SUPELCO

595 North Harrison Road Bellefonte, PA 16823-0048 USA Telephone 800-247-6628 • 814-359-3441 Fax 800-447-3044 • 814-359-3044 email: supelco@sial.com sigma-aldrich.com/supelco

Bulletin 937

Use Discovery to Reduce HPLC Method Development Time

Following the Discovery method development protocol and using the Column Screening Charts (Tables 2 and 3) as a starting point can save time. It allows you to quickly identify the right column and starting conditions to achieve your separation goals. Although each method has its unique objectives for retention and selectivity, we have shown an example of how you can apply the Discovery approach to your work.

1. The Separation Objective

In this example, the goal is to separate phenacetin from codeine (structures appear in Figure A). Codeine is much less concentrated than the phenacetin, so we want codeine to elute first or at least be far enough away that the phenacetin peak doesn't interfere with the quantitation of codeine. The preferred pH is 7.0. We suspect that both compounds have impurities so we want the main peaks to be well separated.

2. Narrowing the Discovery Column Choice

The pH 7 Column Screening Chart (Table 3) shows that the compounds have the right elution order (codeine then phenacetin) on all but the Discovery HS F5 column (if the preferred elution order was reversed, the HS F5 would be the best choice!).

3. Adjusting the Starting Mobile Phase Conditions

Estimate the k' for the two compounds at the same % acetonitrile. The Column Screening Chart shows phenacetin at 20% CH₃CN and codeine at 15%. A general guideline is that k' doubles for every 5% decrease in %organic. Therefore, decreasing the % acetonitrile to 15% would double the k' of phenacetin. A concentration of 15% would be a good start. (See Table 1.)

Table 1. Predicted vs. Actual Retention

4. Predicting Retention and Selectivity

Table 1 shows that all four phases resolved and retained the compounds. The Discovery RP-AmideC16 gave the largest selectivity. The Discovery C18 and C8 selectivity and retention were very similar. Here we would recommend doing the actual screening on three or four Discovery columns: Discovery C18 (or C8), Discovery RP-AmideC16, and Discovery Cyano.

5. Verifying the Predicted Results

In Figure A (see page 4) the sample is shown on the four Discovery columns under the mobile phase conditions predicted using the Column Screening Chart. When k' was calculated, the results were very close to predicted (see Table 1).

6. Choosing the Discovery Column for the Method

Figure A shows that the Discovery RP-AmideC16 gave the right elution order and retention, and the greatest selectivity (peak spacing). It is the best choice for this method.

Conclusions

The Discovery column screening procedure provides the analyst with several good starting points that can save significant time during method development. In this case, four Discovery columns gave the right elution order, but one of the four gave the wide peak spacing, an important requirement for this sample method. Note that each method has its unique requirements and that other Discovery columns might prove to be the best choice. The Discovery method can choose the best column no matter what the requirements of the method are.

Column	Analyte	Predicted k'	Actual k'	Selectivity
Discovery C8	Codeine Phenacetin	3.6 8.2	3.6 6.7	1.86
Discovery CN	Codeine Phenacetin	1.1 2.6	1.0 1.6	1.60
Discovery RP-AmideC16	Codeine Phenacetin	3.3 9.6	2.8 8.4	3.00
Discovery C18	Codeine Phenacetin	4.4 9.4	3.3 7.7	2.33

Table 2. Guidelines for Narrowing Down the Candidate Discovery Functionalized Reversed-Phase Column for Operation at pH 2

pH 2 Operation

Use this chart as a starting point to choose one, two, three or more Discovery silica-based functionalized reversed-phase columns.

