VOL 20.3

The Reporter

sigma-aldrich.com/TheReporter

The Effect of Ion Pair Reagent on Reversed-Phase Selectivity of Polypeptide Separations

Reversed-phase high performance liquid chromatography (RP-HPLC) has been a preferred method for resolution of peptides by virtue of its resolving power and suitability of aqueous samples. Peptides typically contain multiple charges, which can be of opposite polarity, and which therefore present a unique challenge for their chromatographic separation on silica-based reversed-phase supports. Within historic pH limitations of silica supports (pH 2 – 8) charged functional groups of polypeptides may include carboxylates (pK_as ~2, ~4), sulfhydryls (pK_a ~8), amines (pK_as ~6, 9-10, or ~11), and guanidines (pK_a ~12-13). Traditionally, reduction in the effective charge has been accomplished by two simultaneous means: 1) reduction of pH (typically near 2) to minimize the charge on the carboxylates (at pH 2 the only group on the polypeptide not fully protonated should be the α-carboxylate of the C-terminus) and 2) inclusion of an ion-pairing reagent that not only masks the positive charges (by pairing with them) but also enhances the hydrophobicity of the peptide. Inclusion of strongly acidic ion-pairing reagents provides both functions – reduction of pH and pairing with remaining basic groups. As such, they provide another tool by which to manipulate selectivity – not just by effects of concentration on selectivity, but more importantly by choice of the reagent, since they can differ significantly in hydrophobicity.

By far the most common ion-pair reagent for RP-HPLC of polypeptides is trifluoroacetic acid (TFA), traditionally used at concentrations of 0.1% (*v*/*v*) though lower concentrations are commonly employed, especially for LC/MS. It is the smallest of the fluorosubstituted organic acids employed for this function. The fluorosubstitutions enhance the acidity and hydrophobicity of the reagent as compared to the unsubstituted acid. A less common ion-pairing reagent for RP-HPLC, but particularly useful for less hydrophobic peptides, is heptafluorobutyric acid (HFBA).

In the example (Figures A and B), we compare the selectivity of a commercial peptide mixture as affected by ion pairing reagents – in Figure A is included TFA and in Figure B is included pentafluoropropionic acid (PFPA) – an ion pairing reagent with properties intermediate between the typical TFA and less common HFBA. While absolute retention cannot be compared in these two runs (since the initial conditions are not the same), some

(continued on page 4)

LC issue

Figure A. Synthetic Peptide Mixture Chromatographed with TFA Mobile Phase

Column: Discovery BIO Wide Pore C18, 15cm x 4.6mm, 5µm
Cat. No.: **568222-U**
Mobile Phase A: 80:20, (water/0.1%TFA) : (MeCN/0.1%TFA)
Mobile Phase B: 66:34, (water/0.1%TFA) : (MeCN/0.1%TFA)
Flow Rate: 1.0mL/min; **Det.:** 215nm
Temp.: 30° C
Inj.: 10µL (~25µg ea. Peptide)

Peak	Peptide	Amino Acid Sequence
1	Arg^8-vassopressin	CYFQNCPRG-amide; disulfide
2	Bradykinin, fragment 1-5	RPPGF
3	Oxytocin	CYIQNCPLG-amide; disulfide
4	LHRH*	pEHWSYGLRPG-amide **
5	Met-enkephalin	YGGFM
6	Bradykinin	RPPGFSPFR
7	Leu-enkephalin	YGGFL
8	Bombesin	pEQRLGNQWAVGHLM-amide **
9	Substance P	RPKPQQFFGLM-amide

* Luteinizing Hormone Releasing Hormone
** pE is pyroglutamate

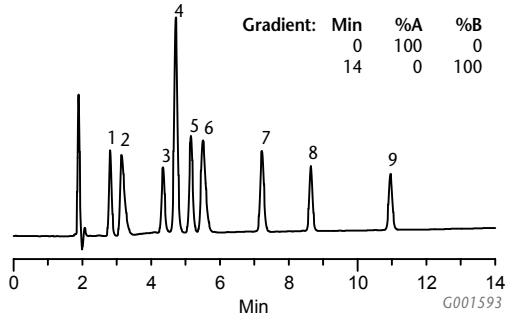
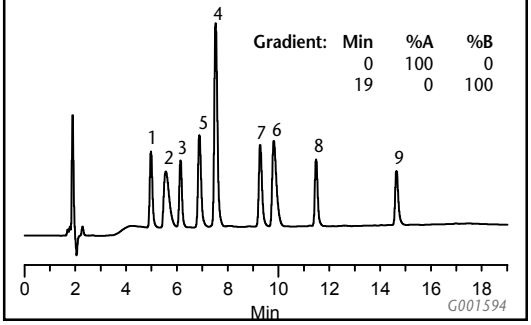


