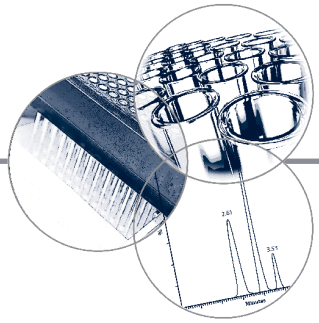

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Hydrophilic interaction chromatography (HILIC) for LC–MS/MS analysis of monoamine neurotransmitters

Background: Hydrophilic interaction chromatography (HILIC) is becoming an increasingly popular alternative to traditional reversed-phase chromatography for the analysis of polar compounds. The ability to retain the most polar compounds in HILIC makes it attractive for the analysis of certain large groups of compounds, such as monoamines, which are inherently very polar. **Results:** This paper details the development of a HILIC LC–MS/MS method for the analysis of monoamine neurotransmitters. The emphasis is on method development; in particular, the factors influencing sensitivity, peak shape and resolution. Mobile-phase ionic strength, temperature and stationary phase functionality are shown to be key parameters for the successful development of HILIC methods. **Conclusion:** HILIC is shown to be an appropriate and suitable method for the analysis of monoamine neurotransmitters and an attractive alternative to reversed-phase analysis. The most polar analytes, which are essentially unretained by reversed-phase chromatography, demonstrate superior retention and resolution when analyzed by HILIC.

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Hydrophilic interaction chromatography (HILIC) is increasingly becoming a method of choice for the analysis of polar compounds [1–5]. Many of the problems associated with analyzing very polar compounds by reversed phase LC (RPLC) stem from their lack of retention on the stationary phase. First and foremost is the lack of chromatographic resolution that can make unequivocal identification difficult. In some cases, this can necessitate LC conditions that use 100% aqueous mobile phases. Without an analytical column designed for such conditions, pore dewetting can occur, further compromising peak shape and resolution [6]. In the past, the most common method of improving retention under RPLC conditions has been the use of ion-pairing agents, such as octyl sulfate or lauryl sulfate to increase the retention of these compounds [7–9]. Unfortunately, these ion-pairing agents are not compatible with MS detection, so identification is limited to nonspecific detectors, such as electrochemical detection or fluorescence detection. Volatile ion-pairing reagents, which are compatible with MS detection, can also be used to increase retention of polar compounds by reversed-phase chromatography [10,11].

Another problematic issue concerns signal intensity in MS analyses. When compounds elute under high aqueous conditions, desolvation is less efficient, and signal intensity is not as high as it is under higher organic elution conditions [12]. Matrix effects may also become more common, as poorly retained compounds can elute with many unretained matrix components, such as salts and buffers. This can cause significant signal suppression or enhancement, compromising the ability to accurately quantify target analytes [13,14].

One set of polar analytes that poses particular challenges are the monoamine neurotransmitters, dopamine (DA), serotonin (5-HT), epinephrine (EP) and norepinephrine (NE). These compounds play a significant role in mood, movement and neurological disorders, such as depression, anxiety, schizophrenia and Parkinson's disease [15–17]. These neurotransmitters also play a critical role in the effects and toxicity of recreational drugs [18–20].

The most widespread method for the analysis of monoamine neurotransmitters is HPLC coupled to electron-capture dissociation. While this is a well-established method that has been refined over the years, it still has significant limitations. These limitations include its inability to unequivocally identify eluting peaks interference from closely eluting components and the need for ion-pairing reagents to improve retention [21].

These limitations have caused many researchers to develop various LC–MS methods for the

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Key Terms

Hydrophilic interaction chromatography (HILIC): A form of LC similar to normal-phase chromatography. Bare silica (or silica-hybrid) or polar-modified (e.g., amide) stationary phases are used, combined with high organic content (acetonitrile) mobile phases. Analytes are eluted based upon increasing polarity.

UPLC: A chromatography system consisting of sub-2-µm particle size packing materials and a chromatography system characterized by extremely low system volume coupled with high pressure tolerances.

Bridged ethylene hybrid: A hybrid polymer chromatographic particle consisting of a polyethoxysilane containing embedded ethyl groups. The resulting hybrid is characterized by low surface silanol activity, pH stability and very high pressure tolerances.

identification and quantification of these compounds, but nearly all use traditional reversed-phase retention mechanisms [21–26]. This work presents the application of HILIC for the analysis of monoamine neurotransmitters using a 2.5 µm hybrid particle bonded with an amide moiety. Retention is substantially improved when compared to reversed-phase analysis, especially for EP and NE, the most polar of these compounds. Separation and chromatographic resolution are also improved, allowing unequivocal identification of these closely related compounds in a short analysis time.

Methods

Chemicals & reagents

Formic acid and heptafluorobutyric acid (HFBA) were purchased from Fluka (Buchs, Switzerland). Pure water was produced in-house using a Millipore Elix® water purification system (MA, USA). Acetonitrile, methanol, ammonium acetate and ammonium hydroxide were obtained from Fisher Scientific (PA, USA). Hydrochloric acid was obtained from JT Baker (PA, USA). EP-HCl, NE-HCl, 5-HT-HCl, *N*-methylserotonin (NMS), Nonafluoropentanoic acid (NFPA) and ascorbic acid were all obtained from Sigma-Aldrich (MO, USA).

Standard preparation

Initial stock solutions of 1.0 mg/ml for all target analytes were prepared in methanol containing 5% HCl (to facilitate dissolution and prevent oxidation) and stored at -30°C. A working stock solution of 10 µg/ml 5-HT, DA, EP, NE and 1 µg/ml NMS was prepared in methanol containing 0.2% ascorbic acid. Working solutions of 100 ng/ml (10 ng/ml NMS) were freshly prepared daily in starting mobile phase conditions.

LC

All separations were performed on a Waters ACQUITY® **UPLC** system equipped with an ACQUITY Sample Manager and column manager from Waters Corp. (MA, USA). HILIC separations were performed using Waters XBridge™ BEH Amide XP and XBridge BEH HILIC XP columns (2.5 µm, 2.1 × 75 mm) at a flow rate of 0.5 ml/min. Mobile phase A (MPA) consisted of 95:5 water:acetonitrile containing either 10, 20, 50 or 100 mM ammonium formate buffered to pH 3.0. Mobile phase B (MPB) consisted of varying combinations of acetonitrile, water and ammonium formate (pH 3.0) that were adjusted to maximize the content of ammonium formate without adversely affecting the miscibility of the solution. The precise mobile phase compositions are detailed in Table 1. The concentrations of ammonium formate listed in Table 1 refer to the total concentration in the mobile phase, not just the aqueous portion. Initial mobile-phase conditions were 100% MPB. The percentage of MPA was increased to 30% over 2.5 min. The percentage of MPB was returned to 100% over 0.1 min and held there for 1.4 min. The total cycle time was 4.0 min. The injection volume was 20 µl. With the exception of experiments in which column temperature was changed, all separations were performed at 30°C.