Scrooning Conditions:		Delizoic aciu
Columns: 15cr	n x 4 6mm ID	m-nitrobenzoic acid
5um	particles	o-nitrobenzoic acid
Mobile Phase	paratitio	o-toluic acid
Buffer: 25m	M Phosphoric Acid,	phthalic acid
adju	sted to pH 2.0 with	p-nitrobenzoic acid
Amn	nonium Hydroxide	sorbic acid
(buff	er was not used in	25% CH CN
liter	ionic compounds	acetamide
were	screened)	anisole
Mobile Phase		benzaldebyde
Organic Modifier: CH ₃	CN	benzamide
Flow Rate: 1mL	/min	benzyl alcohol
Temperature: 30°C	;	methyl benzoate
Noto: A k' of E is approvir	notoly 10 minuton	
retention time on a	15cm x 4 6mm ID	phonol
column with a flow	rate of 1mL/min.	prierio
Note: For most DD UDI C	annorationa	papaverine
Note: For most RP-HPLC	separations,	
every 5% increase	in % organic	30% CH ₃ CN
	in /o organior	dipnennydramine
		furosemide
		salicylic acid
		35% CH ₃ CN
		nordoxepin
		doxepin
		protriptyline
		desipramine
		imipramine
		nortriptyline
		amitriptyline
		trimipramine
		40% CH,CN
		butyl paraben
		ethyl paraben
		methyl paraben
		propyl paraben
		50% CH CN
		bromobenzene
		chlorobenzene
		fluorobenzene
		nitrobenzene
		nitrosobenzene
		fluoxetine
		ibuprofen
		norfluoxetine
		55% CH_CN
		1.3.5-tribromobenzene
		1.3-dinitrobenzene
		1-chloro-2-fluorobenzei
		2-chloronitrobenzene
		4-bromochlorobenzene
		4-nitrophenol
		hevafluorohonzono

