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Nota de aplicación

Hydrophilic Interaction Chromatography Using Silica Columns for the Retention of Polar Analytes and Enhanced ESI-MS Sensitivity

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Abstract

Hydrophilic interaction chromatography (HILIC) is a useful technique for the retention of polar analytes that offers a difference in selectivity compared to traditional reversed-phase chromatography. This paper examines the use of a silica column for HILIC, including mechanistic studies of analyte retention on silica in aqueous–organic mobile phases. The authors compare a silica column and C_{18} column to demonstrate the increased sensitivity of using HILIC with electrospray ionization mass spectrometry (ESI-MS). They also report a quantitative LC–MS method for the analysis of acetylcholine, choline, and choline-trimethyl-d_9, and describe a simplified solid-phase extraction (SPE) procedure that allows the direct injection of extracted organic fractions from SPE devices onto a silica column.

Introduction

The retention and separation of polar analytes is an on-going challenge for chromatographers.[1-3] Ion exchange or ion pairing,[4] mobile-phase pH manipulation,[5] and reversed-phase chromatography with specially designed columns[6] are techniques that traditionally have been used for the retention of polar analytes. However, each of these techniques has certain drawbacks. Ion exchange or ion pairing work well only if the analytes of interest are ionizable. In addition, ion pairing is difficult to use with mass spectrometry (MS) because ion pairing reagents will cause signal suppression.[7] The precision and accuracy of quantitative bioanalytical methods can be affected adversely by these suppression effects.[8] Manipulation of mobile-phase pH is a technique that also works well for ionizable compounds, because the retention characteristics of ionizable compounds are a function of pH of the mobile phase.[5,9] The key to gaining retention is to manipulate the pH so the analyte is in its uncharged or neutral state (that is, a basic compound at alkaline pH). Manipulation of mobile-phase pH, however, is not always successful in retaining analytes that are very polar. Also, some compounds might not be stable outside the neutral pH range. Reversed-phase chromatography is the most widely used technique in the pharmaceutical industry because of its versatility and ability to retain and resolve a number of different types of compounds. However, the retention of polar analytes often requires a highly aqueous mobile phase to achieve retention, which can cause a number of issues such as dewetting of the stationary phase[6,10] and decreased sensitivity in electrospray ionization mass spectrometry (ESI-MS).[11,12] Manufacturers of high performance liquid chromatography (HPLC) columns have made attempts to address the issue of phase dewetting

by introducing stationary phases that contain an embedded polar group to maintain wetting of the pores.[2,6,13–16] These packing materials offer unique selectivities and solve the issue of phase dewetting but often show decreased retention. Another approach is the use of alkyl chain columns designed specifically to retain polar analytes. These columns resist dewetting by incorporating the right blend of pore size, ligand density, surface area, and bonding techniques.[6] Although such columns can be used with MS, the highly aqueous mobile phases that are required for polar retention on these phases are not ideal for mobile phase desolvation by ESI-MS, and thus result in poor sensitivity.[11,12] Additionally, many of these columns still do not retain extremely polar molecules.

Hydrophilic interaction chromatography (HILIC) is a mode of chromatography that can address these issues. HILIC requires mobile phases that are highly volatile (>80% organic), are ideal for compound ionization by ESI-MS, and can retain highly polar analytes. The term hydrophilic interaction chromatography was first coined by Andrew Alpert to distinguish this technique from normal-phase chromatography.[17] HILIC is run on polar stationary phases such as silica,[1,12,18] amino,[19–23] diol,[24] polyhydroxyethyl aspartamide, and cyclodextrin-based packings.[17,21] A high-organic, low-aqueous mobile phase is used to retain analytes with increasing orders of hydrophilicity. According to Alpert, retention is proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase.[17] On silica columns, several retention mechanisms are in effect. A combination of hydrophilic interaction, ion-exchange, and reversed-phase retention result in a unique selectivity that allows for polar retention.[17,25–28] This article examines the use of a silica column for HILIC, including mechanistic studies of analyte retention on silica in aqueous–organic mobile phases. We describe the benefits of using HILIC for the increased retention of polar analytes. A silica column and C_{18} column are compared to demonstrate the benefits of using HILIC for increased sensitivity when utilizing ESI-MS and to examine the selectivity differences between HILIC and reversed-phase LC. A quantitative LC–MS method for the analysis of acetylcholine, choline, and cholinetrimethyl-d_9 is reported additionally, and a simplified solid-phase extraction (SPE) procedure in which evaporation and reconstitution steps can be eliminated with the direct injection of extracted organic fractions from SPE devices onto a silica column is described.

Experimental

Equipment

The LC–MS system consisted of a model 2795 Alliance HT Separations Module (Waters, Milford, Massachusetts) with a ZQ 2000 Single Quadrupole Mass Spectrometer. Data collection and

management was performed by Empower Build 1154 Software (Waters) as well as MassLynx v3.5 Software (Waters). The LC–UV system consisted of a model 2795 Alliance HT Separations Module and model 2996 Photodiode Array Detector (Waters). Data collection and management was performed by Empower Build 1154 Software. The reversed-phase column was an Atlantis dC_{18} Column (3 µm d_p), and the HILIC column was an Atlantis HILIC Silica Column (3 µm d_p). Both columns were in the following dimensions: 50 mm x 4.6 mm and 50 mm x 2.1 mm. SPE was performed on Oasis WCX in a 96-well low-elution device format. All instrumentation, software, sample preparation devices, and columns were supplied by Waters Corp.

Reagents and Chemicals

HPLC grade acetonitrile, methanol, dimethyl sulfoxide, isopropanol, and ammonium acetate salt were obtained from J.T. Baker (Phillipsburg, New Jersey). Water was purified using a MILLI-Q Academic System (Millipore, Bedford, Massachusetts). Formic acid and phosphoric acid were obtained from E.M. Science (Gibbstown, New Jersey). Choline chloride 99%, and choline-trimethyl-d_9 98% was obtained form Aldrich (Milwaukee, Wisconsin). Uracil, 5-fluorouracil, cytosine, 5-fluorocytosine, morphine, morphine-3β-glucuronide, formic acid ammonium salt, acetylcholine chloride 99%, bamethan sulfate salt, salbutamol, acenaphthene, 5-fluoroorotic acid, and allantoin were obtained from Sigma (St. Louis, Missouri). Sodium chloride, potassium chloride, magnesium chloride, calcium chloride, and sodium phosphate were obtained from Fluka (Zurich, Switzerland). See Figure 1 for structures of the analytes.

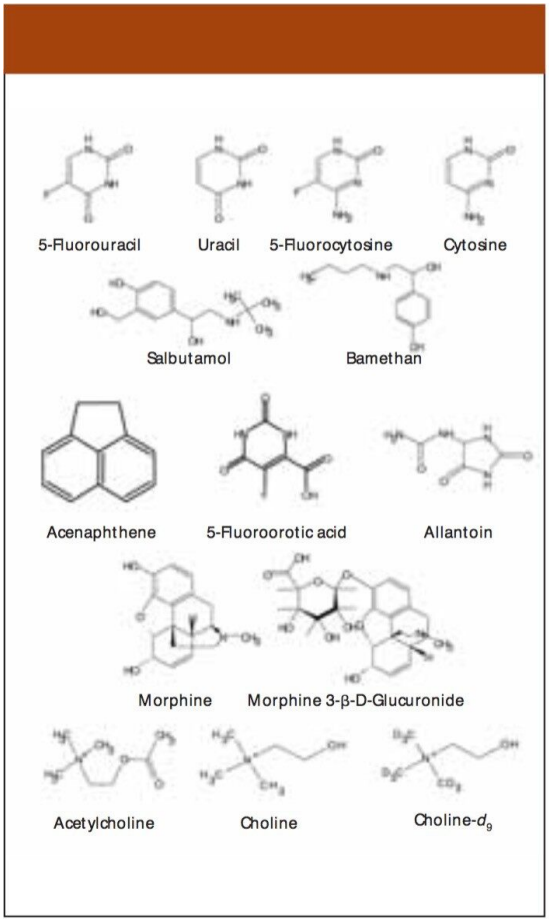

Figure 1. Structures of the compounds used for analysis.

Preparation of Mobile Phase Buffers and Samples

A 1 L volume of 200 mM ammonium formate buffer was prepared by weighing out 12.6 g of formic acid ammonium salt and adding it to 980 mL of water in a 1 L volumetric flask. Formic acid was added to the solution to adjust the final pH to 3.0, and water was then added to the 1 L mark. A second, 1 L volume of 200 mM ammonium formate buffer was prepared identically to the pH 3.0 solution with the exception of adjusting the final pH to 4.0. A 1 L volume of 200 mM ammonium acetate buffer was prepared by weighing out 15.42 g of ammonium acetate salt and adding it to 980 mL of water into a 1 L volumetric flask. Acetic acid was added to the solution to adjust the final pH to 5.0, and water was then added to the 1 L mark.

