides modifier composition all the same chromatographic conditions were used as in the gradient experiment. The values for each standard's peaks corresponding to methanol plus DEA were normalized to 1 for relative comparison with retentions achieved using the other additives, NH_3 and DMEA. Fig. 3b illustrates this comparison of the resulting normalized retention times relative to DEA from these isocratic chiral separations.

Only three of the 60 compounds analyzed exhibited a retention shift relative to DEA of >5% with either NH_3 or DMEA, two of which were enantiomers of sulfinpyrazone. This is consistent

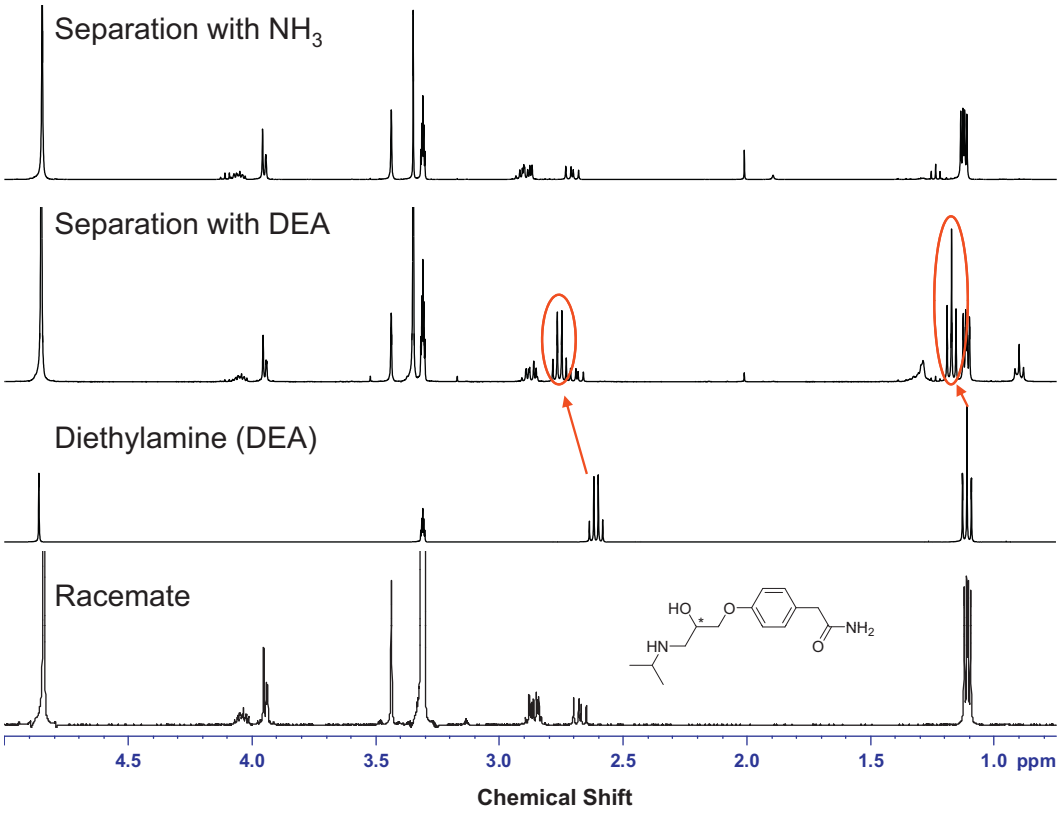

Fig. 4. Comparison of ^{1}H NMR spectra for one enantiomer fraction following chiral SFC purifications of atenolol using 20 mM NH_3 and DEA, respectively, in methanol modifier relative to spectra for DEA and pure atenolol racemate below.

with the variability observed in the associated the gradient data. Since sulfinpyrazone is acidic, given that the highest retention was observed with DMEA, mid-level in basicity between NH_3 and DEA, the retention difference cannot be explained based on relative pKa effects. It is expected that this result is influenced by the molecular level interaction between the solute, mobile phase with additive, and the chiral selector. Overwhelmingly however, the data indicate a high degree of similarity between these mobile phase additives for the purpose of SFC chiral separations. With such slight differences in retention, for the purpose of purification method development, it appears that all additives evaluated provide similar selectivity for scale up separation even if final methods turn out to have slight differences.

