Enantiomer separation of acidic compounds

Using Daicel CHIRALPAK QN-AX and QD-AX columns and the Agilent 1260 Infinity Analytical SFC System

Application Note

Pharmaceuticals

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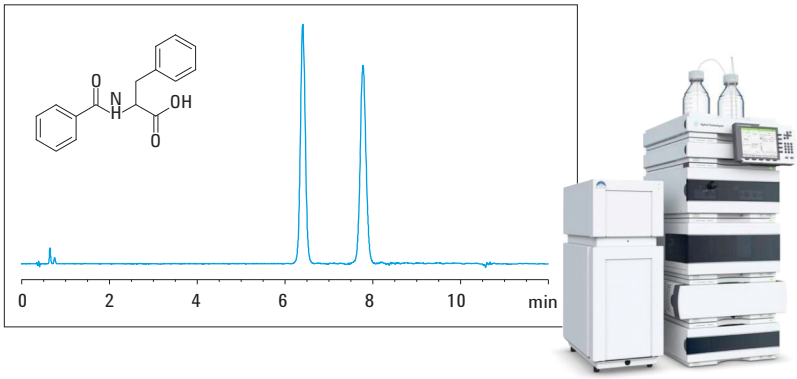

Abstract

CHIRALPAK QN-AX and QD-AX are anion exchanger chiral stationary phases providing specific enantioselectivity for acidic compounds. In this Application Note, we demonstrate that the application of these columns can be extended to super-critical fluid chromatography (SFC) for enantiomer separation of acidic compounds. Using the advantages of the advanced technology of the Agilent 1260 Infinity Analytical SFC system, we were able to assess the influence of a series of para-meters such as the mobile phase additives and the flow rate to gain insight into the general approaches for method development and optimization.

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Introduction

CHIRALPAK QN-AX and CHIRALPAK QD-AX (Figure 1) are anion exchanger chiral stationary phases providing specific enantioselectivity for acidic compounds. The chiral selectors are O-9-tert-butylcarbamate of Quinine (QN) and Quinidine (QD) respectively and immobilized on 5 µm spherical silica gel. The enantiomer recognition mechanism is based on the ionic exchange between the protonated tertiary nitrogen of the quinuclidine moiety of the chiral selector and the anionic analytes. Such an ion-pairing is accompanied by additional intermolecular interactions including hydrogen bonding, dipole-dipole, π-π, hydrophobic as well as steric interactions[1-2].

These chiral columns have been exhaustively investigated in HPLC with aqueous- and non-aqueous polar organic mobile phases and show remarkable performance in enantiomer resolution of a wide variety of acidic compounds[2-8]. The investigation of these columns for enantiomer separation by SFC is a current area of research[9].

This Application Note demonstrates that the application of these columns can be extended to supercritical fluid chromatography (SFC) for enantiomer separation of acidic compounds. Using the advantages of the advanced technology of the Agilent 1260 Infinity Analytical SFC system, we were able to assess the influence of a series of parameters such as the mobile phase additives and the flow rate to gain insight into the general approaches for method development and optimization on CHIRALPAK QN-AX and CHIRALPAK QD-AX by SFC.

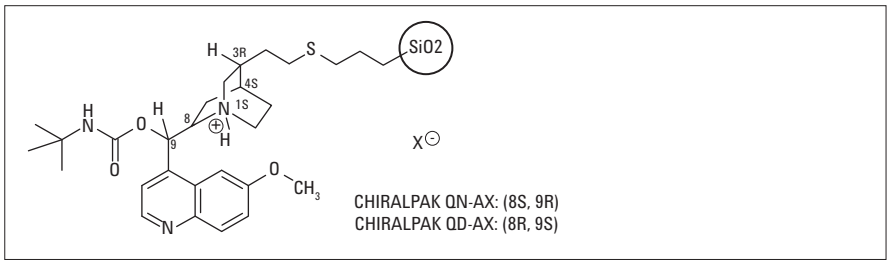

Figure 1
The chiral stationary phases.