Samples for analysis by HILIC were prepared in 75:25 (v/v) 0.2% formic acid acetonitrile–methanol. Samples eluted from an SPE device were prepared in 5.0% formic acid in 60:40 (v/v) isopropanol–acetonitrile. Samples for analysis for reversed-phase chromatography were prepared in 0.2% formic acid in water. Samples for

infusion for LC–MS optimization were prepared in 2.0% formic acid in 60:40 (v/v) isopropanol–acetonitrile.

LC–MS Optimization

LC–MS parameters were optimized by performing infusions of 1 ng/µL solutions of each of the analytes by introducing them at 20 µL/min via a mixing tee into the mobile phase pumped by the HPLC system at 200 µL/min.

Artificial Cerebral Spinal Fluid Preparation

Several stock solutions were prepared to mimic the salt content of cerebral spinal fluid. 1450 mM sodium chloride (8.474 g/100 mL), 27 mM potassium chloride (0.201 g/100 mL), 10 mM magnesium chloride (0.203 g/100 mL), 12 mM calcium chloride (0.176 g/100 mL), and 20 mM dibasic sodium phosphate salt adjusted to pH 7.4 with phosphoric acid, (0.284 g/100 mL) stock solutions were prepared. A 1 mL volume of each stock solution was combined in a 10 mL volumetric flask and diluted with 5 mL of 18.2 MΩ-cm MILLI-Q water.

SPE procedure for choline, acetylcholine, and choline-trimethyl-d_9 in artificial cerebral spinal fluid: A solution spiked with a concentration of 2.5 ng/mL was prepared. A 25 µL volume of a mixture of acetylcholine, choline, and choline-trimethyl-d_9 (500 ng/mL each) in water was added to 4875 µL of artificial cerebral spinal fluid. A 100 µL volume of ammonium hydroxide was then added to basify the spiked solution at a final concentration of 2% (v/v). An artificial cerebral spinal fluid blank was prepared by adding 100 µL of ammonium hydroxide to 4900 µL of artificial cerebral spinal fluid to yield a final concentration of 2% (v/v) ammonium hydroxide. SPE was performed using Oasis WCX (Waters) polymeric mixed-mode cation-exchange material in a 96-well low-elution device format that contained 2 mg/well of sorbent. The sorbent was conditioned with 200 µL of methanol and then equilibrated with 200 µL of MILLI-Q water. A 40 µL volume of the spiked solution was loaded onto the sorbent. The sorbent was washed with 100 µL of MILLI-Q water followed by a second wash containing 100 µL of methanol. Finally, the analytes of interest were then eluted with 40 µL of 5% (v/v) formic acid in 60:40 (v/v) isopropanol–acetonitrile. The eluent then was injected directly onto the silica column. The experiment was conducted with six replicates. To calculate the absolute recovery, 270 µL of an extracted artificial cerebral spinal fluid blank was pooled from 12 wells and spiked with 30 µL of a 25 ng/mL mixture of acetylcholine, choline, and choline-trimethyl-d_9 in water to yield a final concentration of 2.5 ng/mL. The following equation was used to calculate the absolute recoveries:

$$R_{abs} = 100(A_{extr}/A_{spike})$$

where R_{abs} is the absolute recovery, A_{extr}, is the average peak area of the extracted quantity, and A_{spike} refers to the average peak area of the extract spiked with a known quantity of the compound. LC–MS conditions for salbutamol and bamethan: Analytical column: Atlantis HILIC Silica and Atlantis dC_{18}, 50 mm x 2.1 mm, 3 µm

d_p, at ambient temperature; mobile phase: acetonitrile–water gradient from 0 to 50% acetonitrile (reversed phase) and 90 to 50% acetonitrile (HILIC) containing 10 mM ammonium formate adjusted to pH 3.0; flow rate: 0.2 mL/min; injection volume: 10 µL; run time: 5 min; mass spectrometer: Waters ZQ 2000; ionization: positive-ion electrospray (ESI+); mode: SIR, salbutamol (*m/z* 239.8), bamethan (*m/z* 209.9); cone gas flow rate: 50 L/h; desolvation gas flow: 450 L/h; capillary voltage: 3.5kV; cone voltage: 20V; extractor voltage 3.0V; RF lens: 0.3V; source temperature: 150 °C; desolvation temperature 200 °C (HILIC), 300 °C (reversed-phase).

LC–MS conditions for choline, acetylcholine, and choline-trimethyl-d_9: Analytical column: Atlantis HILIC Silica, 50 mm x 2.1 mm, 3 µm, at ambient temperature; mobile phase: 86:14 (v/v) acetonitrile–water containing 10 mM ammonium formate adjusted to pH 3.0; flow rate: 0.3 mL/min; injection volume: 20 µL; run time: 7 min; retention time: acetylcholine: 3.2 min; choline: 4.7 min; choline-trimethyl-d_9: 4.8 min; mass spectrometer: Waters ZQ 2000; ionization: positive ion electrospray (ESI+); mode: SIR, acetylcholine (*m/z* 146.20), choline (*m/z* 103.9), choline-trimethyl-d_9 (*m/z* 113.10); cone gas flow rate: 50 L/h; desolvation gas flow: 700 L/h; capillary voltage: 1.0kV; cone voltage: acetylcholine: 15V; choline: 30V; choline-trimethyl-d_9: 30V; extractor voltage: 3.0V; RF lens: 0.3V; source temperature: 150 °C; desolvation temperature 350 °C.

Results and Discussion

Retention mechanisms and characteristics of silica columns with aqueous–organic mobile phase

Retention mechanisms are multimodal on silica.[26] A combination of hydrophilic interaction, ion-exchange, and reversed-phase retention result in a unique selectivity. The HILIC mechanism involves partitioning between the adsorbed polar component of the mobile phase and the remaining hydrophobic component of the mobile phase. In this mechanism, the polar analyte partitions into and out of the adsorbed water layer on the negatively charged silica surface.[17] Additionally, depending upon the pH of the mobile phase, a positively charged (basic) analyte can undergo cation exchange with the negatively charged silanol groups. Therefore, the second retention mechanism is ion exchange.[27] A third mechanism that can occur is reversed-phase retention by interaction with the siloxane bridges on the silica surface. This interaction is rather weak compared to the retention on C_{18} bonded phases.[28] As organic content is increased above 5%, retention is lost. The most retention is observed in mobile phases containing greater than 70% acetonitrile. An increase in the aqueous content would decrease retention because water is a very polar solvent and is the strongest elution solvent under HILIC conditions.[26] The combination of these mechanisms results in enhanced polar

retention. The retention characteristics of acidic, basic, and neutral analytes were examined at pH 3, 4, and 5. Figure 2 demonstrates the influence of acetonitrile concentration on the retention of cytosine, 5-fluoroorotic acid, and acenaphthene on silica. As the aqueous content of the mobile phase is increased, the retention of the acidic and basic analytes decreases while the retention of the neutral analyte increases slightly. The greatest retention was observed above 70% acetonitrile for cytosine and 5-fluoroorotic acid. Mobile-phase pH offered little selectivity difference for these analytes due to the combination of a lack of change in the ionization state of the analyte in the examined pH range and consistent hydrophilic interaction between the analyte and the adsorbed aqueous layer. Only cytosine was affected by the interaction with free silanols on the silica surface due to its positive charge.