0	%		C18	RP-AmideC16	C8	Cyano	HS F5
Compound Name	Organic	рн	K'	K	K′	K′	K'
5% CH ₃ CN							
aniline	5	pH 2	0.7	0.5	0.7	0.4	1.5
benzyl amine	5	pH 2	1.4	0.8	1.3	0.5	3.1
nizatidine	5	pH 2	1.6	1.0	1.3	0.7	2.4
o-aminopenzoic acid	5 5	p⊓∠ nH2	0.2	4.0	0.0 0.6	1.0	0.3 3.0
pyridine	5	pH 2	0.7	0.0	0.0	0.4	0.5
10% CH_CN	•	p=	0.2	0.2	0.2	0.0	0.0
codeine	10	pH 2	2.0	1.2	1.7	0.7	2.8
hydrochlorothiazide	10	pH 2	3.0	4.3	2.7	3.1	2.3
lidocaine	10	pH 2	5.9	3.0	5.1	1.0	3.0
phentermine	10	pH 2	4.8	2.6	4.3	0.8	3.5
	10	pH 2	2.1	1.4	1.9	1.0	8.7
benzoic acid	20	nH 2	41	5.2	40	13	54
m-nitrobenzoic acid	20	pH 2	5.4	8.1	0 5.1	2.0	12.4
o-nitrobenzoic acid	20	pH 2	2.8	3.9	2.8	1.3	6.2
o-toluic acid	20	pH 2	8.4	10.3	7.8	1.8	9.7
phthalic acid	20	pН 2	1.1	1.4	1.2	0.7	2.3
p-nitrobenzoic acid	20	рН 2	6.1	9.0	5.7	2.2	15.1
sorbic acid	20	pH 2	4.1	4.3	3.8	1.1	4.5
25% CH ₃ CN							
acetamide	25	no buffer	0.1	0.1	0.2	0.3	0.1
anisole	25	no buffer	10.1	8.1	8.0	1.8	*
benzanide	25 25	no buffer	3.6	3.2	3.Z	1.2	4.8
benzyl alcohol	25	no buffer	0.0	0.7	0.7	0.0	1.0
methyl benzoate	25	no buffer	94	7.8	7.7	0.0	10.4
o-cresol	25	no buffer	4.4	6.1	4.2	1.5	5.6
phenol	25	no buffer	2.0	2.9	2.0	1.0	2.8
papaverine	25	pH 2	1.7	1.1	1.5	0.8	4.5
phenacetin	25	pН 2	2.7	3.0	2.4	1.0	1.2
30% CH ₃ CN							
diphenhydramine	30	pH 2	2.7	1.5	2.5	1.2	11.0
furosemide	30	pH 2	5.7	6.3	3.5	2.0	5.7
salicylic acid	30	pH 2	2.4	4.4	2.2	1.1	5.0
35% CH ₃ CN	25	<u>ьЦ 0</u>	15	1.0	1 1	*	10.1
dovenin	35	p⊓∠ nH2	1.5	1.0	1.4	*	10.1
protriptyline	35	pH 2	2.5	1.0	2.1	*	*
desipramine	35	pH 2	2.5	1.5	2.1	*	*
imipramine	35	pH 2	2.8	1.5	2.4	*	13.4
nortriptyline	35	pН 2	3.0	1.8	2.6	*	12.2
amitriptyline	35	pH 2	3.4	1.9	2.9	*	14.2
trimipramine	35	pH 2	3.9	2.0	3.3	*	15.2
40% CH ₃ CN							
butyl paraben	40	no buffer	4.8	7.9	4.0	1.3	4.4
ethyl paraben	40	no buffer	1.4	2.5	1.4	0.8	1.9
nronyl paraben	40	no buffer	2.6	1.5	0.9 2.4	1.0	2.0
50% CH_CN	40	no banci	2.0	-11	2.4	1.0	2.0
bromobenzene	50	no buffer	3.8	3.2	2.8	1.0	3.2
chlorobenzene	50	no buffer	3.3	2.8	2.5	1.0	3.0
fluorobenzene	50	no buffer	2.0	1.8	1.7	0.8	2.3
nitrobenzene	50	no buffer	1.4	1.4	1.3	0.8	1.9
nitrosobenzene	50	no buffer	1.6	1.6	1.5	0.8	2.1
fluoxetine	50	pH 2	2.1	1.2	0.8	0.6	13.4
Ibuproten	50	pH 2	4.3	4.9	3.4	1.0	2.9
	50	рпz	1.0	1.2	0.7	0.6	11.1
1 3 5-tribromobenzene	55	no buffer	13.0	9.4	6.0	1 1	5.0
1.3-dinitrobenzene	55	no buffer	1 0	1.0	1.0	0.7	1.5
1-chloro-2-fluorobenzene	e 55	no buffer	2.3	2.1	1.9	0.7	2.3
2-chloronitrobenzene	55	no buffer	1.4	1.4	1.3	0.7	1.9
4-bromochlorobenzene	55	no buffer	4.5	3.8	2.9	0.9	3.1
4-nitrophenol	55	no buffer	0.5	1.0	0.6	0.5	0.8
hexafluorobenzene	55	no buffer	2.6	2.1	2.2	0.7	3.1
pentachlorobenzene	55	no buffer	18.1	12.4	8.0	1.3	7.5
60% CH ₃ CN	60	no huffer	1 0	1.0	4 4	0.0	10
penzene butyl bonzono	00 60	no buffer	1.2 6 4	1.0	3.0	0.6	1.2
ethyl benzene	60	no buffer	0.4 2 6	4.4 2 1	3.9 1 0	0.0	3.∠ 1 0
propyl benzene	60	no buffer	4.1	3.0	2.7	0.7	2.5
toluene	60	no buffer	1.8	1.5	1.4	0.6	1.6
						-	-

* meaningful data could not be obtained due to coelution or other problem

Table 3. Guidelines for **Narrowing Down the Candidate Discovery Functionalized Reversed-Phase Column** for Operation at pH 7

pH 7 Operation

Screening Conditions: Columns: 150

Mobile Phase

Mobile Phase

Flow Rate:

Temperature:

Organic Modifier:

Buffer:

Use this chart as a starting point to choose one, two, three or more Discovery silica-based functionalized reversed-phase columns.