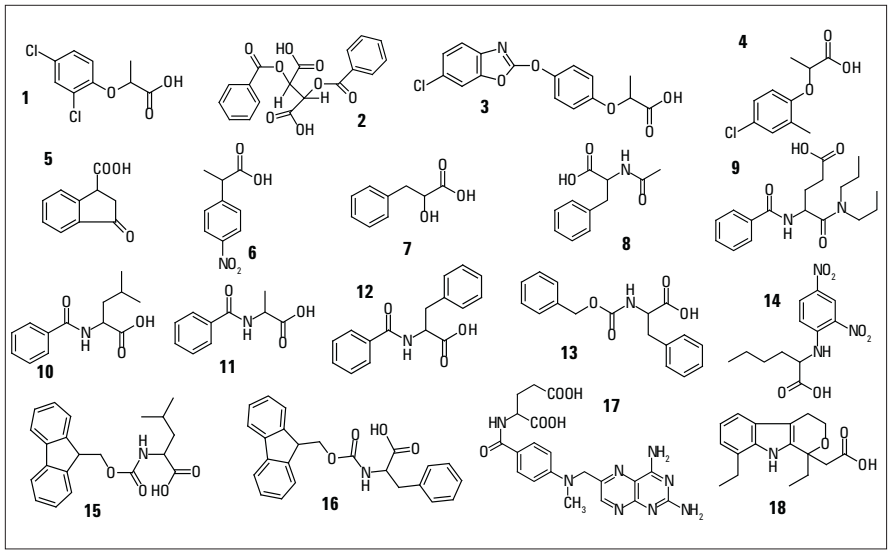

Figure 2
Structures of the acidic analytes.

Experimental

Chemicals

The chiral columns CHIRALPAK QN-AX and CHIRALPAK QD-AX used in this study are products of Daicel Group, Tokyo, Japan. They are manufactured at Chiral Technologies Europe, Strasbourg, France. Columns with dimensions of 4.6 × 100 mm and packed with 5 µm particles of the CSPs were used.

The CO_2 supply was from a cylinder of B50 containing the liquid carbon dioxide of industrial quality 4.8. Methanol (MeOH) of HPLC quality was used as the bulk modifier of the supercritical fluid carbon dioxide (SF-CO_2). Formic acid (FA) or acetic acid (HOAc) was employed as the acidic additive. They were matched respectively by ammonium formate (NH_4OOCH) and ammonium acetate (NH_4OAc) to balance the analyte retention times. The acidic compounds from Sigma-Aldrich (Figure 2) were dissolved in methanol for 5 µL injections.

Instrumentation

All SFC experiments were carried out on an Agilent 1260 Infinity Analytical SFC System (G4309A) consisting of the following modules:

- Aurora SFC Fusion A5 module for CO_2 pre- and post-conditioning
- Agilent 1260 Infinity SFC Binary Pump for accurate and constant metering of the mobile phase
- Agilent 1260 Infinity Degasser
- Agilent 1260 Infinity Autosampler
- Agilent 1260 Infinity Diode Array Detector with high pressure SFC flow cell

In addition, the Agilent SFC Method Development Kit was integrated into the system consisting of two Agilent 1290 Infinity Thermostatted Column Compartments with built-in valve drives and the Method Development Valve Kit.

Chromatographic conditions

Unless specifically indicated, the flow rate was conventionally set at 3.0 mL/min, the temperature of the column compartments at 40 °C and the back pressure of SF-CO_2 at 150 bar.

Results and discussion

In HPLC, methanol is proved to be a versatile mobile phase on CHIRALPAK QN-AX and CHIRALPAK QD-AX columns. Owing to its pronounced protic properties, methanol provides efficient solvation of all the ionized species involved in the ion-exchange equilibriums. Used as a single solvent in HPLC, the eluotropic strength of methanol can be adjusted by the concentration of the counter-ion (acidic additives) and by the ionic strength (acidic and salt additives).

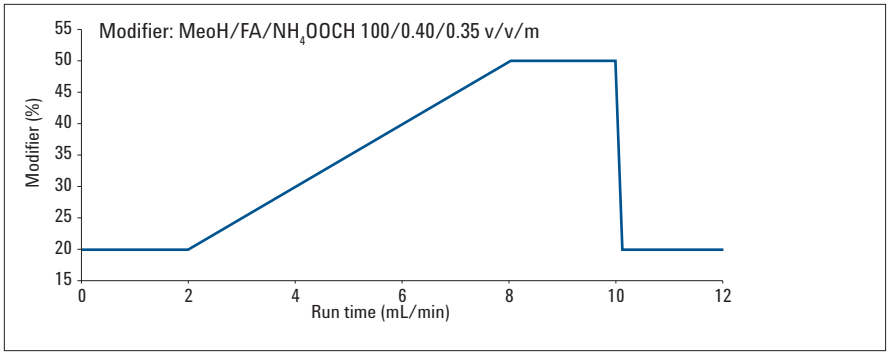

Figure 3
The gradient program.