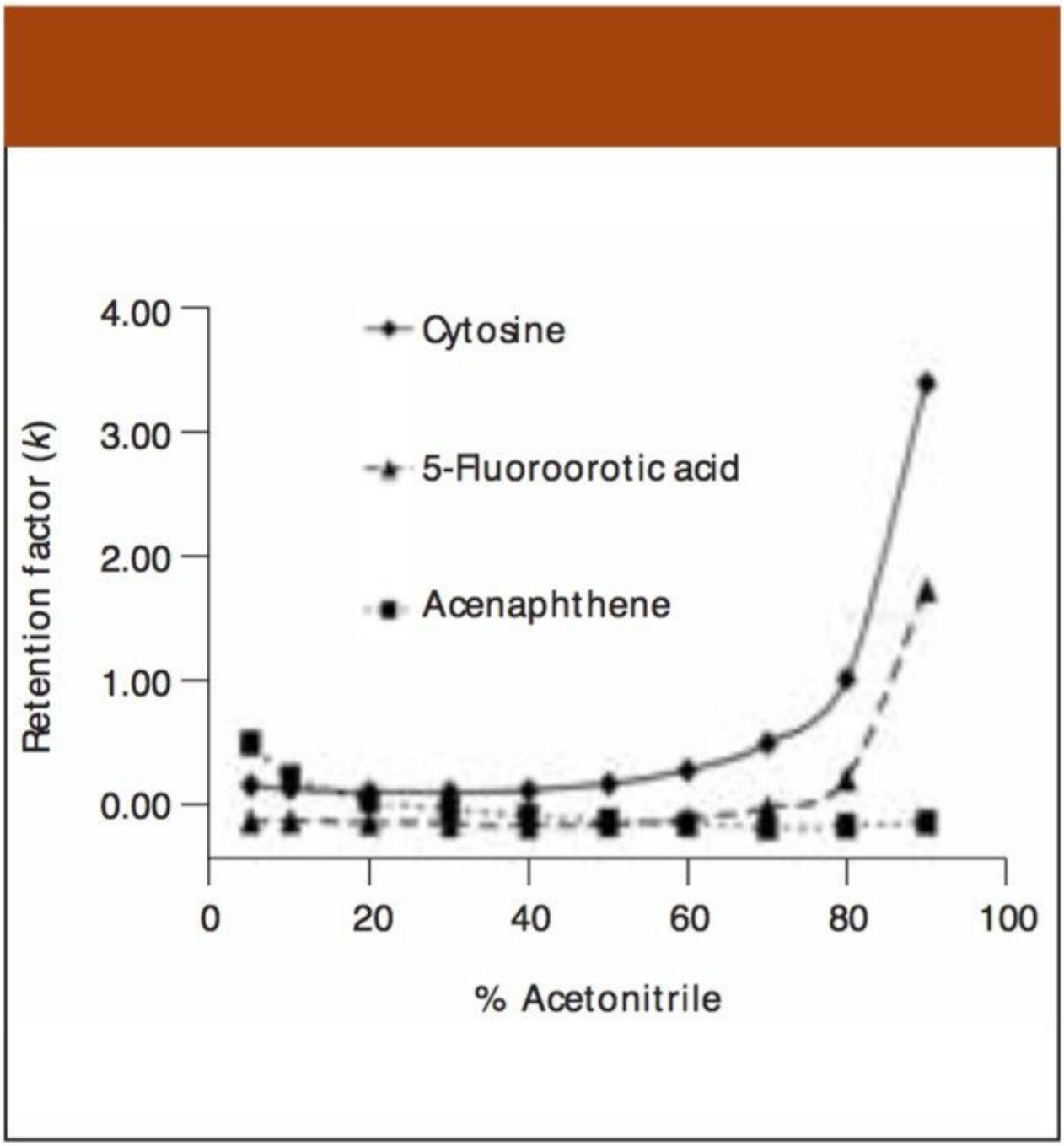

Figure 2. Influence of mobile phase acetonitrile concentration on retention (k) of cytosine, 5-fluoroorotic acid, and acenaphthene. The average retention of each analyte at pH 3, 4, and 5 is summarized into a single line. Column: 50 mm x 2.1 mm, 3 µm d_p Atlantis HILIC Silica; mobile phase: acetonitrile–water, containing 10 mM ammonium formate (pH 3.0 and 4.0) or ammonium acetate (pH 5.0), where acetonitrile varied from 0 to 90%.

In summary, the retention of polar analytes in acidic mobile phases is possible due to the strong hydrophilic interaction between the adsorbed aqueous layer on the silica surface and the analyte. Because of

this interaction, the greatest retention will be observed above 70% acetonitrile. As the aqueous content of the mobile phase is increased, the observed retention of the analyte will decrease due to the strong elution strength of water in HILIC mode.

Selectivity: HILIC versus reversed-phase

When developing analytical methods, a number of reversed-phase columns can be used that differ in selectivity to obtain a desired separation. HILIC offers a selectivity that is complementary to traditional reversed-phase methods and often aids in the retention of analytes that are not retained by reversed-phase columns. The retention and separation of morphine and its polar metabolite morphine 3-β-glucuronide was achieved by both HILIC and reversed-phase chromatography to demonstrate the complementary selectivity that HILIC offers. As shown in Figure 3, both techniques offer acceptable peak shape and retention for these two analytes. A reversal in elution order is obtained on the HILIC column; the polar metabolite is eluted after morphine.

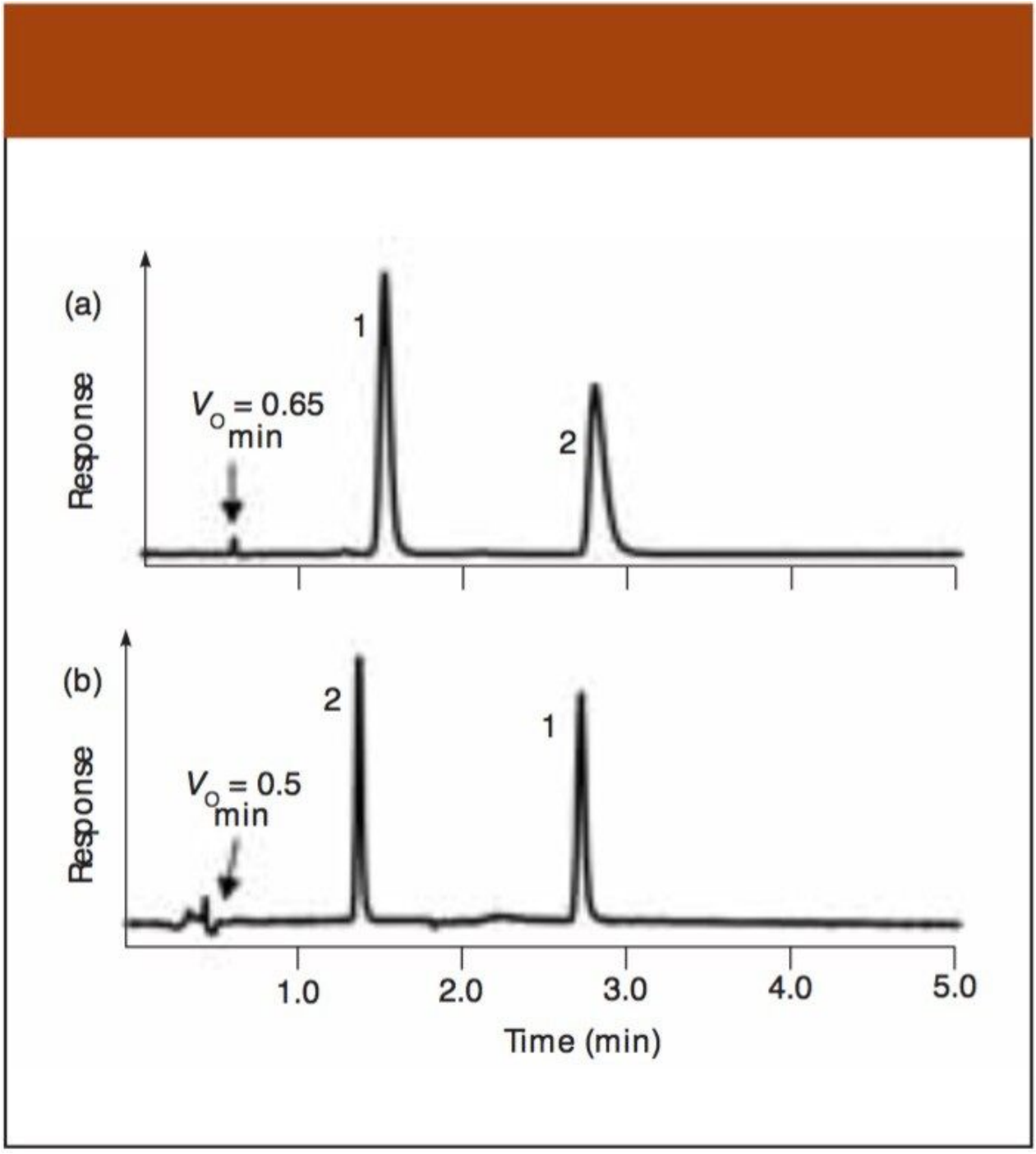

Figure 3. Chromatograms comparing the selectivity differences of HILIC to reversed-phase LC. (a) Column: 50 mm x 4.6 mm, 3 µm d_p Atlantis dC_{18}; mobile phase: 2:98 (v/v) acetonitrile–water containing 10 mM ammonium formate, pH 3; flow rate: 1.4 mL/min; injection volume: 5 µL; sample: 125 µg/mL mixture of morphine 3-β-glucuronide and morphine; column thermostated at 30 °C; detection: UV absorbance at 280 nm. (b) Column: 50 mm x 4.6 mm, 3 µm d_p Atlantis HILIC Silica; mobile phase: 5 min gradient from 90 to 50% acetonitrile (the mobile phase contained a constant 10 mM concentration of pH 3 ammonium formate); flow rate: 2.0 mL/min; injection volume: 5 µL; sample: 125 µg/mL mixture of morphine and morphine 3-β-glucuronide; column temperature: thermostated at 30 °C; detection: UV absorbance at 280 nm. Peaks: 1 = morphine 3-β-glucuronide, 2 = morphine.