Initial reversed-phase analysis was performed on a Waters XBridge C_{18} column (2.5 µm, 2.1 × 75 mm), which was chosen to match the base particle composition and dimensions of the HILIC columns exactly. MPA was MilliQ water with 0.1% formic acid and MPB consisted of acetonitrile with 0.1% formic acid. Initial mobile phase conditions were 100% MPA. For analysis on the 2.5 µm particle column, following a 0.5 min. hold, the percentage of MPB was changed in a linear gradient from 0 to 30% over 1.5 min. The percentage of MPB was then returned to 0% over 0.1 min and held there for the duration of the analytical run. The total cycle time was 4 min and the flow rate was 0.5 ml/min.

Reversed-phase analysis was also conducted using a Waters Atlantis® T3 column (3.0 µm, 2.1 × 100 mm). Various aqueous mobile phases were investigated, including formic acid and 10 mM ammonium formate (pH 3.0). Acetonitrile was used for the organic mobile phase.

For the investigation of ion-pairing reagents, 0.01% of either NFPA or HFBA was added to MPA and MPB used for reversed phase analysis. The resulting mobile phases contained 0.1% formic acid and 0.01% of either NFPA or HFBA. The solvent gradients started at 90% MPA:10% MPB. Following a 0.5 min hold, the percentage of MPB was increased to 80% over 2.5 min. The mobile phase was then returned to starting conditions (90:10, MPA:MPB) over 0.1 min and held at initial conditions for 1.4 min. The total cycle time was 4.5 min.

MS

MS detection was performed using a Waters Xevo™ TQ-S triple-quadrupole MS system (Waters Corp.) equipped with an ESI interface. The source block temperature was 100°C.

Desolvation gas flow (nitrogen) was 900 l/h. Cone gas flow was 150 l/h. Desolvation temperature was 350°C. The capillary voltage was 2.0 kV. Argon was used as the collision gas at a flow of 0.25 ml/min. The precursor ion for each compound was the protonated molecule $(M+H)^+$ with the exception of NE. For this molecule, the $(M+H)^+$ molecule lost an ammonia group in the ion source so the precursor molecule was $(M-NH_3)^+$. Multiple reaction monitoring transitions for target analytes are listed in Table 2. Data were acquired and analyzed using MassLynx Software (V4.1; SCN 810).

Results & discussion

The goal of this study was to evaluate and optimize the performance of HILIC chromatography for the separation of monoamine neurotransmitters and to develop an understanding of the influence of various chromatographic parameters on HILIC separations in general. We used NE, EP, DA, 5-HT and NMS as test compounds. Table 3 shows the structures of the compounds evaluated in this study. All are bases, with amine groups that are ionized at low pH and hydroxyl groups that increase their polarity.

Both XBridge HILIC and XBridge amide columns were evaluated, as well as traditional reversed-phase C_{18} columns. Both HILIC columns and one of the reversed-phase columns had the same base particle (Waters **Bridged ethylene hybrid**), particle sizes (2.5 µm), and dimensions (2.1 × 75 mm). An alternative reversed-phase column, a Waters Atlantis T3 column, was also chosen for evaluation based upon its tolerance for high aqueous mobile phases and enhanced retention for basic compounds. The dimensions of this column (3.0 µm, 2.1 × 100 mm) were as close a match as was available to the bridged ethylene hybrid columns used in this study.

■ Optimization of mobile-phase composition

For the initial separation, MPA and MPB each contained 10 mM ammonium formate. This separation can be seen in Figure 1A using the Waters XBridge BEH Amide XP column (2.5 µm; 2.1 × 75 mm). While peak shapes and resolution are acceptable for NMS and 5-HT, the three most polar analytes, DA, EP and NE, all show significant tailing. Retention mechanisms for HILIC are complex. While HILIC retention has mainly been attributed to partitioning of analytes between the mobile phase

Table 1. Compositions of mobile phase B.

Mobile phase B	ACN (%)	Water (%)	Ammonium formate (mM)
B1	95	5	10
B2	91.7	8.3	19
B3	90	10	20
B4	85	15	30

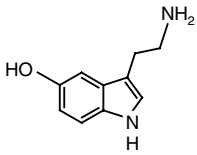
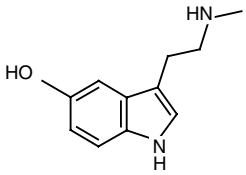
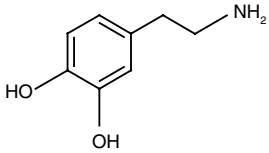
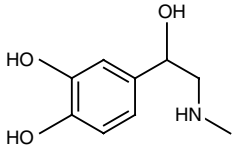
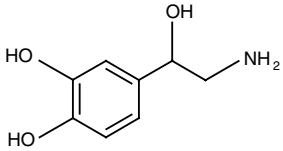
ACN: Acetonitrile.

Table 2. MS parameters used for analysis of monoamine neurotransmitters under hydrophilic interaction chromatography conditions.

Analyte	MRM transition (*m/z*)	Cone voltage (V)	Collision energy (V)
NMS	191.1 > 160	30	15
5-HT	177.0 > 160	14	8
DA	154 > 137	18	8
EP	184 > 166	12	8
NE	152 > 107	30	14

5-HT: Serotonin; DA: Dopamine; EP: Epinephrine; MRM: Multiple reacion monitoring; NE: Norepinephrine; NMS: N-methylserotonin.

Table 3. Chemical structures and LogP values for monoamine neurotransmitters.