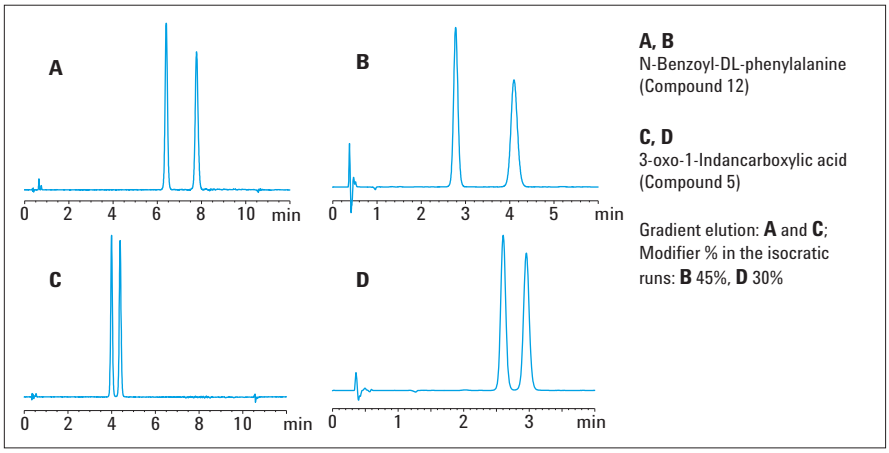

Figure 4
Straightforward method transfer from gradient to isocratic.

The same principles can be applied in SFC regarding the choice of the bulk modifier and the acid-salt additives. Throughout the current study, FA (up to 0.6% by volume) and NH_4OOCH (up to 0.50% by mass) were added into methanol and the resulted mixtures were used as the modifier of SF-CO_2.

Gradient to isocratic transfer

The first screening of the acidic compounds was carried out by running a gradient program (Figure 3) on the single column CHIRALPAK QN-AX using a mixture of MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m as the modifier. The results from the gradient runs allowed targeting the modifier percentage to be used in isocratic mode (Table 1) so that the compounds were eluted in a reasonable time window. The chromatograms in Figure 4 demonstrate the direct transfer from the gradient to the isocratic method. The optimization steps, if needed, were then carried out in isocratic mode.

$^*t_{grad.}$ (min)	Modifier %
2–3	20
3–4	25
4–5	30
5–6	35
6–7	40
7–8	45
8–10	50

$^*t_{grad.} = (t_{r1}+t_{r2})/2$

Table 1
Determination of modifier % for isocratic run from the gradient results.

Effect of the acidic additive

In HPLC, the typical working conditions on CHIRALPAK QN-AX and CHIRALPAK QD-AX are with weakly acidic mobile phases (pH 5–7)[4]. Under such conditions, the chiral selector is protonated at the quinuclidine ring (Figure 1) and the acidic analytes are dissociated. An ionic exchange mechanism is thus activated between the positively charged chiral selector and the negatively charged analyte molecules.

It seems that the ionic exchange mechanism in SFC can be established owing to the mild acidity of SF-CO_2 itself. As shown in Table 2, the chiral recognition happened with no need of external addition of the acidic additive ([FA] =0%). The enantioselectivity was mostly unaffected by the absence or presence of the acidic additive and its concentration (Figure 5B). Except in the case of Compound 9 (proglumide), the addition of FA did not induce notable loss in resolution (Table 2, Figure 5B). Especially when exceeding the level of 0.20%, the presence of FA in the mobile phase provided the benefits of reducing the analysis times to a significant extent (Figure 5A). It can be presumed that FA played the role of the counter-ion, induced competitive effect and favored the mass transfer kinetics of the chromatographic process.