	hydrochlorothiazide	10	pH 7	3.0	4.2	2.7
ns:	nizatidine	10	рН 7	6.1	4.3	4.9
15cm x 4.6mm ID,	phentermine	10	bH 7	5.3	4.0	4.8
sµm particles	15% CH CN		P	0.0		
25mM Phosphoric Acid	codeine	15	nH 7	11	33	36
adjusted to pH 7 with		15	pri	4.4	5.5	5.0
Ammonium Hydroxide	20% CH ₃ CN	00		47	4.0	
(buffer was not used in	pnenacetin	20	рн /	4.7	4.8	4.1
the mobile phase when	25% CH ₃ CN					
non-ionic compounds	acetamide	25	no buffer	0.1	0.1	0.2
were screened)	anisole	25	no buffer	10.1	8.1	8.0
	benzaldehyde	25	no buffer	3.6	3.2	3.2
CH ₃ CN	benzamide	25	no buffer	0.6	0.7	0.7
1mL/min	benzyl alcohol	25	no buffer	1.4	1.5	1.5
30°C	methyl benzoate	25	no huffer	94	7.8	77
	o-cresol	25	no buffer	11	6.1	12
proximately 10 minutes	phonol	25	no buffor	2.0	2.0	4.2
on a 15cm x 4.6mm ID	prierioi	25		2.0	2.9	2.0
flow rate of 1mL/min.	Turosemide	25	рн 7	1.8	1.7	1.7
HPLC separations,	salicylic acid	25	рн 7	0.4	0.4	0.5
old decrease in k' for	30% CH ₃ CN					
ease in % organic.	papaverine	30	pH 7	5.9	5.8	4.9
	quinidine	30	pH 7	1.5	2.2	1.4
	40% CH_CN					
	butyl påraben	40	no buffer	4.8	7.9	4.0
	ethyl paraben	40	no buffer	1 4	2.5	14
	methyl paraben	40	no buffer	0.8	1.5	0.0
	nietityi paraben	40	no buffor	0.0	1.5	0.3
	propyr parabern	40		2.0	4.4	2.4
	dipnennydramine	40	рн 7	2.0	1.9	1.9
	fluoxetine	40	рн 7	2.6	3.4	2.6
	ibuprofen	40	pH 7	0.8	0.8	0.9
	lidocaine	40	pH 7	4.4	3.6	3.3
	norfluoxetine	40	pH 7	2.1	3.3	2.1
	50% CH,CN					
	bromobenzene	50	no buffer	3.8	3.2	2.8
	chlorobenzene	50	no buffer	3.3	2.8	2.5
	fluorobenzene	50	no buffer	2.0	1.8	17
	nitrobenzene	50	no buffer	1 /	1.0	13
	nitroochanzona	50		1.4	1.4	1.5
		50	no buller	1.0	1.0	1.5
	55% CH ₃ CN		. "			
	1,3,5-tribromobenzene	55	no buffer	13.0	9.4	6.0
	1,3-dinitrobenzene	55	no buffer	1.0	1.0	1.0
	1-chloro-2-fluorobenzene	55	no buffer	2.3	2.1	1.9
	2-chloronitrobenzene	55	no buffer	1.4	1.4	1.3
	4-bromochlorobenzene	55	no buffer	4.5	3.8	2.9
	4-nitrophenol	55	no buffer	0.5	1.0	0.6
	hexafluorobenzene	55	no buffer	2.6	2.1	2.2
	pentachlorobenzene	55	no buffer	18.1	12.4	8.0
	amitriptyling	55		2.0	17	1.9
	devenin	55	p117	2.0	1.7	1.0
	doxepin	55	ρ Π /	1.2	1.1	1.2
	Imipramine	55	рн 7	1.4	1.3	1.4
	nordoxepin	55	pH 7	0.4	0.6	0.5
	nortriptyline	55	pH 7	0.6	1.0	0.7
	protriptyline, desipramine	55	pH 7	0.5	0.8	0.6
	trimipramine	55	pH 7	3.0	2.3	2.2
	60% CH_CN					
	benzene	60	no buffer	1.2	1.0	1.1
	butyl benzene	60	no buffer	64	4.4	39
	ethyl benzono	60	no buffor	26		1.0
		60		2.0	2.1	1.8
		60		4.1	3.0	2.1
	toluene	60	no putter	1.8	1.5	1.4
	* meaningful data could not be c	obtained	I due to coelution	or other p	oroblem	