Compund	Structure	LogP
Serotonin		0.48
N-methylserotonin		0.69
Dopamine		0.03
Epinephrine		-0.43
Norepinephrine		-0.68

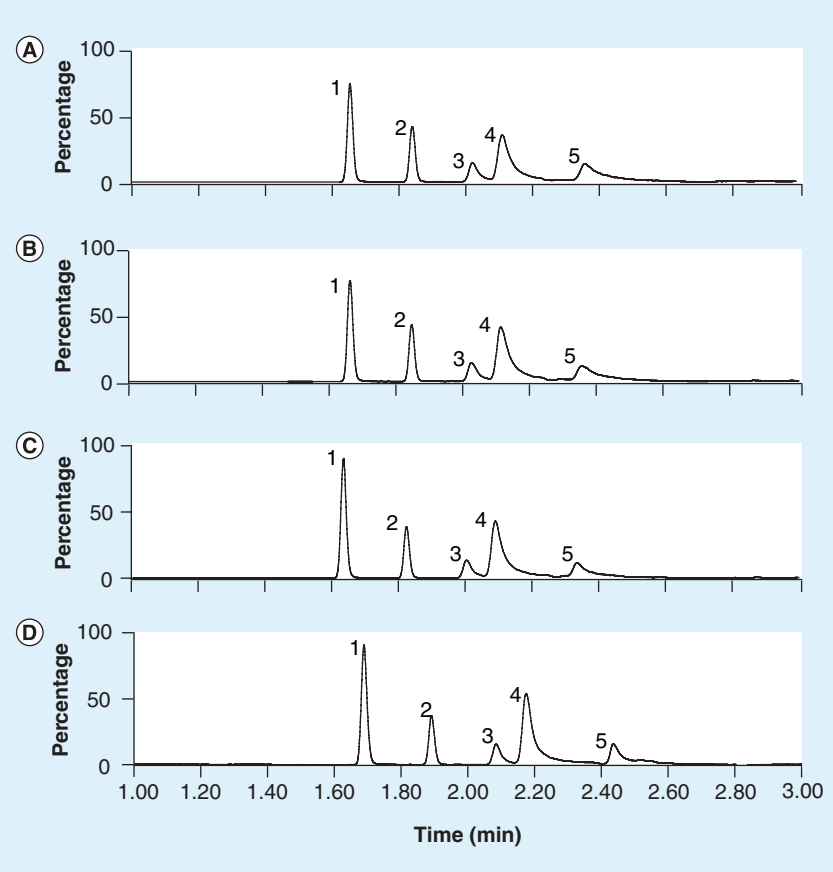


Figure 1. Effect of increasing mobile phase A buffer concentration on monoamine chromatography. Column: Waters bridged ethylene hybrid amide 2.5 µm; 2.1 × 75 mm. Ammonium formate concentrations in MPA were 10, 20, 50 and 100 mM in **(A)**, **(B)**, **(C)** and **(D)**, respectively. Mobile phase B consisted of 95:5 acetonitrile:water containing 10 mM ammonium formate. Column temperature was 30°C.
Analyte key: 1: *N*-methylserotonin; 2: serotonin; 3: dopamine; 4: epinephrine; 5: norepinephrine.

Table 4. Peak properties resulting from increases in mobile phase A ionic strength.

Molarity (mM)	RT (min)	Peak height	Width (s)	Tailing (b/a)
NMS				
10	1.66	1.48E+07	2.39	1.25
20	1.66	1.38E+07	2.4	1.29
50	1.64	1.63E+07	2.39	1.51
100	1.62	1.66E+07	2.43	1.38
5-HT				
10	1.84	8.15E+06	2.38	1.3
20	1.84	7.93E+06	2.41	1.34
50	1.83	7.45E+06	2.39	1.29
100	1.81	7.26E+06	2.42	1.3
DA				
10	2.02	2.64E+06	8.24	5.31
20	2.02	2.37E+06	7.13	7.16
50	2.01	2.51E+06	7.21	7.96
100	2.00	2.76E+06	6.26	6.84
EP				
10	2.11	6.93E+06	9.34	3.19
20	2.11	6.75E+06	10.13	4.15
50	2.09	7.99E+06	10.29	8.88
100	2.09	9.49E+06	8.64	6.33
NE				
10	2.35	2.32E+06	12.59	7.15
20	2.35	1.90E+06	12.45	7.45
50	2.34	1.89E+06	12.16	4.84
100	2.34	2.65E+06	10.25	8.36

5-HT: Serotonin; DA: Dopamine; EP: Epinephrine; NE: Norepinephrine; NMS: N-methylserotonin; RT: Retention time.

and a water-rich layer immobilized on the surface of the stationary phase [27,28], there is also ample evidence for other mechanisms, such as ion exchange, hydrogen-bond formation or dipole–dipole interactions [2,5,29]. We theorized that some of these other interactions between the more polar compounds (DA, EP and NE) and the stationary phase could be responsible for the poor peak shape seen. In an attempt to reduce some of these secondary interactions with the stationary phase, we increased the ionic strength of MPA while keeping all other conditions constant. Panels B, C, and D of Figure 1 show the chromatography resulting from ammonium formate concentrations of 20, 50 and 100 mM in MPA, respectively. Selected peak properties are shown in Table 4. Peak widths were calculated at 5% of peak height. Peak tailing factors were calculated by MassLynx by dividing the width of the tailing half of the peak by the leading half of the peak (b/a). The results from these experiments show that, with the exception of some slight increases in peak height for DA, EP and NE and reductions in peak width for the same three analytes at 100 mM ammonium formate, increasing the ionic strength on the aqueous mobile phase had little effect on monoamine chromatography.

The lack of effect observed after increasing the ionic strength of the aqueous mobile phase could be related to the mobile-phase composition at the time of elution. The maximum aqueous content of this gradient is only 30%, so all of these analytes elute at relatively low proportions of aqueous mobile phase. Therefore, the lack of effect from increasing the ionic strength of MPA could be attributed to the fact that there was simply not a high enough proportion of

aqueous mobile phase present during the chromatographic separation to significantly change the ionic strength of the adsorbed water layer around the particles. Without this change in the stagnant water layer, it would not be possible to disrupt any secondary interactions with the stationary phase that could be contributing to the poor peak shape. If this is indeed the case, then a more effective strategy would be to increase the ionic strength of MPB. The challenge with this approach is that at the aqueous proportions required for adequate retention of these compounds, increasing the concentration of ammonium formate can decrease the miscibility of water in acetonitrile, resulting in phase separation between the aqueous and organic components of MPB. In an attempt to increase the buffer concentration, while still maintaining miscibility, we added concentrated (400 mM) ammonium formate to acetonitrile and then gradually added water until the aqueous phase remained in solution. The resulting solutions were then paired with MPA containing 100 mM ammonium formate. The chromatographs of these experiments are seen in Figure 2 and selected peak properties are listed in Table 5.