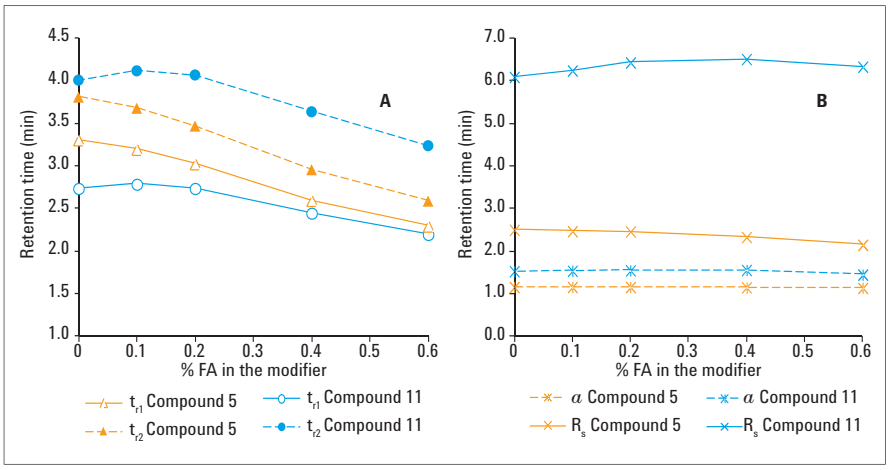

Figure 5
Dependence of chromatographic results on percentage of FA.

Compound	Modifier (%)	[FA]=0% t_{r1}	t_{r2}	α	Rs	[FA]=0.4% t_{r1}	t_{r2}	α	Rs
5	30	3.31	3.81	1.17	2.51	2.60	2.96	1.16	2.34
6	20	3.97	4.29	1.09	1.60	2.87	3.07	1.08	1.46
9	10	3.25	3.69	1.16	2.51	2.56	2.84	1.13	1.29
11	35	2.74	4.01	1.54	6.10	2.45	3.64	1.57	6.52
15	35	3.26	4.92	1.57	6.36	2.48	3.68	1.56	6.27
20	20	4.55	5.10	1.13	2.13	3.20	3.57	1.13	2.18
Average		**3.51**	**4.30**	**1.28**	**3.54**	**2.69**	**3.29**	**1.27**	**3.34**

Table 2
Chromatographic results with and without FA in the modifier.
Column: CHIRALPAK QN-AX, Modifier: MeOH/NH_4OOCH 100/0.35 v/m + FA.

Effect of the salt and its concentration

The ion-exchange chromatographic process is not only dependent on the ionization state of the chiral selector and the analyte molecules but also on the ionic strength of the mobile phase. Adding a certain amount of a suitable salt into the mobile phase can efficiently modulate the ionic strength of the mobile phase and regulate the absorption-desorption process between the analyte and the chiral selector. In practice, it is preferable to choose a salt of high solubility in the modifier or the mobile phase, high volatility for the potential LC/MS hyphenation and high UV transparency to ensure good UV detection of the analytes. NH_4OOCH meets all these requirements.

NH_4OOCH was added into the methanolic modifier at two different concentrations: 0.35% and 0.20%. As shown in Table 3, the enantioselectivity was unaffected by the concentration of NH_4OOCH. The higher concentration of NH_4OOCH led to reduced retention times at about 10% with minor decrease in resolution of the enantiomers. A concentration of 0.35% NH_4OOCH in MeOH seems to be a good compromise for generic method development.

Because of the unknown solubility of the salt in SF-CO_2, caution would be needed if significantly higher salt concentrations are to be used.

It is worth noting that the binary mixture of methanol/FA with no salt is not a stable system due to the rapid esterification reaction. It would lead to longer and longer sample retention over time for a given analysis. The presence of the salt NH_4OOCH in the modifier plays the role of the "stabilizer" of the system and is essential for reproducible chromatographic results.

Compound	Modifier (%)	[NH_4OOCH] = 0.35% t_{r1}	t_{r2}	α	Rs	[NH_4OOCH] = 0.20% t_{r1}	t_{r2}	α	Rs
3	40	2.70	2.85	1.07	0.76	3.30	3.50	1.07	0.91
4	35	2.33	2.58	1.12	1.74	2.71	3.00	1.13	1.85
5	30	2.60	2.96	1.16	2.34	2.85	3.25	1.16	2.39
6	20	2.87	3.07	1.08	1.46	3.03	3.25	1.08	1.48
9	10	2.56	2.84	1.13	1.29	2.59	2.88	1.13	1.34
11	35	2.45	3.64	1.57	6.52	2.80	4.21	1.58	6.72
13	35	3.54	3.90	1.11	1.57	4.04	4.47	1.12	1.65
15	35	2.48	3.68	1.56	6.27	2.69	4.02	1.57	6.42
18	20	3.20	3.57	1.13	2.18	3.33	3.72	1.13	2.19
Average		**2.75**	**3.23**	**1.22**	**2.68**	**3.04**	**3.59**	**1.22**	**2.77**

Table 3
Effect of the salt concentration.
Column: CHIRALPAK QN-AX; Modifier: MeOH/FA 100/0.40 v/v + NH_4OOCH.

