VOL 20.5

TheReporter

sigma-aldrich.com/TheReporter

Ionic Additives and LC/MS Conditions for Polypeptide Analysis

In issue 20.3 of The Reporter the role of ion-pairing reagents (e.g. TFA) in reversed-phase high performance liquid chromatography (RP-HPLC) of polypeptides was presented. In any RP-HPLC separation, ionic additives (like ion-pairing agents) in the mobile phase serve one or more of the following functions: pH control (buffering), suppression of adverse ionic interactions that can occur between silanols and basic analytes and between analyte molecules, or complexation with oppositely charged ionic groups to enhance RP retention. When using an ionic additive, consideration of its effect on LC/MS response must also be made.

While ion-pairing reagents have a very dramatic beneficial effect on RP-HPLC of polypeptides, they generally cause considerable problems for MS detection. In the case of trifluoroacetic acid (TFA), the reasons include: 1) typical concentrations (0.1% *v/v*) of TFA prevent efficient spray formation due to the high surface tension of the mobile phase and 2) TFA ions in the gas phase ion-pair with the polypeptide basic groups suppressing ionization and reducing sensitivity (A. Apffel, *et al*, Journal of Chromatography, vol. 712, pg. 177, 1995). Therefore, ionic reagents other than TFA that are still volatile, can provide pH control, and do not exhibit the strong ion-pairing exhibited by the perfluorinated organic acids, are desirable.

While pH control below pH 3 can still keep protein carboxylate groups neutral (other than the C-terminal carboxylate) to maximize retention and chromatographic performance, the weak ion-pairing afforded by simple, small organic acids, like formic acid, leaves the polypeptide basic groups susceptible to silanol interactions. These interactions lead to peak tailing and decreased resolution and sensitivity. Using a highly efficient and inert RP-HPLC column like Discovery BIO Wide Pore C18, especially in the case of basic analytes, overcomes this dilemma (see the Performance Tip and Case Study of this issue for demonstrations of this principle).

Although TFA increases retention and can improve peak shape, the improvement comes at the expense of MS signal (response or sensitivity). To demonstrate the effect of TFA on MS signal we ran a series of peptides at three different TFA concentrations and measured the MS signal. The data is presented in Figure A. Comparison of Figures A1, A2, and A3, which have the same scale, shows that maximum MS signal is achieved with no TFA in the mobile phase. (In Figure A1 formic acid was added to the mobile phase to control pH).

(continued on page 4)

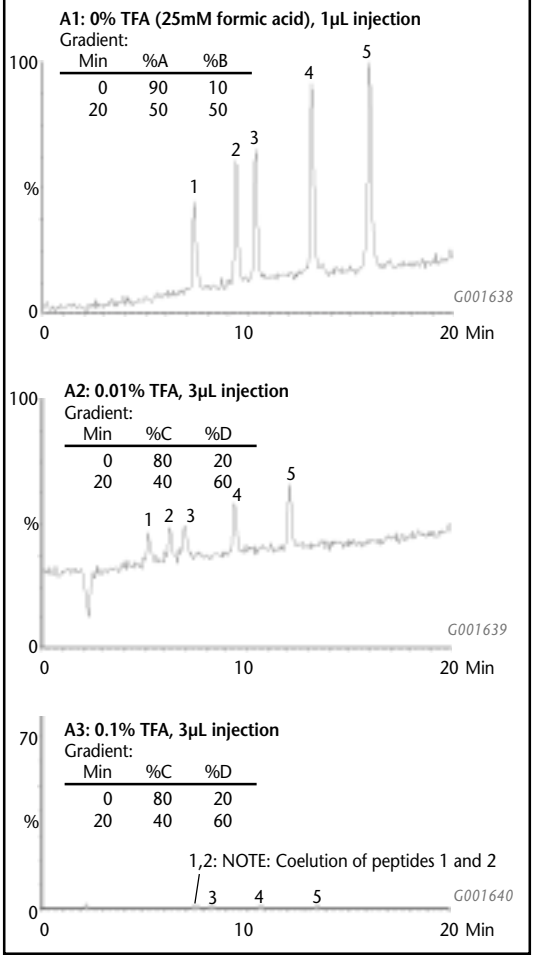
LC issue

Figure A. Effect of Chromatographic Conditions on MS Signals of Peptides

Column: Discovery BIO Wide Pore C18, 15cm x 2.1mm, 3µm; **Cat. No.:** 567202-U; **Mobile Phase A:** 25mM formic acid in water, **Mobile Phase B:** 50:50 (25mM formic acid in water) : (20mM formic acid in MeCN)[a], **Mobile Phase C:** 0.01 or 0.1% TFA in water, **Mobile Phase D:** 50:50 (0.01 or 0.1% TFA in water) : (0.01 or 0.1% TFA in MeCN); **Flow Rate:** 0.208mL/min[b]; **Det.:** +ES; **Temp.:** ambient; **Inj.:** 1µL or 3µL; **Sample:** RP Peptide Performance Standard, p/n RPS-P0010 (Alberta Peptide Institute)

a) molarity of formic acid adjusted to provide minimum baseline drift
b) linear velocity equal to 1mL/min on 4.6mm ID columns