The changes made in MPB composition clearly had a dramatic, positive effect on monoamine chromatography. Peak height increases ranged from two- to fourfold over the initial values obtained using an organic mobile-phase containing 10 mM ammonium formate and 5% water. Peak widths and tailing were also significantly reduced, especially for the more polar analytes – DA, EP and norpeinephrine. For EP and NE, peak widths were reduced from 8.64 and 10.25 s to 5.07 and 3.76 s, respectively. Peak tailing factors decreased from 6.33 and 8.36 to 2.34 and 1.31, respectively. Initially, the combination of 19 mM ammonium formate combined with an aqueous composition of 8.7% appeared to give excellent results in terms of sensitivity and resolution (Figure 2B). Unfortunately, it was not possible to consistently duplicate this mobile phase composition so that the aqueous and organic portions remained dissolved within each other. Increasing the aqueous proportion to 10% resulted in complete and consistent miscibility. However, as can be seen in Figure 2C, the peak shape for NMS was asymmetrical. To further increase the solvent strength of MPB, the aqueous proportion was increased to 15%. This was sufficient to consistently dissolve 30 mM ammonium formate without separation of the organic and aqueous phases. The resulting chromatography is seen in Figure 2D. This mobile-phase composition resulted in symmetrical, baseline resolved peaks with minimal tailing. There is some loss of sensitivity for 5-HT, DA and EP compared with the mobile phase containing 10% water and 20 mM ammonium formate. This could be a result of a loss of ionization efficiency due to the increased aqueous content of the mobile phase, a decrease in peak height due to slightly wider peaks or a combination of these two factors. Overall, changing the composition of the organic portion of the mobile phase had a much greater impact on the chromatography than what was seen with the changes in the aqueous mobile phase, significantly improving both sensitivity and peak shape.

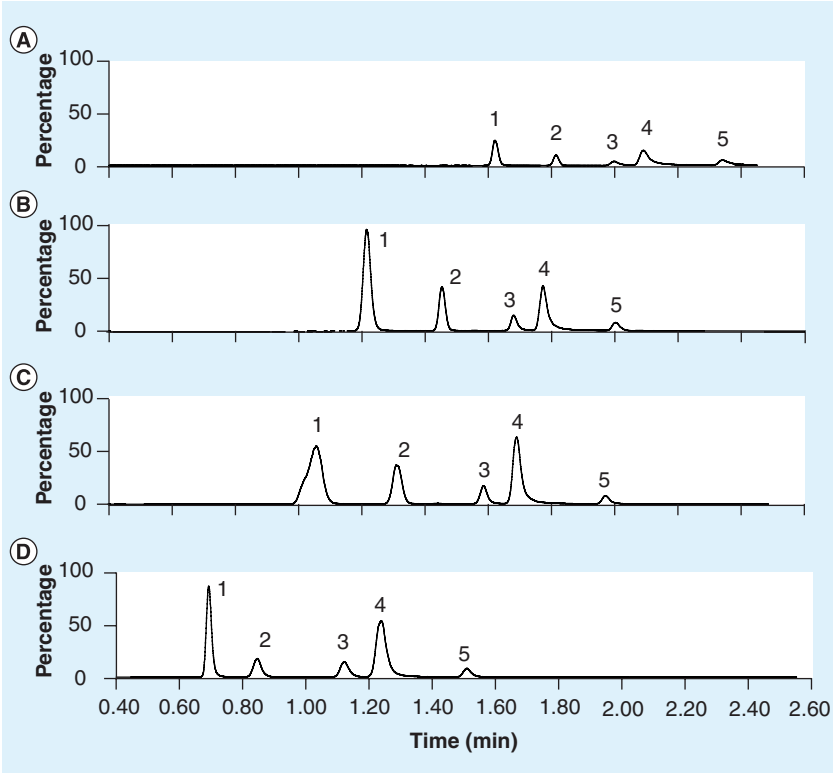


Figure 2. Effects of modifying the ionic strength and aqueous content of mobile phase B on monoamine chromatography. Panels **(A)**, **(B)**, **(C)** and **(D)** correspond with mobile phase compositions B1–B4 listed in Table 1. Mobile phase A consisted of 95:5 water:acetonitrile containing 100 mM ammonium formate (pH 3.0). Column temperature was 30 °C. Column: Waters bridged ethylene hybrid amide 2.5 µm; 2.1 × 75 mm.
Analyte key: 1: *N*-methylserotonin; 2: serotonin; 3: dopamine; 4: epinephrine; 5: norepinephrine.

In an attempt to fully characterize the differential effects of mobile phase ionic strength and aqueous content, additional experiments were performed in which each variable was changed independently. Figure 3 & Table 6 demonstrate

Table 5. Peak properties resulting from changes in mobile phase B composition.

Molarity (mM)/aqueous (%)	RT (min)	Peak height	Width (s)	Tailing (b/a)
NMS				
10/5	1.62	1.66E+07	2.43	1.38
19/8.7	1.19	5.34E+07	3.94	1.23
20/10	1.07	3.43E+07	6.26	1.05
30/15	0.72	5.11E+07	2.34	1.66
5-HT				
10/5	1.81	6.93E+06	2.42	1.3
19/8.7	1.45	2.66E+07	3.06	1.24
20/10	1.32	2.27E+07	3.87	1.25
30/15	0.86	1.08E+07	3.65	1.48
DA				
10/5	2	2.76E+06	6.26	6.84
19/8.7	1.69	8.91E+06	4.47	2.57
20/10	1.59	1.08E+07	4.07	2.7
30/15	1.11	7.53E+06	4.22	1.66
EP				
10/5	2.09	9.49E+06	8.64	6.33
19/8.7	1.79	2.70E+07	5.6	5.53
20/10	1.69	4.08E+07	4.95	3.65
30/15	1.22	2.80E+07	5.07	2.34
NE				
10/5	2.34	2.65E+06	10.25	8.36
19/8.7	2.07	4.55E+06	4.23	2.82
20/10	1.97	4.83E+06	4.52	3.32
30/15	1.47	5.38E+06	3.76	1.31

5-HT: Serotonin; DA: Dopamine; EP: Epinephrine; NE: Norepinephrine; NMS: N-methylserotonin; RT: Retention time.

the changes in chromatography and peak properties observed when the aqueous content of MPB was increased without changing the ionic strength. The most obvious effects of this change are a predictable decrease in the retention for all compounds and some fairly dramatic changes in sensitivity. The changes in retention time are consistent with partition models of HILIC in which water is the strong elution solvent. While sensitivity is increased, especially for NMS, peak widths are nearly doubled for most analytes, to the point where NE is no longer separated from EP, but is essentially lost in the tail of the EP peak. EP, in particular demonstrates a prominent shoulder on the backside of the peak that could compromise quantitation.