Elution of strongly retained compounds

From the experimental data, the mixture of MeOH/FA/NH_4OOCH at the proportion of 100/0.40/0.35 v/v/m represents a good compromise in terms of elutropic strength and achievable resolution and can be used as a starting point for SFC method development. However, a few of the acidic compounds under investigation (such as Amethopterin (Compound 17), 2,3-Dibenzoyl-DL-tartaric acid (Compound 2) and N-DNP-DL-norleucine (Compound 14)) have strong retention on the columns and could not be (completely) eluted with such a methanolic modifier in the gradient as described above.

Compound	Modifier (%)	Flow rate (mL/min)	t_{r1}	t_{r2}	α	Rs
2	60	5	16.39	21.17	1.30	2.43
7	50	3	2.05	2.37	1.19	2.13
14	50	3	4.20	5.74	1.40	4.68
16	60	5	1.16	1.47	1.38	2.52
17	60	5	3.17	5.95	1.99	5.01

Table 4
Enantiomer separation employing strong eluting conditions.
Column: CHIRALPAK QN-AX; Modifier: MeOH/FA/NH_4OOCH 100/0.60/0.50 v/v/m.

In this scenario, efficient elution could be achieved by employing a high percentage of the modifier (50–60%) containing slightly higher concentrations of the acid and salt additives and, if necessary, increasing the flow rate. Some chromatographic results obtained under the enhanced eluting conditions are presented in Table 4.

Effect of flow rate

As previously mentioned, the enantiomer separation on CHIRALPAK QN-AX and CHIRALPAK QD-AX involves multiple specific interactions of high affinity, particularly the long range electrostatic interaction. These combined forces ensure the enantioselectivity for acidic compounds of various molecular structures. However, these effects may be too strong for the dissociation or desorption process even in the copresence of the counterion (the acidic additive) and the salt.

This will unavoidably result in slow mass transfer kinetics as confirmed by a large C-term in the Van Deemter plot (Figure 6A) and the drastic variation of the resultant resolution degree according to the flow rate (Figure 6B).

This disadvantage can actually be converted into effective means to improve the enantiomer separation by reducing the flow rate. As depicted in Figure 7, the just base-line resolution of Compound 1 (Dichlorprop) was significantly increased by reducing the flow rate from 5.0 mL/min to 1.0 mL/min.

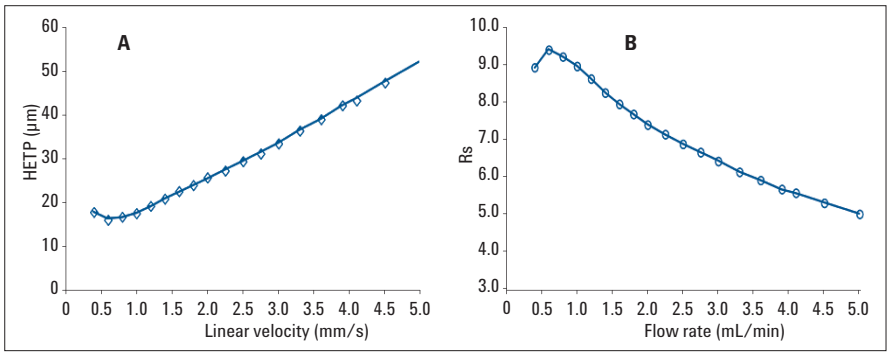

Figure 6
Dependence of peak efficiency and resolution degree on flow rate.
Compound 11 (N-benzoyl-DL-alanine; (A) peak-2
Column: CHIRALPAK QN-AX; Modifier: 35% (MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m).