To demonstrate the retention benefits of HILIC, allantoin (commonly used in cosmetic creams[29]) was analyzed by both HILIC and reversed-phase chromatography. Figure 4 shows that allantoin was not retained

under reversed-phase conditions, even with 100% aqueous mobile phase conditions. However, retention was achieved by using a silica column under HILIC conditions, yielding a retention factor of 1, demonstrating the retention benefits of this technique.

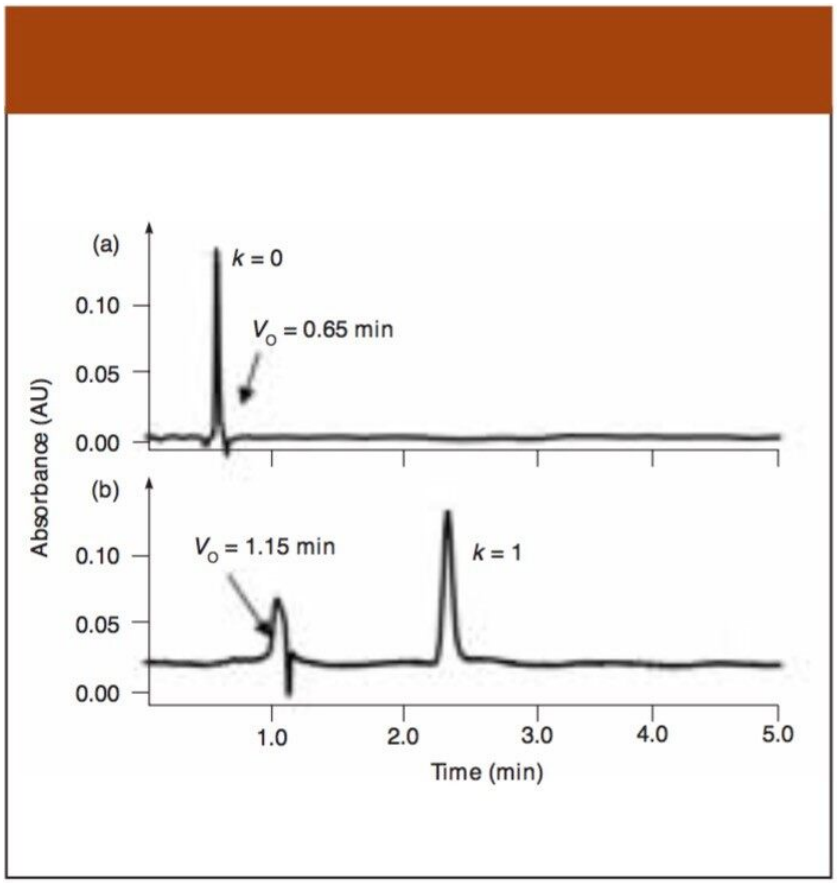

Figure 4. Chromatograms comparing the retention of allantoin on Atlantis HILIC Silica and Atlantis dC_{18} Columns. (a) Column: 50 mm x 4.6 mm, 3 µm d_p Atlantis dC_{18}; mobile phase: 10 mM ammonium formate, pH 3; flow rate: 1.4 mL/min; injection volume: 5 µL; sample: 0.25 mg/mL; column temperature: thermostated at 30 °C; detection: UV absorbance at 205 nm. Shows no retention (k = 0) of allantoin. (b) Column: 50 mm x 4.6 mm, 3 µm d_p Atlantis HILIC Silica; mobile phase: 95:5 (v/v) acetonitrile–water containing 10 mM ammonium formate, pH 3; flow rate: 1.0 mL/min; injection volume: 5 µL; sample: 0.25 mg/mL; column temperature: thermostated at 30 °C; detection: UV absorbance at 205 nm. Shows retention (k = 1) of allantoin.

Mobile phase considerations for optimizing performance of a silica column for HILIC

When optimizing the chromatographic performance of silica columns for use with organic–aqueous mobile phases, several factors should be considered. Typically, we have used acetonitrile as the weak solvent. Initial mobile phase conditions should not be more than 95% or less than 70% acetonitrile. At least 5% of the mobile phase should be a polar solvent (such as water or methanol) due to the partitioning mechanism explained previously. In addition, it is necessary for the mobile phase to contain 5% water if using a buffer due to the solubility of most buffer salts in organic solvents.

For optimum performance and reproducibility when using buffers or additives, at least a 10 mM buffer concentration or 0.2% additive concentration should be introduced on column. This is important to yield the best possible peak shapes and reproducible retention times. Phosphate buffers are not recommended due to their insolubility in high organic mobile phases (>60% organic) as well as their lack of volatility, rendering them incompatible with ESI-MS.

Bonded silica has a typical operating range from pH 2 to 8. Mobile phases above pH 8 will result in dissolution of the silica particle and below pH 2 results in ligand hydrolysis. Silica columns without a bonded phase have an operating pH range extendsing well into the acidic pH range to below pH 1. Additives such as phosphoric acid, formic acid, and trifluoroacetic acid have been used at concentrations ranging up to 1.0% on column. Buffers such as ammonium formate at pH 3 and ammonium acetate at pH 5 have been used at concentrations ranging from 5 mM to 20 mM on column. It is important to note that unbonded silica is more susceptible to particle dissolution at midlevel pH and should not be used above pH 6.

When developing a HILIC method for analytes not retained by reversed-phase chromatography, first run an initial scouting gradient from 90 to 50% acetonitrile to see where the analytes are eluted over the course of the gradient. If the analytes are not retained, then an isocratic method of 95% acetonitrile can be run. To further increase the retention of the analytes, replacing some of the water in the mobile phase with another polar solvent such as methanol or isopropanol can lead to increased retention as shown in Figure 5. These solvents exhibit weaker elution strength than water under HILIC mode. Additionally, replacing acetonitrile with acetone as the primary solvent can help manipulate retention and selectivity. A unique solvent selectivity on silica under HILIC mode can be observed. The solvent elution strength (from weakest to strongest) under HILIC conditions on silica is: tetrahydrofuran < acetone < acetonitrile < isopropanol < ethanol < methanol < water, where water is the strongest elution solvent. This unique selectivity can be used as a powerful tool in method development.