Peptide 1: RGAGGLGLGK-amide
Peptide 2: ac-RGGGGLGLGK-amide
Peptide 3: ac-RGAGGLGLGK-amide
Peptide 4: ac-RGVGGLGLGK-amide
Peptide 5: ac-RGVVGLGLGK-amide



CONTENTS

SUPELCO
a member of the Sigma-Aldrich family

T202005

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, T402038, T401097, T401099 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 is available 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 request T402052, T402053, T402054 or 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

Confirmation of Peptide Identification by LC/MS

The peptides angiotensin I, II, and III have been shown to be resolved on Discovery BIO Wide Pore RP-HPLC (Reporter 20.3). We chose to take the method, adapt it to conditions amenable for LC/MS, and inspect the MS data. The former phosphate-buffered pH 7.0 mobile phase was substituted with ammonium acetate, pH 7.0. While this is not buffered at pH 7.0, it does provide for adequate pH control to resolve the three species. Figure C1 shows the separation (total ion chromatogram) of the three angiotensin peptides. Figures C2, C3, and C4 show the scans of the three individual peaks. In all three mass scans, the large peak of lowest m/z (or in the case of panel C4, the only peak) corresponds to $[M+2H]^{2+}$ ions, while the other major peaks of larger m/z value likely correspond to $[M+H]^{+}$ ions. Results of all three scans confirm the peak identities. Obviously, this is a simple case. However, in the case of a complex sample of unknowns, combined with adequate mass accuracy of the detector, peptides can often be identified by mass alone when searched against an appropriate database. This is referred to as peptide mass fingerprinting. Another advantage shown in this application are the use of very narrow ID columns, in this case 0.32mm ID capillaries, to greatly enhance sensitivity and decrease sample consumption.

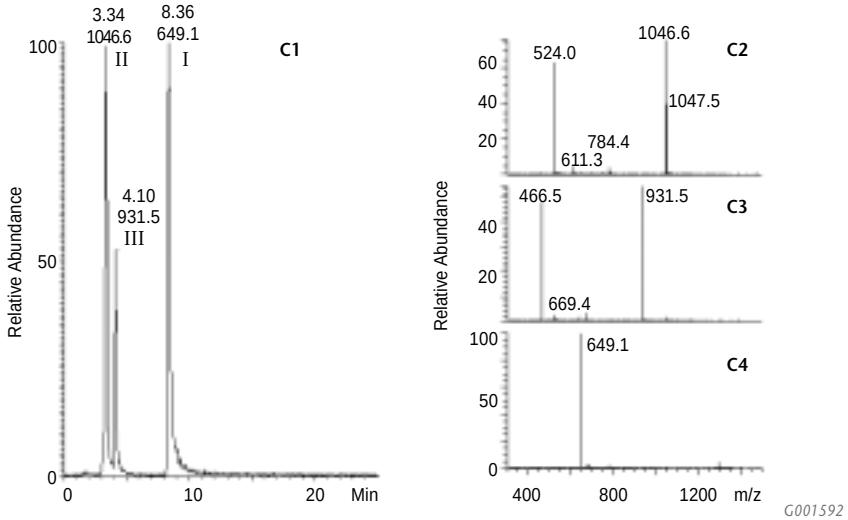
For more information request T402038, T402051.

Figure C. LC/MS of Angiotensin Mixture

Column: Discovery BIO Wide Pore C18, 10cm x 0.32mm, 3µm; **Cat. No.:** 65527-U; **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:** x:y, 65:35; **Mobile Phase B:** x:y, 25:75; **Flow Rate:** 6µL/min; **Det.:** +ES, source 2.5kV, capillary 12V, 130° C; **Temp.:** ambient; **Inj.:** 50pmol

Gradient:

Min	%B
0	0
12.5	100



LIQUID CHROMATOGRAPHY PERFORMANCE TIP

Maximizing Peak Efficiency in LC/MS

When used at traditional concentrations, typically above 0.05% (*v*/*v*), TFA can effectively mask silanol interactions. Under those conditions, most modern RP-HPLC columns will give good peak shape. However, when TFA is absent or used at very low concentrations, with polypeptide separations (for the purpose of maximizing MS sensitivity), the inertness, or lack of silanol activity, of the column becomes increasingly relevant to attaining the best chromatographic performance. Differences in column inertness are obvious under low- or no-TFA conditions. Figure D shows the separation of four basic peptides on Discovery BIO Wide Pore C18 (D1) and two other modern wide pore C18 silica columns (D2, D3). Results show that under the same conditions, Discovery BIO Wide Pore C18 provides superior resolution of the most basic and hydrophobic peptides. This clearly illustrates that choosing the best column becomes a critical element for developing efficient and sensitive LC/MS methods.

For more information request T402038, T402051.