Figure B. Synthetic Peptide Mixture Chromatographed with PFPA Mobile Phase

Column: Discovery BIO Wide Pore C18, 15cm x 4.6mm, 5µm
Cat. No.: **568222-U**
Mobile Phase A: 81:19, (water/0.1%PFPA) : (MeCN/0.1%PFPA)
Mobile Phase B: 62:38, (water/0.1%PFPA) : (MeCN/0.1%PFPA)
Flow Rate: 1.0mL/min
Det.: 215nm
Temp.: 30° C
Inj.: 10µL (~25µg ea. Peptide)



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SUPELCO
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T202003

Discovery BIO Wide Pore Capillary and Microbore HPLC Columns

Discovery SPE Tubes, SPE-96 Well Plates and Büchner Funnels

NEW PRODUCTS

HPLC Capillary and Microbore Dimensions of Discovery BIO Wide Pore C5 and C18

Increase sensitivity and conserve precious samples on Discovery BIO Wide Pore capillary or microbore dimensions without sacrificing efficiency or resolution.

For protein and peptide chemists, Discovery BIO Wide Pore C5 and C18 is now available in 0.32mm and 0.5mm ID capillaries and 1mm ID microbore HPLC columns. Because samples are diluted over a smaller column volume, capillary or microbore columns give greater peak height (sensitivity) than columns with conventional internal diameters (*e.g.* 4.6mm). These new dimensions, when packed with highly efficient and selective Discovery BIO Wide Pore C5 or C18 phases, give greatly enhanced sensitivity. They are ideal for peptide mapping, proteomics, and other applications were analytes exist at extremely low levels or where sample volume is limited. All Discovery BIO Wide Pore phases are no-bleed and stable at low and high pH making them suitable for LC/MS.

For more information, request T402051 or visit ***sigma-aldrich.com/thereporter.***

Discovery BIO Wide Pore C5 - Capillary and Microbore Dimensions (for proteins and hydrophobic peptides)

Particle Size (micron)	Length (cm)	ID (mm)	Cat. No.
3	5	0.32	**65531-U**
3	10	0.32	**65532-U**
3	5	0.5	**65520-U**
3	10	0.5	**65521-U**
3	5	1.0	**65511-U**
3	10	1.0	**65512-U**
5	15	0.32	**65533-U**
5	15	0.5	**65522-U**
5	15	1.0	**65513-U**

Discovery BIO Wide Pore C18 - Capillary and Microbore Dimensions (for peptides and small molecules)

Particle Size (micron)	Length (cm)	ID (mm)	Cat. No.
3	5	0.32	**65526-U**
3	10	0.32	**65527-U**
3	5	0.5	**65517-U**
3	10	0.5	**65518-U**
3	5	1.0	**65504-U**
3	10	1.0	**65506-U**
5	15	0.32	**65529-U**
5	15	0.5	**65519-U**
5	15	1.0	**65508-U**
5	25	1.0	**65509-U**

Discovery Solid Phase Extraction (SPE) Products for Pharmaceutical Analysis and Purification

Reversed-phase… normal phase… ion exchange… Discovery SPE is the next generation of silica based sorbents designed specifically for the reproducible isolation and recovery of diverse compounds from difficult sample matrices. The multitude of phase chemistries and hardware configurations available within the Discovery SPE line offers a comprehensive level of selection and flexibility required to handle today's increasingly complex and diverse sample prep problems.

A complete array of hardware configurations to better suit your specific application needs.