C18

k

7.1

1.4

1.5

3.5

1.2

1.0

1.7

0.1

3.2

3.0

3.5

1.8

%

Organic

5

5 5

5

5 5

5

pН

pH 7

pH 7

. pH 7

pH 7

. pH 7

pH 7

pH 7

pH 7

pH 7

. рН 7

pH 7

. pH 7

Compound Name

benzoic acid

benzyl amine

o-toluic acid

phthalic acid

procainamide

pyridine

10% CH, CN

sorbic acid

m-nitrobenzoic acid

o-aminobenzoic acid

o-nitrobenzoic acid

p-nitrobenzoic acid

5% CH₂CN

aniline

RP-AmideC16

k'

4.4

1.1

1.2

3.0

1.0

0.7

1.2

0.2

3.1

2.4

2.3

1.3

C8

k'

6.6

1.5

1.4

1.2

1.0

1.8

0.3

2.4

3.5

1.9

HS F5

k'

8.6

2.4

6.7

10.2

0.4

0.9

2.0

0.0

2.4

5.6

2.6

1.9

7.4

3.8

3.0

2.2

0.1

4.8

1.0

1.8

10.4

5.6

2.8

1.3

1.0

2.9

5.0

4.4

1.9

1.3

2.9

6.8

9.0

1.7

3.0

6.4

3.2

3.0

2.3

1.9

2.1 5.0

1.5

2.3

1.9

3.1

0.8

3.1

7.5 8.4

7.8

8.4 6.3 7.6

6.3 9.1

1.2

3.2

1.9

2.5

1.6

Cyano

k'

1.3

0.7

1.0

0.5

*

0.2

1.1

1.0

0.9

3.0

1.2

1.3

1.1

1.3

0.3

1.8

1.2

0.6

0.8

1.7

1.5

1.0

1.0

0.5

1.7

1.3

1.3

0.8

0.7

1.0

1.6

2.4

0.5

1.1

2.0

1.0

1.0

0.8

0.8

0.8

1.1 0.7

0.7

0.7

0.9

0.5

0.7

1.3

*

*

*

0.6

0.8

0.7

0.7

0.6

Note: A k' of 5 is approxim retention time on a column with a flow r

Note: For most RP-HPLC assume a 2-fold dec every 5% increase i

Figure A. Chromatograms from Column Screening



2 0 2 4 Min 0 10 10 20 Min G002111

Discovery RP-AmideC16



Ordering Information

Other dimensions and Discovery phases are available. Please call or visit our web site.

Phase	Cat. No.
Columns: 15cm x 4.6mm ID, 5µm particles	
Discovery C18	504955
Discovery C8	59353-U
Discovery Cyano	59356-U
Discovery RP-AmideC16	505013
Discovery HS F5	59356-U
Discovery HS PEG	505013

Phase	Pack of 2 Cat. No.	Kit ² Cat. No.
Supelguard Cartridges: 2.00 5µm particles	cm x 4.0mm ID,	
Discovery C18 ¹	505137	505129
Discovery C8 ¹	59590-U	59589-U
Discovery Cyano ¹	59585-U	59586-U
Discovery RP-AmideC16 ¹	505099	505080
Discovery HS F5 ¹	567576-U	567577-U
Discovery HS PEG ¹	567476-U	567477-U

¹For 4.0mm ID and 4.6mm ID analytical columns.

 $^2\mbox{Kits}$ include one cartridge, a stand-alone holder, a piece of tubing, and 2 nuts and ferrules.

Trademark Discovery - Sigma-Aldrich Co.

> For expert answers to your questions, contact our Technical Service Department: Phone **800-359-3041**, **814-359-3041** Fax **800-359-3044**, **814-359-5468** E-mail **techservice@sial.com**

To download Supelco's free technical literature visit us at sigma-aldrich.com/supelco-literature



© 2003 Sigma-Aldrich Co. Printed in USA. Supelco brand products are sold through Sigma-Aldrich, Inc. Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse FVT side of the invoice or packing slip.