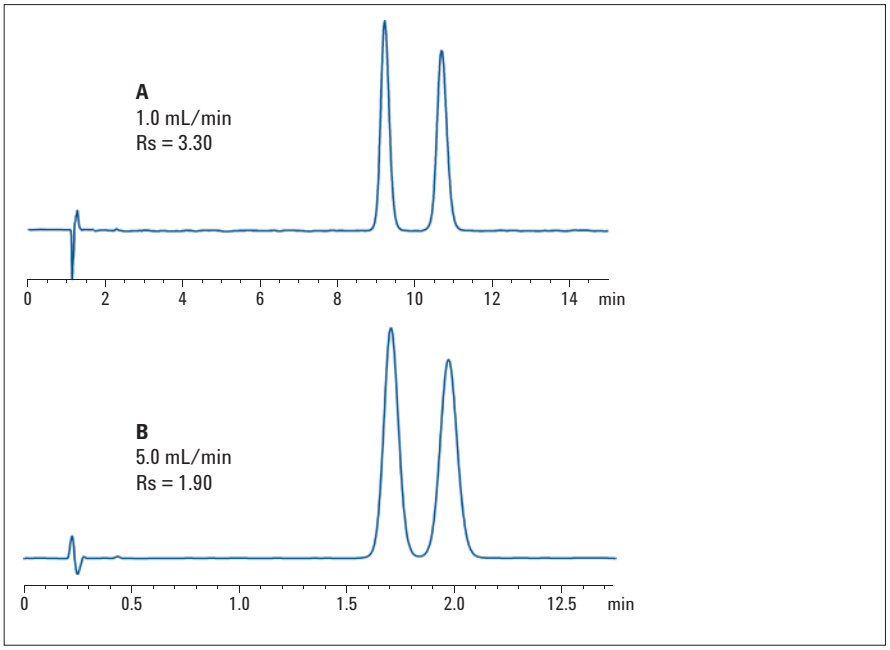

Figure 7
Enantiomer resolution of Dichlorprop (Compound 1) at different flow rates.
Column: CHIRALPAK QN-AX; Modifier: 45% (MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m).

Elution order and complementarity

As shown in Figure 1, QN and QD are diastereomers. Chromatographically, the two CSPs behave as "pseudo-enantiomers" due to the fact that the stereoselectivity is under the control of C8 and C9, which have the opposite configurations[1,6]. Consequently, the elution order (EO) of the enantiomers is reversed on these two columns. In SFC, such a phenomenon was monitored by injecting one of the pure enantiomers, which were available in our laboratory (Table 5). Coincidently, all the four D-enantiomers examined were first eluted on CHIRALPAK QN-AX and secondly eluted on CHIRALPAK QD-AX. The feasibility to choose the column in terms of EO is undoubtedly of value especially for applications in trace analysis of a given enantiomer.

As indicated by the averaged values in Table 5, CHIRALPAK QN-AX and CHIRALPAK QD-AX afforded the same enantioselectivity, but with a stronger retentivity and a slightly improved resolution degree on CHIRALPAK QD-AX. If the individual cases are examined, a complementarity in enantiomer resolution between the two columns can be observed. As exemplified in Figure 8 A-B, CHIRALPAK QN-AX offered the full separation of enantiomers for 3-oxo-1-indancarboxylic acid (Compound 5) whilst a compromised resolution was found on CHIRALPAK QD-AX for the same compound. The trend is inversed with the Fenoxaprop enantiomers (Compound 3) as shown in Figure 8 C-D. This type of complementarity is highly desirable for compounds being difficult to be resolved into enantiomers.

Compound	Modifier (%)	CHIRALPAK QN-AX					CHIRALPAK QD-AX				
		t_{r1}	t_{r2}	α	Rs	EO	t_{r1}	t_{r2}	α	Rs	EO
1	50	2.65	3.05	1.18	2.24		2.66	3.29	1.27	3.35	
3	40	2.70	2.85	1.07	0.76		2.74	3.18	1.18	2.27	
4	35	2.33	2.58	1.12	1.74		2.50	2.81	1.14	2.03	
5	30	2.60	2.96	1.16	2.34		2.72	2.87	1.07	1.02	
8	30	2.64	3.40	1.34	4.30	D/L	2.57	3.46	1.40	5.01	L/D
9	10	2.56	2.84	1.13	1.29		3.05	3.05	1.00	0.00	
10	30	2.24	4.19	2.04	10.21		2.13	3.73	1.90	9.38	
13	35	3.54	3.90	1.11	1.57	D/L	3.71	4.35	1.19	2.68	L/D
15	35	2.48	3.68	1.56	6.27	D/L	2.56	3.61	1.48	5.72	L/D
16	50	3.37	4.35	1.33	3.64	D/L	3.59	4.75	1.36	4.10	L/D
18	20	3.20	3.57	1.13	2.18		3.70	4.36	1.20	3.31	
Average		**2.76**	**3.40**	**1.29**	**3.32**		**2.90**	**3.59**	**1.29**	**3.53**	