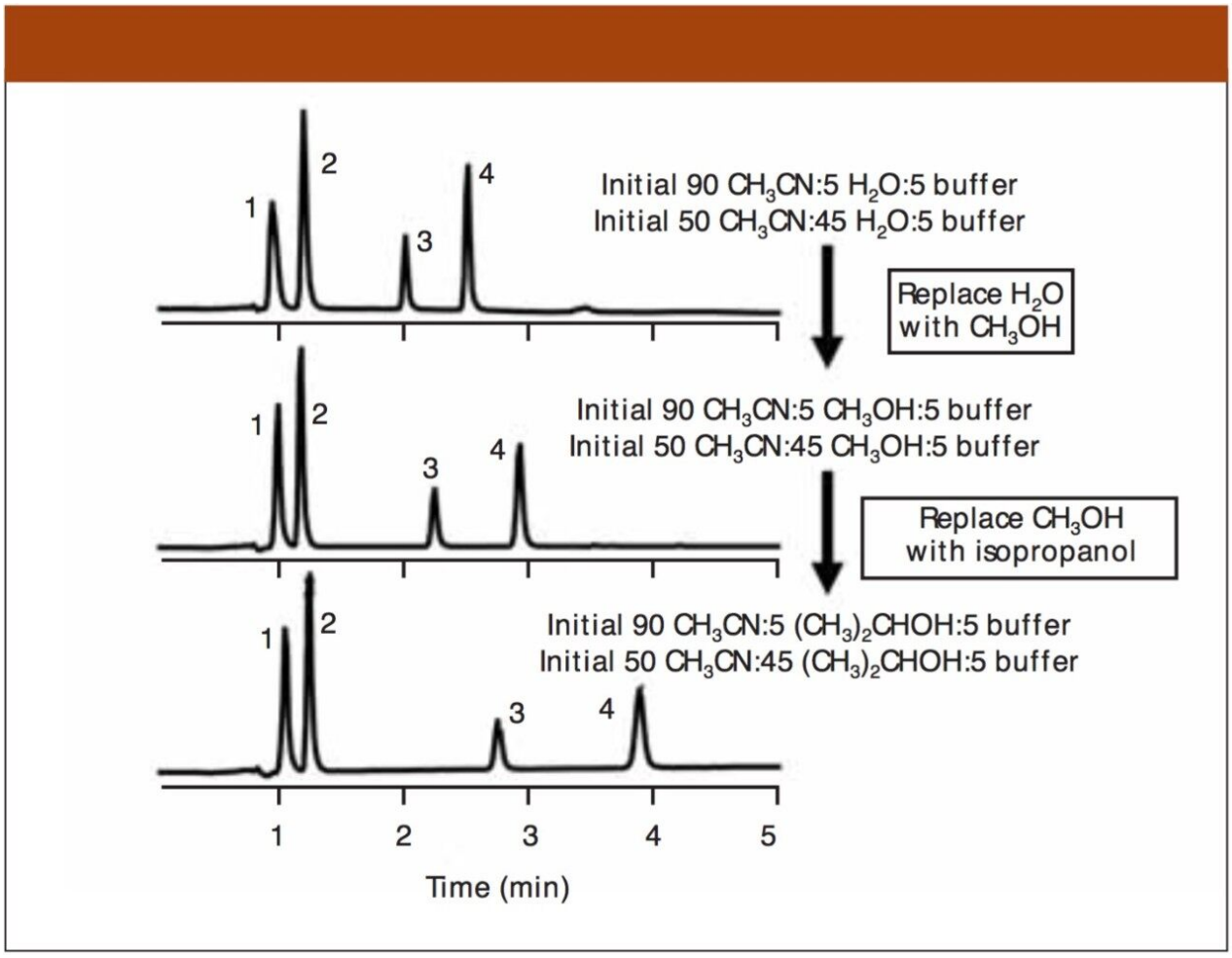

Figure 5. Influence of polar solvents on selectivity. Analyte retention is increased by utilizing a less polar solvent. Column: 50 mm x 4.6 mm, 3 µm d_p Atlantis HILIC Silica; mobile phase: 5 min gradient from 90 to 50% acetonitrile and 5 to 45% water, methanol, or isopropanol (the mobile phase contained a constant 10 mM concentration of pH 3 ammonium formate); flow rate: 1.0 mL/min; sample: 25 µg/mL mixture of 5-fluorouracil (V_o), uracil, 5 fluorocytosine, and cytosine; injection volume: 2 µL; column temperature: thermostated at 30 °C; detection: UV absorbance at 254 nm. Peaks: 1 = 5-fluorouracil (V_o), 2 = uracil, 3 = 5-fluorocytosine, 4 = cytosine.

The importance of the sample diluent on peak shape and solubility

The sample diluent also is an important parameter in HILIC. As in reversed-phase chromatography, the sample diluent can strongly influence the peak shape of the analytes.[30] The sample diluent should be as close to the initial mobile phase conditions as possible. In HILIC, the sample should be dissolved in a high percentage of organic solvent so that it is compatible with the high-organic mobile phase. However, polar

analytes often have low solubilities in organic solvents.

To determine a generic diluent to solubilize a majority of polar analytes for use in HILIC, a mixture of four polar basic analytes was dissolved in a number of combinations of water, acetonitrile, and methanol. Figure 6 shows the influence of several diluents on peak shape and peak area. The peak shape improves as the aqueous content of the diluent is decreased. However, if the aqueous content is removed completely and the sample mixture is prepared in 100% methanol, wide peaks are observed and the resolution of the compounds decreases. As the proportions of the diluent were modified to favor the less polar solvent (acetonitrile), peak shape and solubility improved. It was determined that 75:25 (v/v) acetonitrile–methanol is the best compromise for solubility and peak shape.

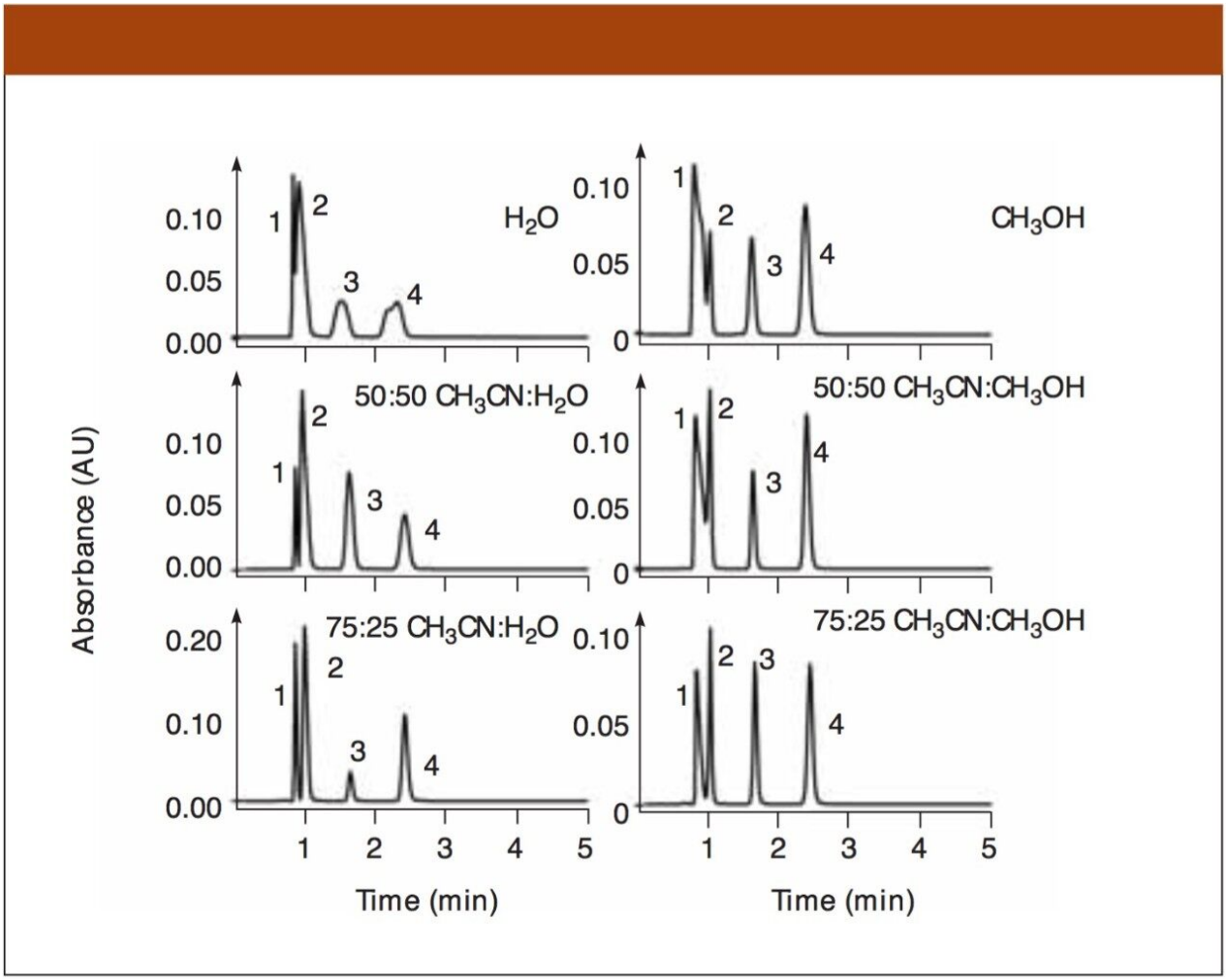

Figure 6. Influence of sample diluent on solubility and peak shape. Column: 50 mm x 4.6 mm, 3 µm d_p Atlantis HILIC Silica; mobile phase: 88:12 (v/v) acetonitrile–water containing 12 mM ammonium formate, pH 3; flow rate: 1.0 mL/min; injection volume: 10 µL; sample: 25 µg/mL mixture of 5-fluorouracil (V_o), uracil, 5-fluorocytosine and cytosine; sample diluent: as stated; column temperature: thermostated at 30 °C; detection: UV absorbance at 254 nm. Peaks: 1 = 5-fluorouracil (V_o), 2 = uracil, 3 = 5-fluorocytosine, 4 = cytosine.