- Protects analytical instrument from unwanted sample matrix components
- Concentrates target analyte(s) for increased sensitivity
- Removes background interferences for improved accuracy and sensitivity
- Improve extraction selectivity through Discovery's narrower pore size distribution
- Ultra clean phases for highly sensitive analyses
- Achieve better reproducibility through tightly controlled physical, chemical, and packing specifications
- Acid washed to reduce metal chelating activity

For more information, email atrinh@sial.com

Discovery SPE Product		Reversed-Phase				Normal Phase				Ion Exchange		
Dimension	Qty.	DSC-18	DSC-18Lt	DSC-8	DSC-Ph	DSC-CN	DSC-Si	DSC-Diol	DSC-NH_2	DSC-SAX	DSC-WCX	DSC-SCX
Discovery SPE Tubes												
50mg/1mL	108/pk	**52601-U**	**52610-U**	**52703-U**	**52723-U**	**52693-U**	**52652-U**	**52747-U**	**52635-U**	**52661-U**	**52737-U**	**52684-U**
100mg/1mL	108/pk	**52602-U**	**52611-U**	**52707-U**	**52725-U**	**52694-U**	**52653-U**	**52748-U**	**52636-U**	**52662-U**	**52739-U**	**52685-U**
500mg/3mL	54/pk	**52603-U**	**52613-U**	**52713-U**	**52727-U**	**52695-U**	**52654-U**	**52751-U**	**52637-U**	**52664-U**	**52741-U**	**52686-U**
500mg/6mL	30/pk	**52604-U**	**52615-U**	**52714-U**	**52728-U**	**52696-U**	**52655-U**	**52752-U**	**52638-U**	**52665-U**	**52742-U**	**52688-U**
1g/6mL	30/pk	**52606-U**	**52616-U**	**52716-U**	**52731-U**	**52697-U**	**52656-U**	**52753-U**	**52640-U**	**52666-U**	**52743-U**	**52689-U**
2g/12mL	30/pk	**52607-U**	**52618-U**	**52717-U**	**Custom**	**52698-U**	**52657-U**	**Custom**	**52641-U**	**52677-U**	**52744-U**	**52690-U**
5g/20mL	20/pk	**52608-U**	**52621-U**	**52718-U**	**Custom**	**52699-U**	**52658-U**	**Custom**	**52642-U**	**52688-U**	**52745-U**	**52691-U**
10g/60mL	20/pk	**52609-U**	**52622-U**	**52722-U**	**Custom**	**52700-U**	**52659-U**	**Custom**	**52644-U**	**52699-U**	**52746-U**	**52692-U**
Bulk packing	100g	**52600-U**	**52623-U**	**57223-U**	**57227-U**	**57222-U**	**52651-U**	**57229-U**	**57212-U**	**57214-U**	**57228-U**	**57221-U**
Discovery SPE-96 Well Plates												
100mg/well	1ea	**575603-U**	**575606-U**	**575627-U**	**575630-U**	**575624-U**	**575609-U**	**575636-U**	**575615-U**	**575618-U**	**575633-U**	**575621-U**
50mg/well	1ea	**575602-U**	**575605-U**	**575628-U**	**575631-U**	**575625-U**	**575608-U**	**575637-U**	**575616-U**	**575619-U**	**575634-U**	**575622-U**
25mg/well	1ea	**575601-U**	**575604-U**	**575629-U**	**575632-U**	**575626-U**	**575607-U**	**575638-U**	**575617-U**	**575620-U**	**575635-U**	**575623-U**
Discovery Büchner Funnels												
55mmID x 30mmH, 12.5g	6 qty/pk	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**	**52591-U**	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**
70mmID x 40mmH, 25g	6 qty/pk	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**	**52592-U**	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**
90mmID x 48mmH, 50g	6 qty/pk	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**	**52593-U**	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**
110mmID x 66mmH, 100g	6 qty/pk	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**	**52594-U**	**Custom**	**Custom**	**Custom**	**Custom**	**Custom**

NEW APPLICATIONS

Resolution of Cytochrome c Homologs

To inspect the resolving power of the new Discovery BIO HPLC product line, we sought a sample of biological macromolecules of substantial sequence identity. Based on availability, we chose cytochrome c from various sources. These six homologous proteins of ~11,600 molecular weight (104 amino acids) remarkably retain a >88% sequence identity. As shown in Figure D, all six proteins were resolved, albeit by application of a very shallow gradient. Within the parameters investigated (bonded phase, pH, temperature, and ion pairing reagent) the chromatogram shown is the optimized separation. It is of particular interest to note that resolution of the various proteins is not correlated with overall sequence similarity. This is not surprising, since the location of a given amino acid substitution is independent of the chromatographic contact surface of the protein. Rather, what is remarkable is that such small changes in amino acid sequence can have significant effects on retention, and that none of the proteins coelute. However, perhaps the two most similar, pigeon and chicken, which differ in three conservative substitutions and one nonconservative substitution are, not surprisingly, the least resolved.