3.4. Removal/analysis of basic additives after SFC purification

Assuming similar performance for use in preparative SFC, any of the additives discussed are interchangeable and only considerations other than performance become important in the decision of which additive to use. Most important among these are compound stability in the presence of additive during dry down and issues with the required removal of additive from the compound isolated in SFC separation. With a boiling point of −17 °C, ammonia should be quickly and fairly completely eliminated from the methanolic fraction solution at 40 °C bath temperature on the rotary evaporator. The boiling points of DMEA and DEA are 36 °C and 56 °C, respectively, and even under harsher, higher temperature evaporation conditions these additives can tend to remain in dry fractions due to hydrogen bonding with the drying solute. Note that for acidic compounds any basic amine additive including ammonia is likely to form the salt with the dry compound and require further work up to remove. Since there is not expected to be a chromatographic advantage to using basic additives for acidic compounds, however, neat SFC modifier solvents should be favored for these separations.

In a preparative SFC example, chromatographic consistency was found between separations with either methanol plus 20 mM NH_3 or with 0.2% DEA for the chiral purification of atenolol. The resulting dried fractions from purification with either DEA and NH_3 additives were analyzed by ^{1}H NMR, and spectra were compared to those of the unpurified Atenolol standard and for DEA as referenced in Fig. 4.

Evidence of DEA remains in the spectrum of the fraction labeled “Separation with DEA” as indicated by encircled triplet and quartet signals (slightly shifted due to interactions with solute). The

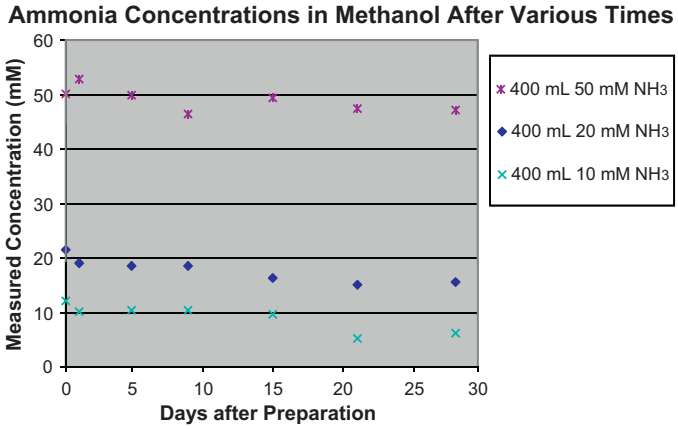

Fig. 5. Concentrations of ammonia in methanol mobile phase bottles (500 mL) with 1/8″ holes in caps in static laboratory environment. Concentration values for bottles initially prepared to 50 mM, 20 mM and 10 mM NH_3 in methanol determined from spectrophotometric ammonia assay at 1-day, 5-day, 8-day, 15-day, 21-day and 28-day time points.