Figure 4 & Table 7 show the result of increasing the ionic strength of MPB alone while keeping the aqueous content unchanged at 15%. As the elution strength of MPB remains consistent, retention times are not altered. As observed in Figure 2, however, the increase in ionic strength results in dramatically improved chromatography. Peak widths for DA, EP and NE were reduced by 65–70% as amonium formate molarity increased from 10 to 30 mM. Peak tailing is also significantly reduced and the irregular shape of the EP peak is eliminated. These changes are all consistent with the theory that increasing mobile phase ionic strength can disrupt secondary interactions with the stationary phase, resulting in improved chromatography.

■ Choice of stationary phase

We compared the performance of the amide column detailed above with an unbonded hybrid particle (XBridge HILIC) column of matching dimensions. Preliminary work with 10 mM ammonium formate in MPA and MPB had shown that the compounds in this study exhibited better separation and resolution on the amide column versus the XBridge HILIC column. Comparison of the two columns using the optimized conditions described above confirmed those initial results. Figure 5 shows chromatograms of monoamine standards analyzed on the XBridge amide column (Figure 5A) and the XBridge HILIC column (Figure 5B). Clearly, retention of nearly all analytes is superior on the amide column as is the resolution between adjacent peaks. In particular, NMS and 5-HT, which have a resolution factor of 3.8 on the amide column, nearly coelute on the XBridge HILIC column and DA and EP are no longer baseline separated. Resolution was 2.1 for DA and EP on the amide column versus 1.2 on the XBridge HILIC column. The superior performance of the amide column may be attributable to its polar functional group. In an acidic environment (pH 3.0), the polar nature of the amide functionality may be more effective at interacting with the aqueous portion of the mobile phase and forming the stagnant water layer required for HILIC chromatography. Regardless of the exact mechanism, use of the amide column consistently resulted in superior performance for the analytes in this study.

■ Effect of temperature on HILIC chromatography

HILIC chromatography can be sensitive to pH and temperature [30,31]. Since monoamines can be unstable at high pH [32], we decided to concentrate on optimizing performance under the optimized acidic conditions described above and investigated the effect of different temperatures on monoamine chromatography. Figure 6 shows

chromatograms of monoamines run under the mobile-phase conditions used in Figure 2D at different temperatures. In general, as temperature increased, the resolution between peaks decreased. At 40°C, there is loss of resolution between DA and EP and at 60°C, baseline separation has clearly been lost. Interestingly, when the column was cooled to 20°C, there was a significant loss of peak shape for NMS. As this figure clearly shows, 30°C provided the optimum balance of speed, resolution and peak shape for all analytes. Experiments looking at the effect of temperature on retention in HILIC show a variety of results that can be highly dependent upon the stationary phase. Many combinations of columns and analytes showed increases in retention in response to temperature increases. However, this may be due to enhanced ion-exchange interactions between the analytes and the stationary phase at elevated temperatures [31]. By contrast, columns with neutral active sites, including amide phases, have shown the more classical result of decreasing retention with increasing temperature [31,33,34]. The combination of the amide functionality, along with the high ionic strength of the mobile phase could serve to reduce ion-exchange interactions with the stationary phase leading to the results seen in this study.

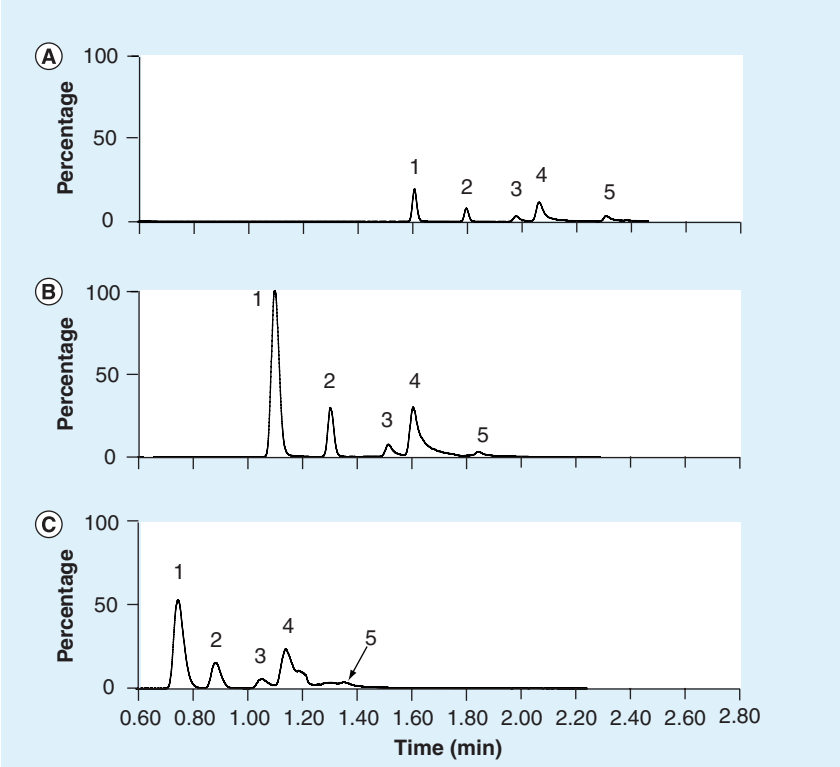


Figure 3. Effects of modifying the aqueous content of mobile phase B alone on monoamine chromatography. The proportions of acetonitrile and water in mobile phase B are **(A)** 95:5; **(B)** 90:10; and, **(C)** 85:15. Mobile phase B contained 10 mM ammonium formate (pH 3.0). Mobile phase A consisted of 95:5 water:acetonitrile containing 100 mM ammonium formate (pH 3.0). Column temperature was 30 °C. Column: Waters bridged ethylene hybrid amide 2.5 µm; 2.1 × 75 mm.
Analyte key: 1: *N*-methylserotonin; 2: serotonin; 3: dopamine; 4: epinephrine; 5: norepinephrine. Vertical axes are linked for comparison of signal intensity.