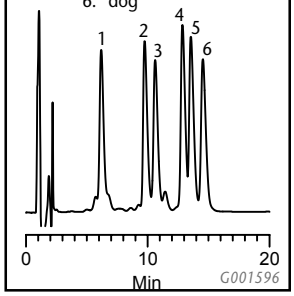
For more information, request T401095, T401096, T401097, T401098, T401099, T401100 or visit ***sigma-aldrich.com/thereporter.***

Figure D. Resolution of Six Cytochrome c Isozymes

Column: Discovery BIO Wide Pore C5, 15cm x 4.6mm, 5µm; **Cat. No.:** 568422-U; **Mobile Phase A:** 70:30, (water/0.1%TFA) : (MeCN/0.1%TFA), **Mobile Phase B:** 64:36, (water/0.1%TFA) : (MeCN/0.1%TFA); **Flow Rate:** 1.0mL/min; **Det.:** 220nm; **Temp.:** ambient; **Inj.:** 12µL (~10µg ea.);

Gradient:	**Min**	**%A**	**%B**
	0	100	0
	30	0	100

1. horse
2. rabbit
3. cow
4. pigeon
5. chicken
6. dog



NEW LITERATURE

Discovery BIO Wide Pore HPLC Columns and Capillaries

Learn about Supelco's newest addition to our premier HPLC column line, new Discovery BIO Wide Pore C5, C8, and C18, in this comprehensive, 16-page brochure.

In it, you will see how Discovery BIO Wide Pore HPLC columns and capillaries solve the challenges facing the biotech and proteomics industries by providing sensitive, stable, efficient, scalable, and reproducible HPLC separations of proteins, synthetic and natural peptides, and peptide maps. The brochure details applications that demonstrate the differences between the Discovery BIO Wide Pore phases, and with competitive phases as well. Because Discovery BIO Wide Pore phases are low-bleed and stable at low and high pH, they are ideal for LC/MS applications. Extensive stability data presented in the brochure demonstrates the enhanced stability of the Discovery BIO Wide Pore C5 over conventional C3 and C4 phases.

For more information, request T402038 or visit ***sigma-aldrich.com/thereporter.***

Reversed-Phase LC/MS Analysis of Three Basic Pharmaceutical Compounds From Serum Using Discovery DSC-18 SPE-96 Plates and Discovery HS C18, 3µm

The use of short, narrow bore, small particle, "no bleed", reversed-phase columns in conjunction with MS-MS analysis and 96-well solid phase extraction provides a powerful tool for the high throughput resolution and quantitation of pharmaceuticals in biological fluids. An assay incorporating these three key technologies was developed for the determination of three anti-ulcer compounds (nizatidine, ranitidine, and cimetidine) spiked in porcine serum. The three basic compounds were extracted from serum using a highly generic SPE protocol on Discovery DSC-18 SPE-96 Plate. Extracted samples were further resolved using a Discovery HS C18, 3µm HPLC column prior to analysis via tandem mass spectroscopy.

For more information, request T401203 or visit ***sigma-aldrich.com/thereporter.***

LIQUID CHROMATOGRAPHY PERFORMANCE TIP

Practical Tips on Buffer Preparation

In this Performance Tip, we offer some of the more practical aspects of buffer preparation for biomolecules.

1) In preparing the buffer, dissolve the solid in ~95% of the final volume desired. After the solution is adjusted to the pH desired, bring the solution to volume.

2) Always calibrate the pH meter either at the final adjusted pH (the final adjusted pH must match the pH of a standard) or calibrate the pH meter at values above and below the final adjusted pH. This is the only way to reliably measure pH with a pH-meter. You cannot reliably measure a value outside the range for which you have calibrated in instrument.

3) For consistent results, pH adjustments to the aqueous solution should be made before addition of organic solvent. True, the actual pH will change after addition of the organic, but there is no good method for reliable measurement of pH after such addition. In this way, the laboratory practice is consistent.

4) All buffer solutions should be filtered before use as mobile phase in HPLC. This is to remove any particulates that may already be in the water or introduced by the solid buffer when dissolved. A hydrophilic 0.45µ filter is recommended. Filtration should be a last step before use as is or before mixing with an organic component.