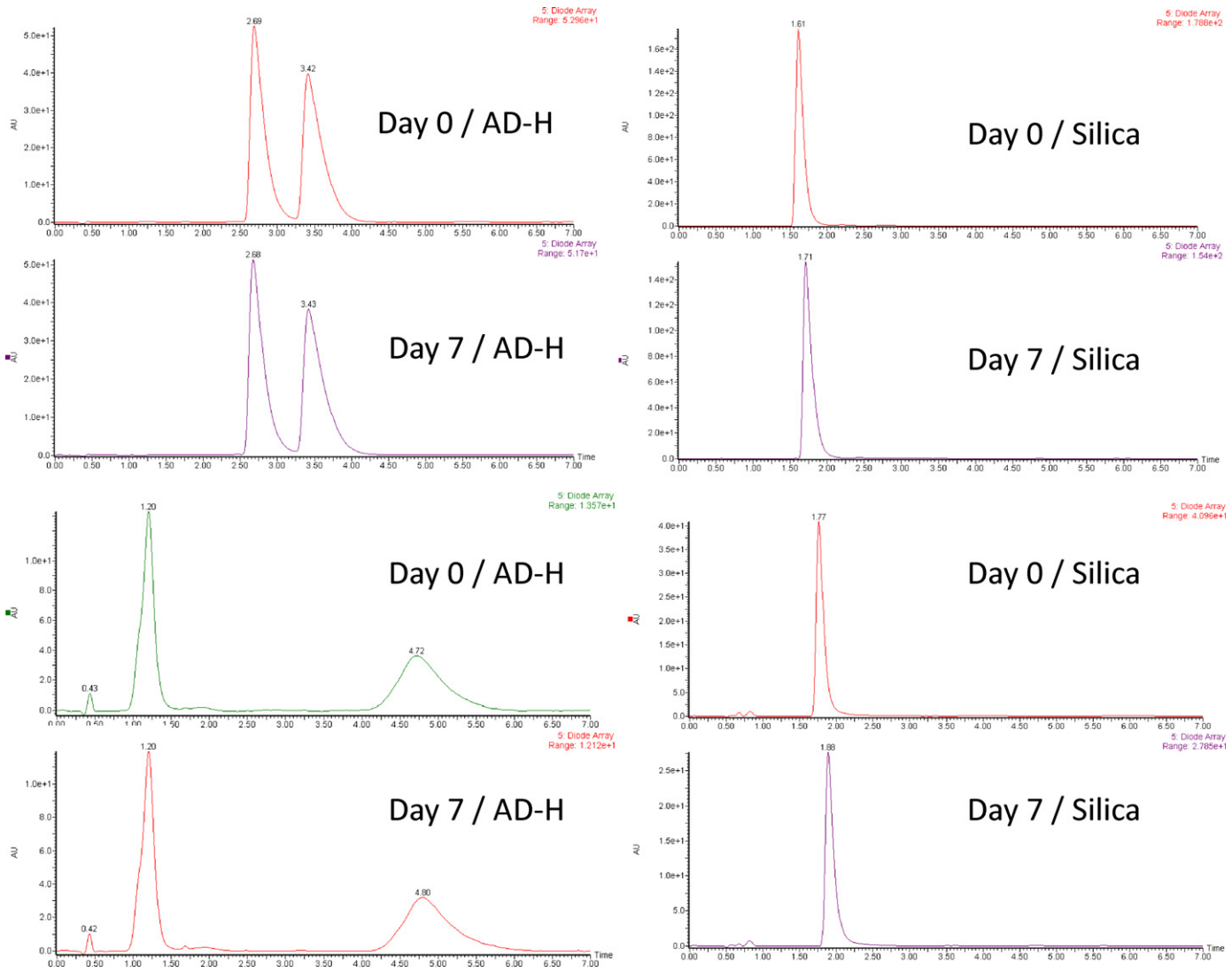

Fig. 6. Chromatograms displaying SFC separation using methanol with 20 mM ammonia additive in an initial run (top) and after 7 days (bottom) for (a) AD-H column with thioridazine, (b) AD-H column with fenoterol, (c) silica column thioridazine and (d) silica column with fenoterol.

fraction spectrum labeled "Separation with NH_3" more closely matches that of the unpurified racemate. A further treatment such as an aqueous workup of the DEA-purified fraction is thus required for complete isolation of the desired enantiomer, which may be time-consuming and result in sample loss. On the other hand, the NH_3-purified enantiomer fraction is ready, after simply drying down, to be moved on to final registration testing, etc. saving time and effort for the chemist.

3.5. Stability of ammonia concentrations in SFC modifier solvent – methanol

While the volatility of ammonia in SFC modifier simplifies compound isolation post-purification, this volatility can also lower its concentration in the modifier over time. This could possibly adversely affect the separation of basic compounds. To model how such loss may occur over time a determination of ammonia concentration in methanol mobile phases exposed to evaporation under common laboratory conditions was performed.

In Fig. 5, the concentrations of ammonia in the three bottles thus measured on each day throughout a 28-day period for bottles corresponding to starting concentrations of 10 mM NH_3, 20 mM NH_3, and 50 mM NH_3 were plotted. The relative standard deviation for each measurement decreased with increasing signal size due to increased signal-to-noise resulting in an RSD of 10% for the 10 mM data points, 6% for the 20 mM and 2% for 50 mM. Measurements were repeated five times for each time point. The data nonetheless indicates that losses in concentration exceeding 10% were not reached until some point after the 8th day of measurement. If such a change results in a negligible effect on separation performance, environmental evaporation would most likely not be a concern in most laboratory applications as mobile phase solvents are regularly consumed and replenished on a weekly or more frequent basis. Evaporation from mobile phase supply containers can even be largely controlled with now available solvent safety caps.