Comparison with RPLC

Early experiments using RPLC proved quite challenging. We found that both NE and EP demonstrated very poor retention on the XBridge C_{18} column and were barely separated. In fact, NE was essentially unretained by RPLC. Similar results had been seen with other attempts to separate EP and NE under reversed-phase conditions [21,23]. Adequate retention is critical, as unretained peaks can be subject to ion suppression from other components in a complex matrix (i.e., salts) that also elute in the void volume. We also attempted traditional reversed-phase analysis on a Waters Atlantis T3 column (see 'Methods') as this column was designed specifically to retain polar compounds and for use with high-aqueous mobile phases. The Atlantis T3 column did appear to improve the retention of NE and EP, but we were unable to achieve consistent and acceptable peak shapes for NE.

In an attempt to achieve a successful reversed-phase separation of these analytes, we investigated the use of ion-pairing reagents on the XBridge C_{18} column (2.5 µm; 2.1 × 75mm) and on the Atlantis T3 column (3.0 µm; 2.1 × 100 mm). Both NFPA and HFBA are volatile ion pairing reagents that are compatible with MS. Each was added to the MPA and MPB at a concentration of 0.01%. Figures 7A & 7B show monoamine chromatography with NFPA and HFBA, respectively, on the XBridge C_{18} column. While the use of ion pairing reagents improves the retention of all of the analytes, certain challenges remain. When using NFPA (Figure 7A) there is no separation of 5-HT and NMS. The use of HFBA, on the other hand, improves the separation of NMA and 5-HT, although they are still not baseline separated. However, DA (peak 3) is split and elutes as two peaks. This phenomenon has been seen with monoamines and sympathomimetic drugs before [35] and may be due to formation of ion-pairs between HFBA and DA in the mobile phase as well as on the stationary phase [35,36]. Finally, NE and EP are not baseline resolved.

One important consideration when developing chromatographic methods, is the limitation

Table 6. Peak properties resulting from changes in mobile phase B aqueous content alone.

Aqueous content (%)	RT (min)	Peak height	Width (s)	Tailing (b/a)
NMS				
5	1.62	1.66E+07	2.43	1.38
10	1.08	9.44E+07	4.05	1.53
15	0.75	4.71E+07	5.28	1.87
5-HT				
5	1.81	6.93E+06	2.42	1.3
10	1.28	2.85E+07	3.16	1.34
15	0.89	1.40E+07	4.98	1.54
DA				
5	2	2.76E+06	6.26	6.84
10	1.5	7.79E+06	7.65	3.95
15	1.07	5.29E+06	12.28	3.44
EP				
5	2.09	9.49E+06	8.64	6.33
10	1.59	3.04E+07	10.75	7.03
15	1.16	2.05E+07	15.71	4.94
NE				
5	2.34	2.65E+06	10.25	8.36
10	1.84	2.65E+06	8.79	4.86
15	1.38	2.38E+06	13.68	6.94

5-HT: Serotonin; DA: Dopamine; EP: Epinephrine; NE: Norepinephrine; NMS: N-methylserotonin; RT: Retention time.

that system back pressure places upon method development. System pressures under the HILIC conditions, described above, ranged from 1900 to 2900 psi over the course of the gradient at a flow rate of 0.5 ml/min. Under the reversed-phase conditions described, using a column of matched dimensions, back pressures ranged from 4900 to 7000 psi over the course of the entire gradient. Traditional HPLC systems, limited to 4000–6000 psi, would already be challenged by the back pressures observed for the reversed-phase conditions, limiting the ability to use longer columns and/or reduced particle sizes. By contrast, the lower back pressures observed under the HILIC conditions in this study would allow the use of even higher flow rates to further reduce analysis time or longer columns to increase separation efficiency. This could allow a single, validated method to be used on multiple-instrumentation platforms if desired. By extension, the use of systems with the advantages of elevated pressure tolerances would allow even greater flexibility in flow rate and column selection.

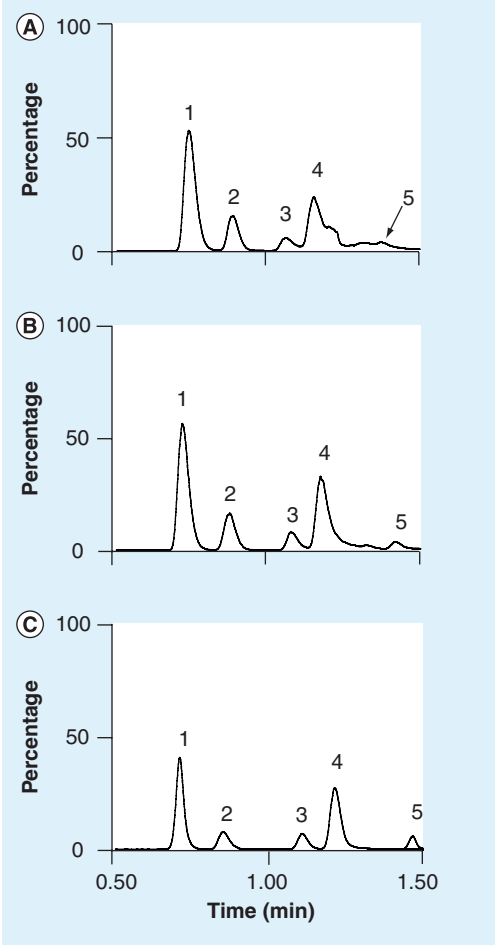


Figure 4. Effects of modifying the ionic strength of mobile phase B alone on monoamine chromatography. The molarity of ammonium formate (pH 3.0) in mobile phase B is in **(A)** 10 mM; **(B)** 20 mM; and, **(C)** 30 mM. Mobile phase B consisted of 85:15 acetonitrile:water with either 10, 20 or 30 mM ammonium formate (pH 3.0). Mobile phase A consisted of 95:5 water:acetonitrile containing 100 mM ammonium formate (pH 3.0). Column temperature was 30°C. Column: Waters bridged ethylene hybrid amide 2.5 µm; 2.1 × 75 mm.
Analyte key: 1: *N*-methylserotonin; 2: serotonin; 3: dopamine; 4: epinephrine; 5: norepinephrine. Vertical axes are linked for comparison of signal intensity.