For more information, request T401012 or visit ***sigma-aldrich.com/thereporter.***

The Effect of Ion Pair Reagent...

(continued from page 1)

changes in selectivity are seen. First, note that some of the sample components are quite similar, confirming the particular resolving power of RP-HPLC, but also of the Discovery BIO Wide Pore C18 column in particular. Peaks 1 and 3 are nonapeptides that differ by one conservative substitution (F vs. I) and one less conservative substitution (R vs. L). Peaks 2 and 6 differ in that the latter contains and additional four amino acid C-terminal extension. Peaks 5 and 7 differ by a conservative substitution in the C-terminal position (M vs. L). In comparing the chromatogram of Figure B to Figure A, elution orders are reversed for two pairs: Peaks 4 and 5; and Peaks 6 and 7. In both cases, it is the more basic peptide that elutes later in the case of PFPA as the ion-pairing reagent. This might be expected since the retention of peptides of greater basicity is predicted to be more greatly effected by the hydrophobicity of an ion-pairing reagent.

Thus we have demonstrated the high resolving power of the Discovery BIO Wide Pore column and the utility of choice of ion pairing reagent to effect selectivity.

For more information, request T402038, T401097, T401098, T401099, T401100 or visit ***sigma-aldrich.com/thereporter.***

CASE STUDY 2

Resolution of Angiotensins I, II, and III on Discovery BIO Wide Pore HPLC Columns at pH 7

Referring to the primary structure of angiotensins in Figure C, angiotensin I possesses the additional C-terminal residues histidine and leucine which give it additional retention by RP-HPLC. However, the difference between angiotensins II and III is that the former contains an additional *N*-terminal aspartate. Under traditional conditions employed for RP-HPLC of polypeptides (pH ~2), the additional *N*-terminal aspartate of angiotensin II confers negligible hydrophobicity, such that angiotensins II and III are not resolved. This has historically been a case for the application of polymeric reversed-phase supports which can be used under alkaline conditions where the *N*-terminal aspartate of angiotensin II confers a substantial reduction in hydrophobicity, and therefore, baseline resolution of angiotensin II and III is possible. However, as shown in Figure C, all three angiotensins are clearly resolved at neutral pH on Discovery BIO Wide Pore C18. The reduction in retention afforded by aspartate at neutral pH as opposed to acidic pH is because at the lower pH, the side chain carboxylate is fully protonated and therefore neutral, but exhibits little retention. In contrast, at neutral pH, the side chain is fully ionized, thus negatively impacting retention.

For more information, request T401095, T401096, T401097, T401098, T401099, T401100 or visit ***sigma-aldrich.com/thereporter.***

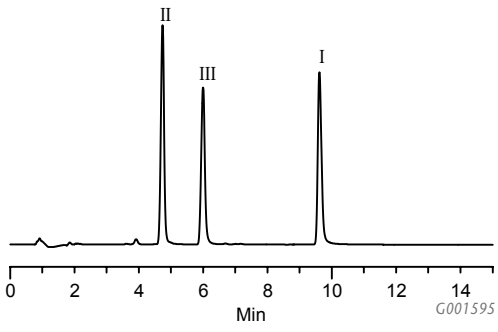
Figure C. Resolution of Three Angiotensins at Neutral pH

Column: Discovery BIO Wide Pore C18, 15cm x 4.6mm, 5µm
Cat. No.: 568222-U
Flow Rate: 1.0mL/min
Det.: 215nm
Temp.: R.T.
Inj.: 6µL (~10µg ea.)
Solution x: 10mM NH_4OAc/NH_4OH, pH 7.0
Solution y: 50:50 (20mM NH_4OAc/NH_4OH, pH 7.0) : MeCN
Mobile Phase A: 65:35, x:y
Mobile Phase B: 40:60, x:y

Gradient:	**Min**	**%A**	**%B**
	0	100	0
	12.5	0	100

angiotensin I	DRVYIHPFHL
angiotensin II	DRVYIHPF
angiotensin III	RVYIHPF

1. Angiogensin I
2. Angiotensin II
3. Angiotensin III



All literature mentioned in this issue can be obtained from the website, ***sigma-aldrich.com/TheReporter,*** *by completing the Literature Request section on the reply card, or by calling our Technical Service Dept.*

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