3.6. Chromatographic effects of evaporative ammonia loss from mobile phase modifier solvent

To demonstrate whether evaporative ammonia loss over the span of one week was significant to chiral or achiral separations an experiment with separation of two basic chiral compounds, thioridazine and fenoterol, was performed. After being used to analyze standards for initial data, a 1 L supply bottle with 20 mM NH_3 in methanol was left idle for 7 days with a 1/8″ hole in the top. In Fig. 6, the AD-H column data (chromatograms on the left) exhibit

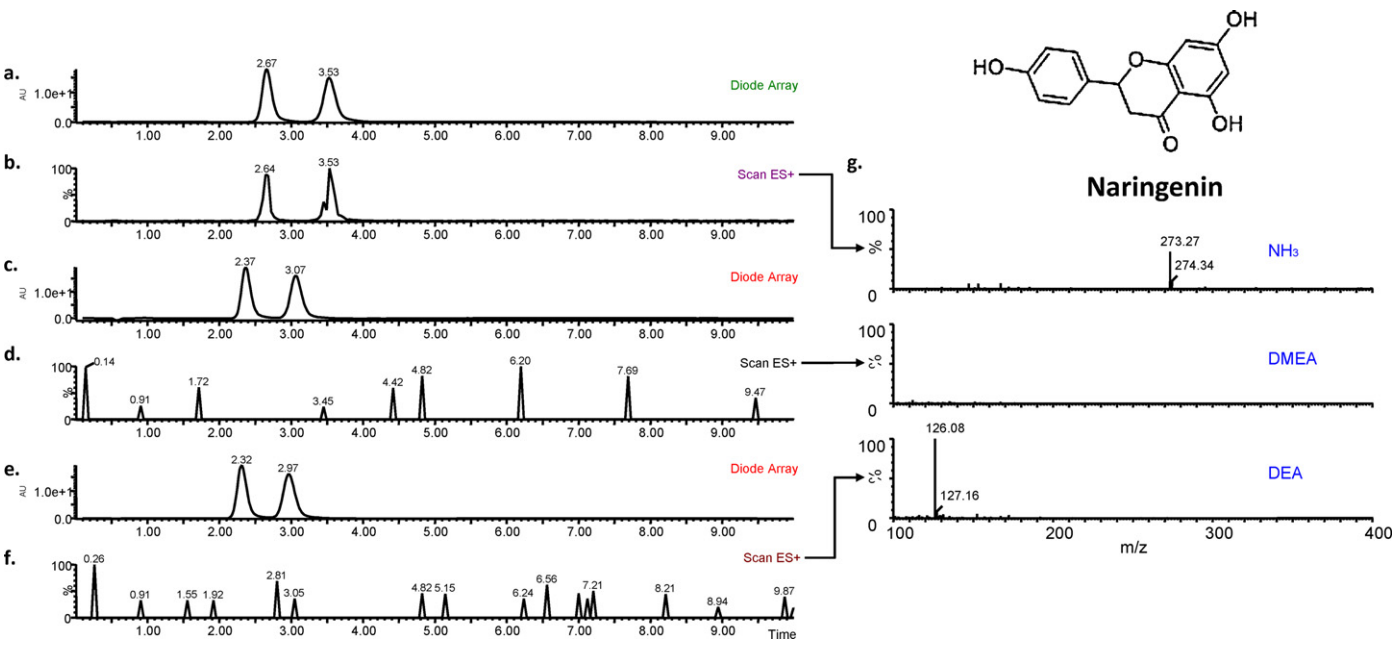

Fig. 7. Photodiode array (PDA) summed signal chromatograms (upper), and ESI+ *m/z* 273 mass chromatograms (lower) for naringenin (separated with 35% modifier at 5 mL/min. on 150 mm × 4.6 mm AD-H column) with each of three modifier additives: (a) 20 mM NH_3 PDA, (b) 20 mM NH_3 ESI+ TIC, (c) 0.2% DMEA PDA, (d) 0.2% DMEA ESI+ TIC, (e) 0.2% DEA PDA, (f) 0.2% DEA ESI+ TIC; (g) right side – overlaid average mass spectra detected during elution of first enantiomer peak corresponding to additives NH_3 (top), DMEA (middle) and DEA (bottom).