Conclusion

This manuscript details the development of HILIC chromatography for the analysis of monoamine neurotransmitters using a 2.5 µm particle HILIC column containing an amide-bonded hybrid-stationary phase. Through the careful balancing of mobile phase ionic strength and solubility, we were able to dramatically improve the chromatographic performance

of the most polar compounds. The superior performance of the amide-bonded stationary phase demonstrates the importance of stationary phase choice considerations during HILIC method development. To the best of our knowledge, this is the first example of an amide-linked stationary phase being used for monoamine analysis. This work also demonstrates the utility and promise of using HILIC chromatography for the analysis of monoamine neurotransmitters. Retention, separation and resolution of even the most polar compounds (EP and NE) were achieved in an analysis time of 4 min. The intermediate length of the column (75 mm) combined with the relatively low back pressures characteristic of HILIC analysis can allow future investigators to improve separation, reduce analysis time, or, if desired, both. The low back pressures also allow flexibility to use the developed method on multiple instrumentation platforms if necessary. Going forward, the increased availability of novel HILIC phases, particle sizes and column options should provide additional options for the analysis of compounds, which can be quite challenging by conventional RPLC methodology.

Table 7. Peak properties resulting from changes in mobile phase B molarity alone.

Molarity (mM)	RT (min)	Peak height	Width (s)	Tailing (b/a)
NMS				
5	0.75	4.71E+07	5.28	1.87
10	0.73	4.57E+07	5.15	1.78
15	0.72	5.11E+07	2.34	1.66
5-HT				
5	0.89	1.40E+07	4.98	1.54
10	0.88	1.33E+07	5.12	1.40
15	0.86	1.08E+07	3.65	1.48
DA				
5	1.07	5.29E+06	12.28	3.44
10	1.08	6.27E+06	6.97	2.73
15	1.11	7.53E+06	4.22	1.66
EP				
5	1.16	2.05E+07	15.71	4.94
10	1.18	2.61E+07	9.28	3.65
15	1.22	2.80E+07	5.07	2.34
NE				
5	1.38	2.38E+06	13.68	6.94
10	1.43	2.69E+06	7.92	2.80
15	1.47	5.38E+06	3.76	1.31

5-HT: Serotonin; DA: Dopamine; EP: Epinephrine; NE: Norepinephrine; NMS: N-methylserotonin; RT: Retention time.

Future perspective

The choice of HILIC for the analysis of polar compounds is becoming increasingly popular in the bioanalytical laboratory. The data provided here demonstrates that HILIC can be an important choice for the most polar compounds, providing an important complement to reversed-phase analysis. As more particle chemistries and column options become available, the potential of HILIC to solve more of these analytical problems should also increase, providing a tool to fill this analytical space.

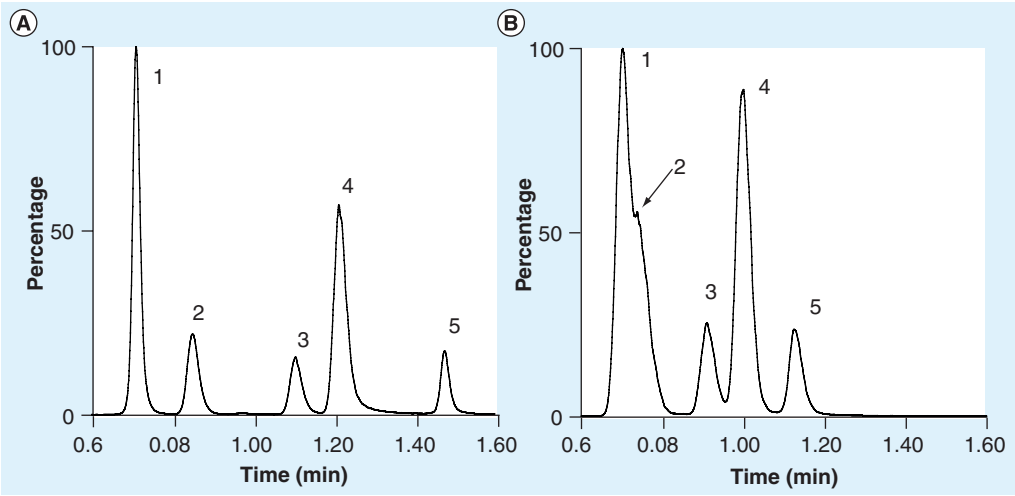


Figure 5. Comparison of monoamine chromatography. Produced using **(A)** Waters XBridge™ Amide and **(B)** XBridge HILIC columns. Mobile phase A consisted of 95:5 water:acetonitrile containing 100 mM ammonium formate (pH 3.0). Mobile phase B consisted of 85:15 water:acetonitrile containing 30 mM ammonium formate (pH 3.0). Column temperature was 30°C. Analyte key: 1: *N*-methylserotonin; 2: serotonin; 3: dopamine; 4: epinephrine; 5: norepinephrine. Vertical axes are linked for comparison of signal intensity.