Dimethyl sulfoxide is a common diluent used in preparative chromatography and combinatorial chemistry due to its ability to solubilize a large number of compounds. Although dimethyl sulfoxide is versatile, extremely poor peak shape was observed when it was used as a diluent under HILIC conditions and is therefore not recommended. Figure 7 illustrates this poor peak shape. The addition of methanol improves peak shape, but the peaks are still wide. If acetonitrile is mixed with dimethyl sulfoxide, an improvement of peak shape can be observed. If it is absolutely necessary to have dimethyl sulfoxide in the diluent, 75:25 (v/v)

acetonitrile–dimethyl sulfoxide will exhibit the best possible results. It should be noted that the solubility in various organic solvent combinations is analyte-dependent and should be optimized accordingly.

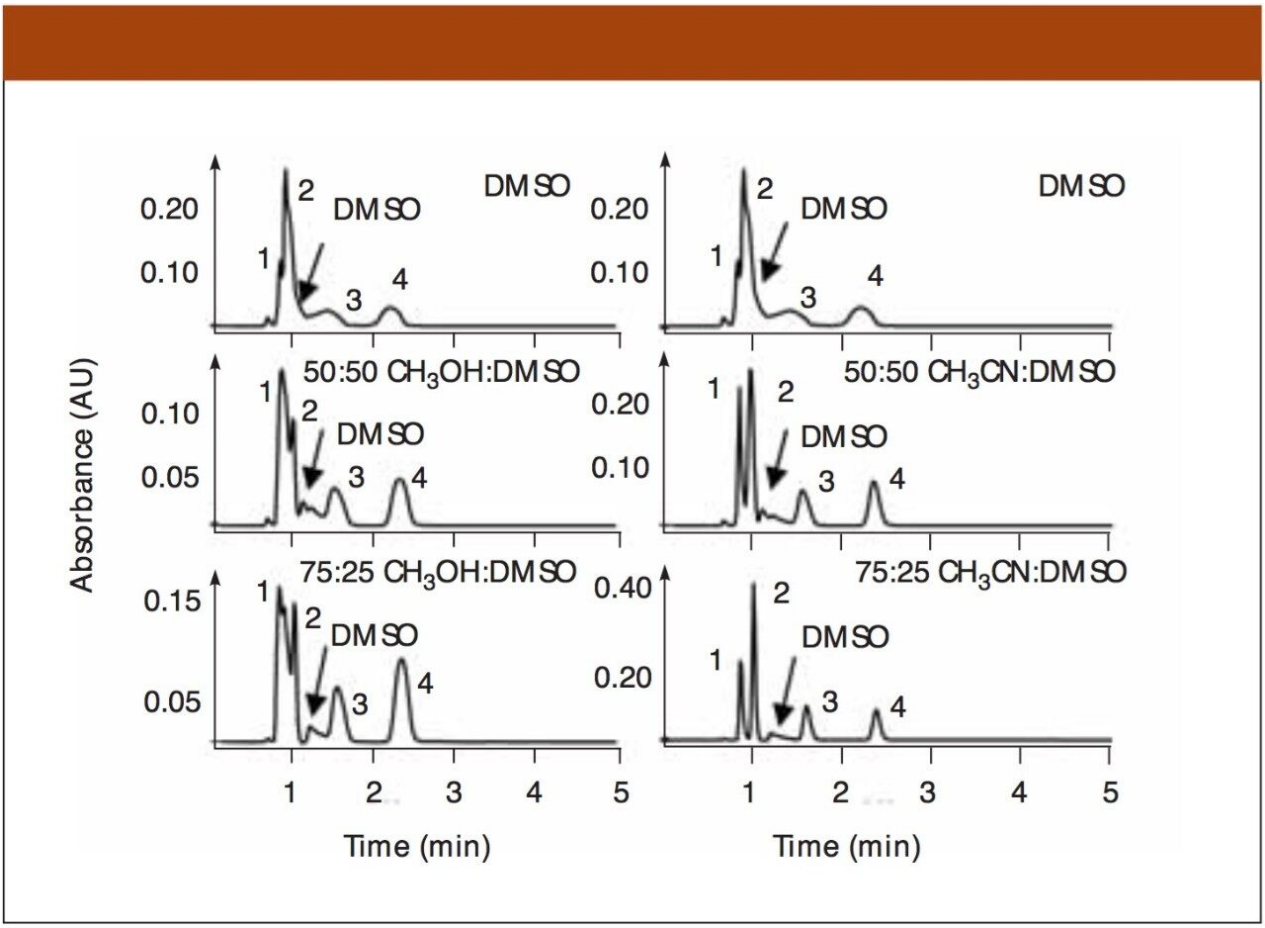

Figure 7. Influence of sample diluent containing dimethyl sulfoxide (DMSO) on solubility and peak shape. Column: 50 mm x 4.6 mm, 3 µm d_p Atlantis HILIC Silica; mobile phase: 88:12 (v/v) acetonitrile–water containing 12 mM ammonium formate, pH 3; flow rate: 1.0 mL/min; injection volume: 10 µL; sample: 25 µg/mL mixture of 5-fluorouracil (V_o), uracil, 5 fluorocytosine and cytosine; sample diluent: as stated; column temperature: thermostated at 30 °C; detection: UV absorbance at 254 nm. Peaks: 1 = 5-fluorouracil (V_o), 2 = uracil, 3 = 5-fluorocytosine, 4 = cytosine.

Enhanced sensitivity in ESI-MS

To study the sensitivity differences between HILIC and reversed-phase chromatography using ESI-MS, the analytes were selected carefully so that retention could be achieved in both modes. Salbutamol and bamethan are compounds that are retained easily by reversed-phase columns and also are retained under HILIC. Salbutamol is a fast-acting bronchodilator used for the treatment of asthma and other lung obstructing

diseases and bamethan is used commonly as its internal standard (28). The extracted ion chromatograms in Figure 8 demonstrate the sensitivity and selectivity differences between the two techniques. Salbutamol (100 ng/mL) and bamethan (50 ng/mL) were analyzed under both HILIC and reversed-phase LC and yield a considerable sensitivity increase by using HILIC-ESI-MS compared with reversed-phase LC-ESI-MS. In Table 1, the peak area for salbutamol and bamethan demonstrates the sensitivity increase of three and four orders of magnitude on the silica column, respectively. This increase in sensitivity is due to the utilization of highly volatile mobile phases, which is ideal for efficient desolvation and compound ionization, in effect enhancing signal response in MS. This enhancement effect will allow for low limits of detection.