virtually no retention change for Thirodazine enantiomer peak, less than 0.01 min difference. Only a modest increase of 0.08 min (<2% retention on the 4.8 min apex) was observed on the more retentive enantiomer of fenoterol. On silica the effects were more pronounced indicating some degree of effect due to evaporation of ammonia as seen on the right hand side of Fig. 6. The retention time of thioridazine increased from 1.61 to 1.71 min., while that for fenoterol increased from 1.77 to 1.88 min. Silica, as shown before, is more sensitive to base additive concentration and would require fresher solution preparation or monitoring if ammonia were used for an achiral separation requiring reproducible performance. However, as described earlier 2-ethylpyridine stationary phases, for example, have been shown to be far more inert to such effects. It should also be noted that in usual laboratory practice more favorable conditions for maintaining ammonia concentration usually exist and this illustration almost represents a worst case scenario. In addition, vapor-free solvent bottle caps now commercially available can help alleviate volatility effects altogether. Nonetheless, the data for the AD-H column are indicative of a sufficiently high degree of stability of the 20 mM ammonia mobile phase for chiral separation applications using laboratory practices appropriate for any common SFC mobile phase supply solvent.

3.7. Comparison of SFC/MS sensitivity between methanol with NH_3, DEA, or DMEA

Online detection by mass spectrometry as detection for analytical SFC is an important component to preparative SFC applications. SFC/MS analyses are useful for product characterization both before and after purification [3,35,39–41]. SFC/MS sensitivity becomes most important after purification with samples at possibly low concentration in collected fractions. Before purification, when sample quantities are not limited for the purpose of analysis, abundant signals are usually obtainable for basic compounds in ESI+ (positive-mode electrospray ionization) with any additive present, even though basic additives will suppress the ionization of solutes to some degree [42,43]. With a low concentration of solute, or with a neutral species difficult to ionize by ESI, ion suppression can be a serious detriment when positive identification of a desired compound or impurity is necessary from among many peaks in a chromatogram. In the example shown in Fig. 7 below, a neutral chiral compound, Naringenin, was analyzed by SFC/MS with each of three additives, ammonia (20 mM), DMEA (0.2%), and DEA (0.2%), in the mobile phase modifier, methanol.

While chiral separations were achieved with all additives, positive mass spectral detection for the ion of interest was only found in the case of ammonia additive (Fig. 7b). This indicates that with ammonia's lower basicity, ion suppression is reduced in positive mode electrospray mode relative to DMEA or DEA, in this case making the difference between detection and non-detection. Though detection sensitivity is less an issue with more basic solutes, it was also observed that signal levels are usually still twice as high or more with ammonia relative to DEA or DMEA. Significantly more abundant signal was also observed in general with APCI (atmospheric pressure chemical ionization) mode for NH_3 relative to either DEA or DMEA.

4. Concluding remarks

This paper has described the properties and benefits of ammonia as a mobile phase additive to methanol in SFC separations, particularly preparative. To our knowledge this is the first time a publication has described the advantages of non-aqueous, free ammonia (NH_3) applied to SFC separations. Basic additives reduce silanol interactions with basic solutes on silica stationary phases, and ammonia was shown to be comparable with DEA and DMEA in terms of performance, especially for chiral separations. The most pronounced effects on retention were seen with bare silica. While each additive studied was generally comparable with regard to selectivity and retention, NH_3 is the least reactive and easiest to remove following SFC separation and fraction collection, simplifying and speeding the downstream treatment following purification.

Ammonia in methanol was also shown to be stable overall and not volatile enough to cause concern regarding its evaporation and resulting loss of concentration in the mobile phase supply solvent on a time scale of one week or more. It was also found to improve mass spectral sensitivity to basic compounds relative to DMEA or DEA additives enhancing an analyst's ability to detect unknown species or more neutral compounds present in sample mixtures analyzed by SFC/MS.

Ammonia as an additive to methanol may not always provide an optimal separation, but accounting for its advantage in simplifying post-purification processing, it is overall the most advantageous additive among those compared for the application of preparative SFC.

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