Table 5
Comparison between CHIRALPAK QN-AX and CHIRALPAK QD-AX.
Modifier: MeOH/FA/NH_4OOCH (100/0.40/0.35 v/v/m).

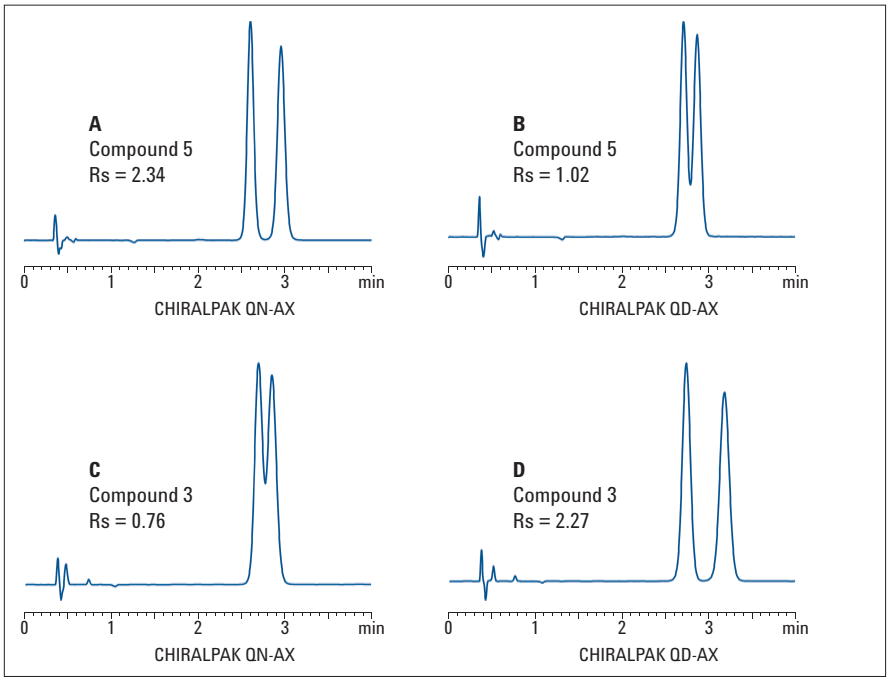

Figure 8
Examples of enantiomer separation on CHIRALPAK QN-AX and CHIRALPAK QD-AX.
Modifier: MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m, 30% for Compound 5; 40% for Compound 3.

Conclusions

CHIRALPAK QN-AX and CHIRALPAK QD-AX are versatile in enantiomer resolution of acidic compounds by SFC using methanolic modifiers. The major factors influencing the chiral separation include the acidic additive, the salt concentration and the flow rate. The mixture of MeOH/FA/NH_4OOCH 100/0.40/0.35 v/v/m is a suitable modifier for the first trials of method development.

Some other parameters such as the temperature, the variation of the modifier percentage and different pair of acid-salt additives (for example, HOAc-NH_4OAc) were investigated as well. It was observed that the enantiomer separations by SFC are mostly unaffected by temperatures in the range between 20 °C and 40 °C. Lower modifier percentage usually lead to longer retention times but has no major effect on enantioselectivity and resolution degree. The additive pair of FA-NH_4OOCH would be the preferred choice over the pair of HOAc-NH_4OAc in terms of efficiency of the enantiomer resolution.

The effect of other polar organic modifiers (such as ethanol, 2-propanol and acetonitrile) instead of methanol was not investigated because of their poor solubility to the salt additives and the resultant potential inconvenience in the balance of eluotropic strength of the mobile phase.

Most of the enantiomer separations on the CHIRALPAK QN-AX and CHIRALPAK QD-AX columns can be obtained within 5 minutes. The advanced technology of the Agilent 1260 Infinity Analytical SFC System makes the method development easy, fast and reliable.

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