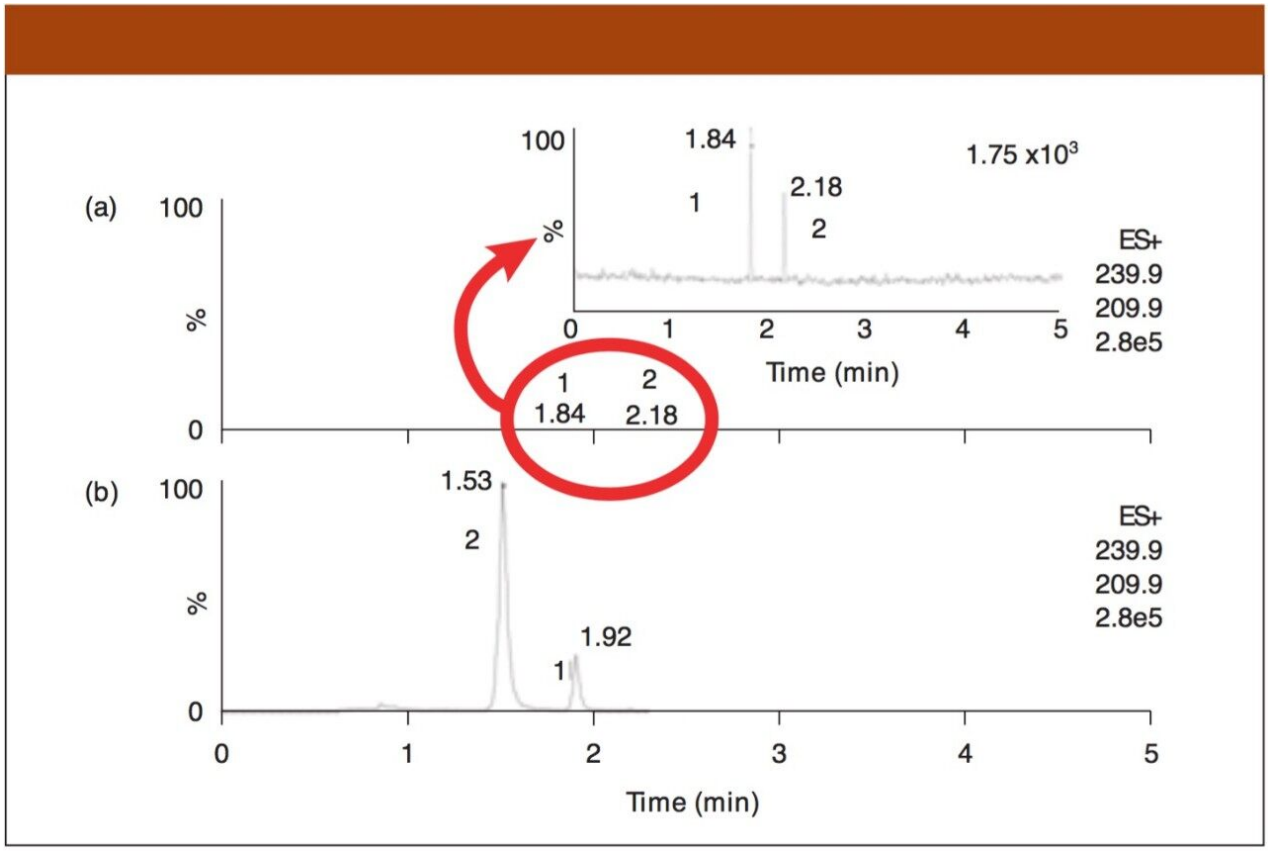

Figure 8. Extracted ion chromatograms demonstrating improved signal response with HILIC. Extracted ion chromatograms for HILIC and reversed-phase LC are set to the same scale. LC–MS total ion chromatograms of SIR channels m/z 239.8 (salbutamol) and m/z 209.9 (bamethan). Sample concentrations: salbutamol: 100 ng/mL; bamethan: 50 ng/mL. (a) Column: 50 mm x 2.1 mm, 3 µm d_p Atlantis dC_{18}; 5 min gradient from 0 to 50% acetonitrile. Inset shows the data normalized to the peak height of salbutamol. (b) 50 mm x 4.6 mm, 3 µm d_p Atlantis HILIC Silica; mobile phase: 5 min gradient from 90 to 50% acetonitrile. See experimental section for details. Peaks: 1 = salbutamol, 2 = bamethan.

Column	Analyte	Concentration	Peak area
Atlantis dC_{18}	Salbutamol	100	(ND)
Atlantis dC_{18}	Bamethan	50	(ND)
Atlantis HILIC Silica	Salbutamol	100	19,567
Atlantis HILIC Silica	Bamethan	50	110,085

Table 1. Peak area comparison.

Low limits of detection

In order to demonstrate low detection limits when running bioanalytical assays in HILIC, analytes were chosen that required detection at extremely low levels. Choline and acetylcholine are two quaternary amine compounds that were selected to demonstrate the increased sensitivity obtained in HILIC. Choline is important for phospholipid formation, normal membrane function, and the synthesis of neurotransmitters such as acetylcholine.[32]

Many methods for the quantitation of low levels of these compounds have been investigated, such as gas chromatography (GC)–MS, LC–MS, ion-pairing, LC–ESIisotope doping MS (IDMS),[32] and LC with electrochemical detection.[33] LC with electrochemical detection is by far the most commonly used technique to detect choline and acetylcholine due to its sensitivity. On column detection limits have been reported that range from 10 fmol to 10 pmol depending upon the method.[33]

An HILIC–ESI-MS method was developed that offers a unique selectivity that retains and resolves choline and acetylcholine. Extracted ion chromatograms demonstrating the retention and selectivity of these analytes are shown in Figure 9. The highly volatile HILIC mobile phase results in a limit of detection (LOD) of 0.1 ng/mL (15 fmol) for acetylcholine on a singlequadrupole mass spectrometer. A number of analyte concentrations were investigated to determine the LOD and limit of quantitation (LOQ) of this method using HILIC–ESI-MS. Data from the calibration curves generated are listed in Table 2. choline-trimethyl-d_9 was used as the internal standard held at a constant 5 ng/mL. Responses for choline and acetylcholine were analyzed over the working range of 0.1–100 ng/mL, using weighted (1/x) linear regression with correlation coefficients of 0.9948 and 0.9993, respectively. Once experimentation was conducted, a limitation of the linear range for choline was observed. Due to this limitation, the LOD and LOQ for choline were determined to be 1.0 ng/mL and 2.5 ng/mL, respectively. A 0.1 ng/mL concentration was the LOD for acetylcholine, based upon an S/N of 3:1. The LOQ for acetylcholine was determined to be 0.25 ng/mL based upon an S/N of 10:1.

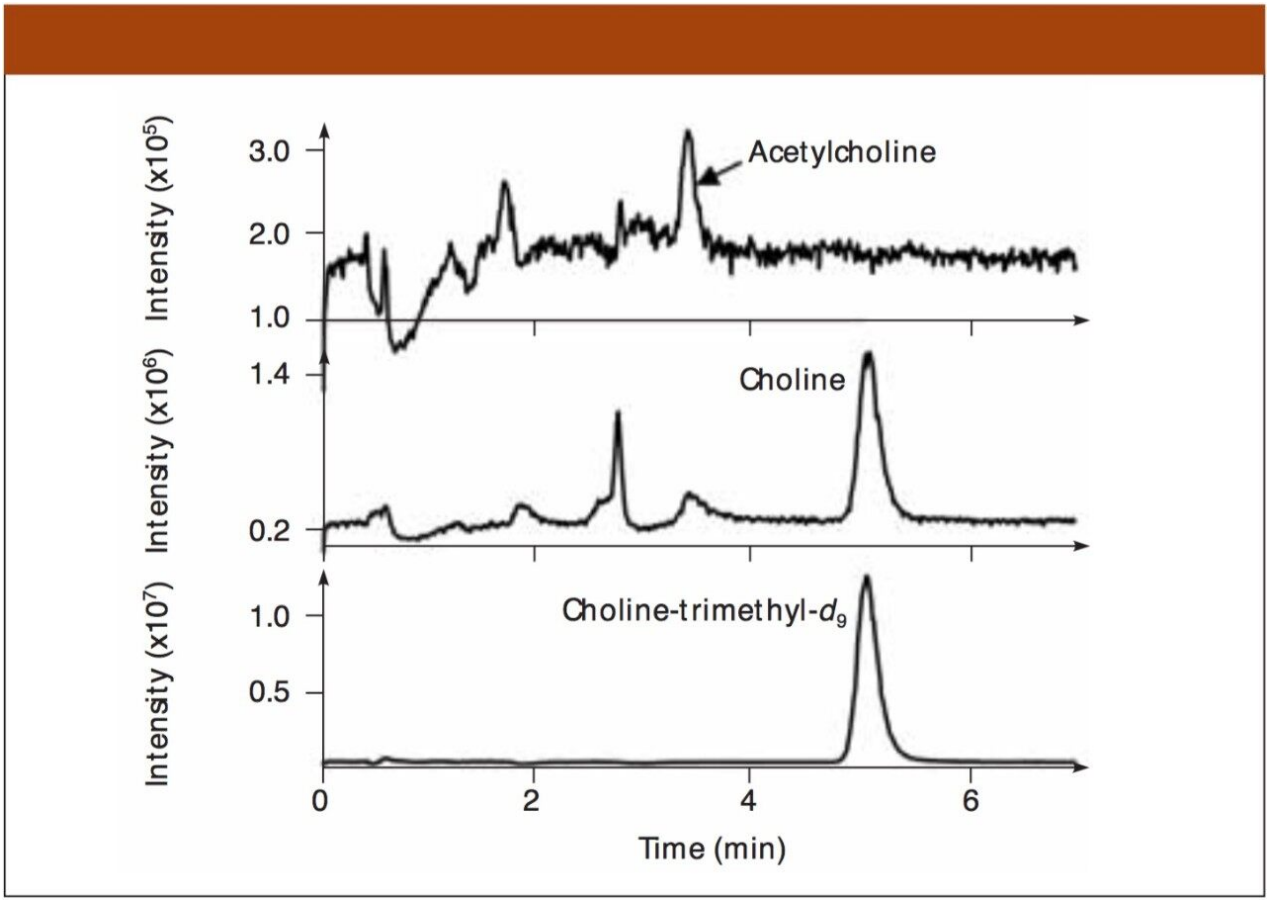

Figure 9. Low limits of detection achievable by a single quadrupole mass spectrometer. Shown are LC–MS SIR extracted ions of acetylcholine (m/z 146.2), choline (m/z 103.9), and cholinetrimethyl-d_9 (internal standard, m/z 113.1). Sample concentrations: acetylcholine, 0.1 ng/mL; choline, 1.0 ng/mL; choline-trimethyl-d_9, 5 ng/mL. See experimental section for details.