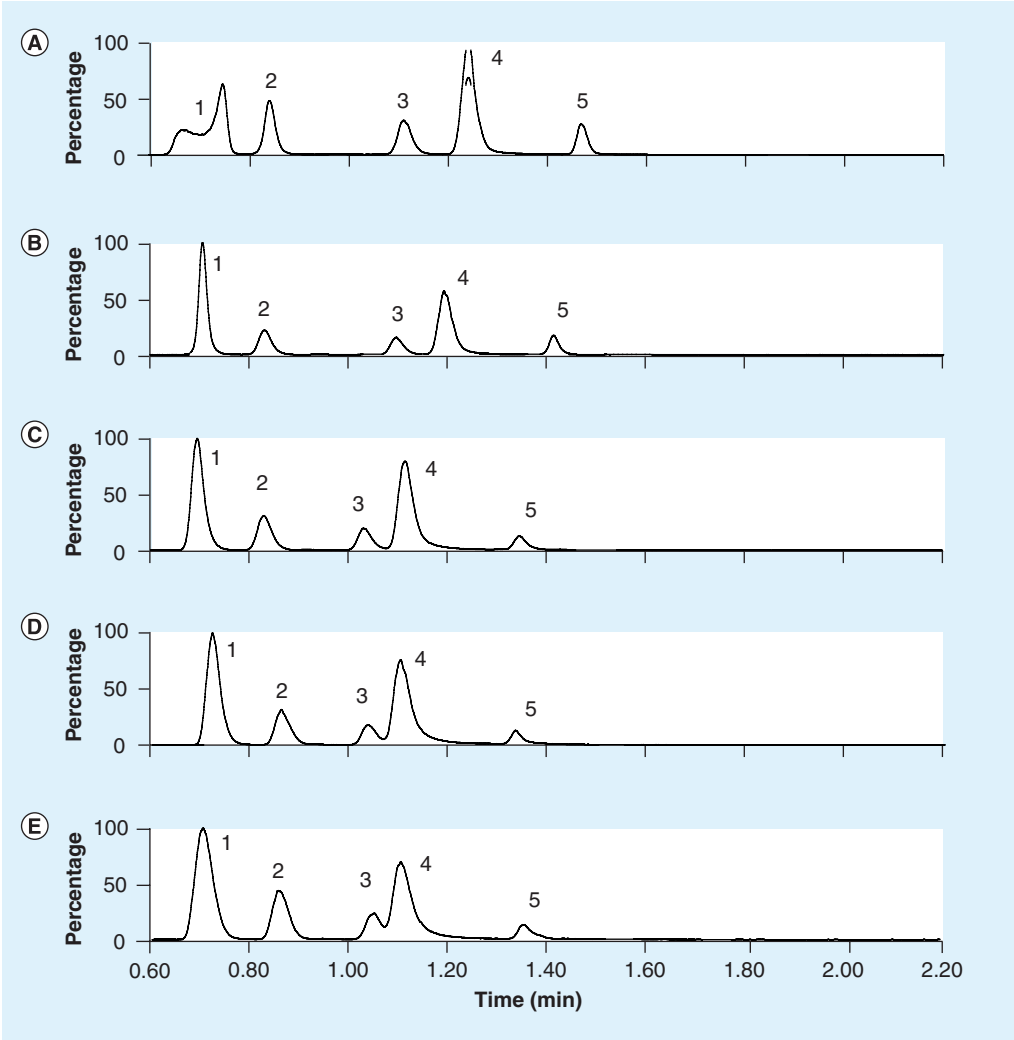


Figure 6. Effect of changes in column temperature on monoamine chromatography. Temperatures were: **(A)** 20°C; **(B)** 30°C; **(C)** 40°C; **(D)** 50°C; and, **(E)** 60°C. Mobile phase A consisted of 95:5 water:acetonitrile containing 100 mM ammonium formate (pH 3.0). Column: Waters XBridge™ amide (2.5 µm; 2.1 × 75 mm). Mobile phase B consisted of 85:15 water:acetonitrile containing 30 mM ammonium formate (pH 3.0). Analyte key: 1: *N*-methylserotonin; 2: serotonin; 3: dopamine; 4: epinephrine; 5: norepinephrine. Vertical axes are linked for comparison of signal intensity.

Executive summary

Background

- Hydrophilic interaction chromatography (HILIC) has recently become a very effective complementary tool to reversed-phase chromatography for the analysis of polar compounds. This manuscript details the development of HILIC conditions for the analysis of monoamines on a 2.5 µm bridged ethylene hybrid column, bonded with an amide functionality.

Results

- The careful optimization of mobile-phase composition is shown to be critical for achieving excellent chromatographic performance for monoamines, especially dopamine, epinephrine and norepinephrine. Increasing the ionic strength of the organic mobile phase resulted in dramatic chromatographic improvements for the most polar compounds. Increases in ionic strength, however, need to be balanced against solution miscibility in high organic mobile phases. Column functionality is also shown to be a critical factor to consider during HILIC method development.

Conclusion

- The increased availability of different HILIC stationary phases and particle sizes will provide more options for the analysis of polar compounds and provides a key complementary method to reversed-phase analysis.

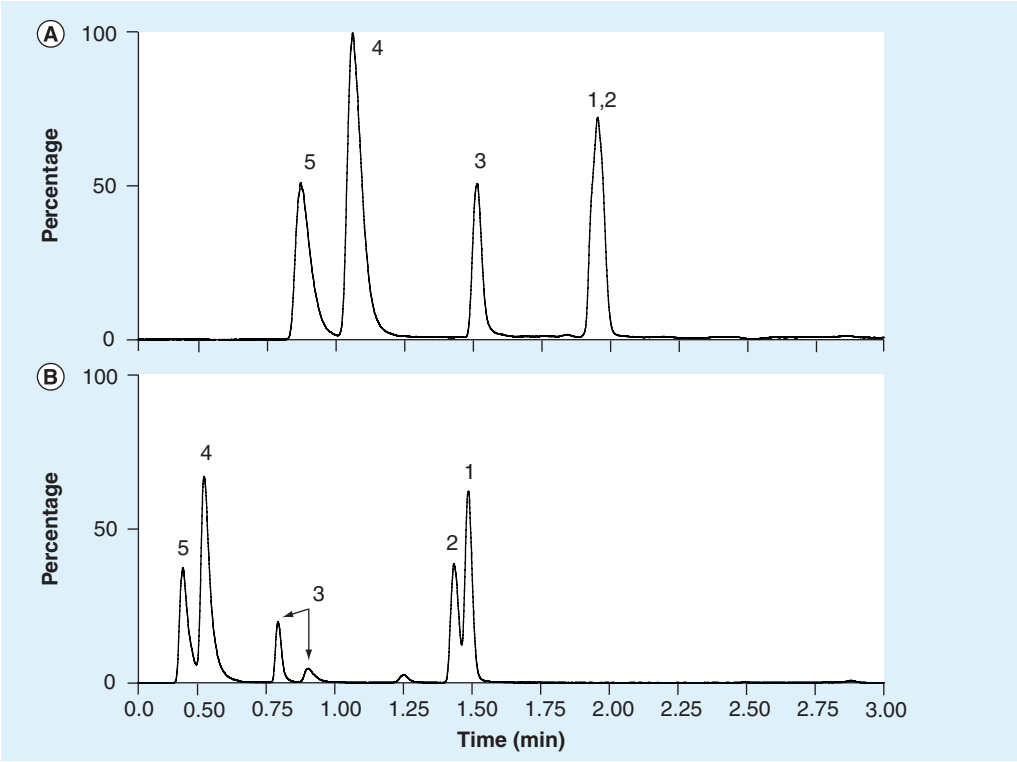


Figure 7. Reversed-phase chromatography of monoamines. Ion pairing reagents are **(A)** nonafluoropentanoic acid and **(B)** heptafluorobutyric acid. Mobile phase compositions and the gradient profile are detailed in 'Methods'. Column temperature was 30°C. Column: Waters XBridge™ C_{18} (2.5 µm; 2.1 × 75 mm).
Analyte key: 1: *N*-methylserotonin; 2: serotonin; 3: dopamine; 4: epinephrine; 5: norepinephrine.

Financial & competing interests disclosure

All authors are employees of Waters Technologies Corporation (MA, USA). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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