Acetylcholine (ng/mL)										
Nominal concentration	0.10	0.25	0.50	1.00	2.50	5.00	10.00	25.00	50.00	100.00
Calculated mean conc.	0.11	0.24	0.47	1.15	2.41	4.71	9.74	24.76	48.63	102.13
RSD (%)	4.88	4.59	0.93	3.26	1.98	1.93	2.45	1.66	1.82	1.05
RE (%)	8.65	-5.06	-5.32	15.38	-3.61	-5.88	-2.60	-0.95	-2.74	2.13
Choline (ng/mL)										
Nominal concentration	1.00	2.50	5.00	10.00	25.00	50.00	100.00			
Calculated mean conc.	1.37	2.43	4.17	8.29	23.47	49.50	104.24			
RSD (%)	0.79	2.22	1.44	0.97	0.53	0.63	1.33			
RE (%)	36.50	-2.63	-16.55	-17.07	-6.13	-1.00	-4.24			

Table 2. Precision and accuracy of calibration standards (N = 6).

The sensitivity gained by utilizing HILIC–ESI-MS allows detection levels obtainable on a tandem MS system with a single-quadrupole mass spectrometer. One could assume that sensitivity would increase at least 10 fold by using an MS-MS system, which would allow for detection limits of 0.01 ng/mL (1.5 fmol) or less for acetylcholine.

Simplified Sample Preparation

Traditional SPE methods often contain an elution step that uses a large concentration (>75%) of an organic solvent. This organic fraction is not always compatible with reversed-phase analysis, especially when the extracted sample has a higher organic content than the mobile phase. To make this extracted sample more compatible with the mobile phase, the high organic eluent is evaporated and then reconstituted in a solvent that more closely matches the mobile phase conditions. This process of evaporating and reconstituting is often the most lengthy and cumbersome of tasks.[34] In HILIC, the high organic eluent can be injected directly onto the column, thus eliminating the need for lengthy evaporation and reconstitution steps and increasing throughput.[18,26] Choline, acetylcholine, and choline-trimethyl-d_9 were extracted from artificial cerebral spinal fluid using a polymeric mixed-mode cation-exchange SPE material in a 96-well low elution device format with 5% formic acid in 60:40 (v/v) isopropanol–acetonitrile as the elution solvent. This elution solvent is weaker than the mobile phase 10 mM ammonium formate in 86:14 acetonitrile–water and was injected directly onto the HILIC column. Figure 10 shows extracted ion chromatograms of choline, acetylcholine, and choline-trimethyl-d_9 extracted from artificial cerebral spinal fluid. Analyte recovery was determined by comparing the area counts of the extracted samples to the area count of postextraction spiked blanks (n = 6), spiked with the same analyte concentration. Recovery results are reported in Table 3. The direct injection of SPE eluents onto an LC–MS system can eliminate potential loss of analytes during the

evaporation step. This not only will make the method more robust, it also will reduce variability.[26]

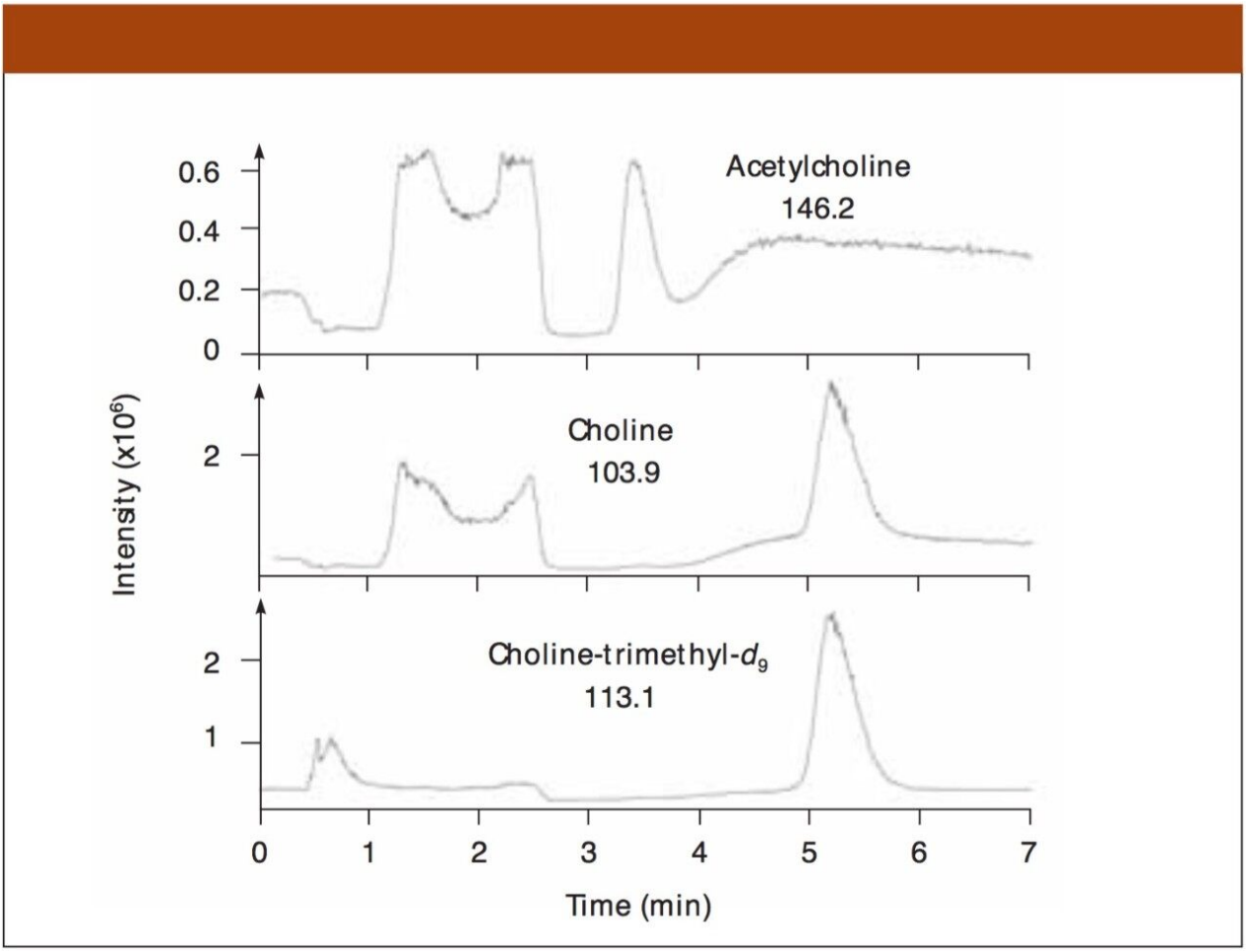

Figure 10. Direct injection of SPE eluent onto HILIC column. Shown are LC–MS SIR extracted ions of acetylcholine (m/z 146.2), choline (m/z 103.9), and choline-trimethyl-d_9 (internal standard, m/z 113.1). Sample concentrations: acetylcholine, 2.5 ng/mL; choline, 2.5 ng/mL; and choline-trimethyl-d_9 (internal standard), 2.5 ng/mL.

	Choline	Acetylcholine	Choline d_9
Nominal concentration (ng/mL)	2.50	2.50	2.50
Absolute recovery (%)	101.4	99.8	97.5
RSD (%)	2.52	1.64	2.78

Table 3. Absolute recoveries of acetylcholine, choline, and choline-d_9 (n = 6).

Conclusion

The use of silica columns for the retention of polar analytes by HILIC is a useful and complementary technique to reversed-phase chromatography. Retention mechanisms of aqueous–organic mobile phases on silica are a combination of hydrophilic interaction, ion-exchange, and reversed-phase retention that result in enhanced retention of polar analytes. Due to the strong hydrophilic interaction between the aqueous layer on the silica surface and the analyte, the greatest retention for basic and acidic analytes can be observed when using greater than 70% acetonitrile in acidic mobile phases. This high organic mobile phase is ideal for analyte desolvation and ionization, which leads to enhanced response in MS compared with traditional reversed-phase methods. This enhancement effect allows for lower limits of detection than in reversed-phase LC. When preparing samples for injection into HILIC mobile phases, the sample diluent must be close to the initial mobile phase conditions. Because water is the strongest elution solvent in HILIC, samples dissolved in high organic content (that is, extracted samples from an SPE device) can be injected directly onto the HILIC column. Potential loss of analytes during the evaporation step of an SPE method can be eliminated with the direct injection of the SPE eluent onto the silica column. This will reduce method variability and improve robustness.

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