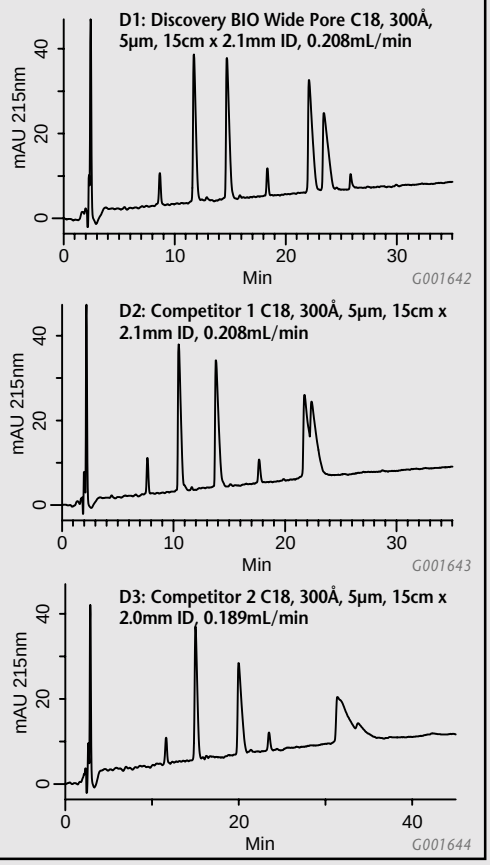
Figure D. Column Performance Differences toward Basic Peptides without TFA

Columns: C18, 300Å, 5µm, 15cm x 2.1 or 2.0 mm ID; **Mobile Phase A:** 25mM formic acid in water; **Mobile Phase B:** 50:50 (25mM formic acid in water) : (20mM formic acid in MeCN); **Flow Rate:** 0.208 (or 0.189) mL/min; **Det.:** 215nm; **Temp.:** 35° C; **Inj.:** 0.5µL (~0.25µg ea peptide); **Sample:** RP Peptide Ionic Interactions Standard, p/n RPS-I0020 (Alberta Peptide Institute)

Gradient:

Min	%B
0	15
45	60

Peptide 1: ac-GGGLGGAGGLK-amide
Peptide 2: ac-KYGLGGAGGLK-amide
Peptide 3: ac-GGALKALKGLK-amide
Peptide 4: ac-KYALKALKGLK-amide



*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.*

Ionic Additives and LC/MS...

(continued from page 1)

This data is also tabulated as S/N (signal to noise) ratios in Table 1. The detrimental effect of TFA on MS signal (sensitivity) is dramatic. Another disadvantage of high concentrations of TFA is that the strong ion-pairing masks small differences between peptides, which can reduce their resolution. In the example shown in Figure A, peptide 2 differs from peptide 1 only by acetylation of the *N*-terminus and one less methyl group (glycine vs alanine). Note that they are resolved at 0 and 0.01% TFA, but not at 0.1% TFA.

The results of this study demonstrate that for detection of low levels of polypeptides by LC/MS, TFA should be avoided. Viable alternatives to TFA for the LC/MS of polypeptides are formic acid or acetic acid. These acids effectively control pH, but do not suppress ionization and LC/MS signal. However, when eliminating TFA to avoid peak tailing, it is desirable to use an inert, efficient RP-HPLC column that gives good peak shape with or without TFA. Discovery BIO Wide Pore meets this non-trivial requirement. Its performance without TFA is compared to competitive RP-HPLC phases in the Performance Tip.

For more information request T402056, T402038.

Table 1. MS Signal-to-Noise Ratio with and without TFA

Signal-to-noise ratio is a measure of analyte peak height relative to the baseline noise and is an indication of sensitivity. Even upon injecting 3-times as much sample, the presence of TFA significantly reduces the signal-to-noise ratio. At 0.1% TFA, peptide peaks were too small to measure.

		S / N Ratio				
Condition	**µL injected**	**Peak 1***	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**
25mM formic	1	39	56	54	78	85
0.01% TFA	1	7	8	10	9	14
0.01% TFA	3	11	13	13	20	26

* Refer to Figure A for the peak identity.

CASE STUDY 3

Predicting Column Performance Under LC/MS Conditions

As the lead article discussed, for optimum LC/MS sensitivity TFA should be used at the lowest possible concentration, if at all. This is because of its severe negative impact on analyte ionization. Traditionally, TFA is used at 0.05% (*v/v*) for purposes of pH control (to neutralize protein carboxylates) and masking of polypeptide basic moieties by ion-pairing with them. As such, it greatly improves the chromatography of these analytes by enhancing retention and reducing silanol interactions. We therefore sought to document in a quantitative way how a reduction in TFA concentration affects RP-HPLC of peptides. Retention of the same peptide sample used in Figure A was measured at different concentrations of TFA. Results in Figure B show that retention is affected by TFA concentration in a predictable fashion.

As TFA concentration is decreased, so does the retention because of reduced ion-pairing. Chromatographic selectivity is also affected, particularly for the first two eluting peptides.

This small study demonstrates that when changing the ionic additives in the mobile phase, particularly if using TFA, the column performance even for highly inert columns can be affected.

An expanded version of this Case Study is available at ***sigma-aldrich.com/thereporter.***

For more information request T402056, T402038.

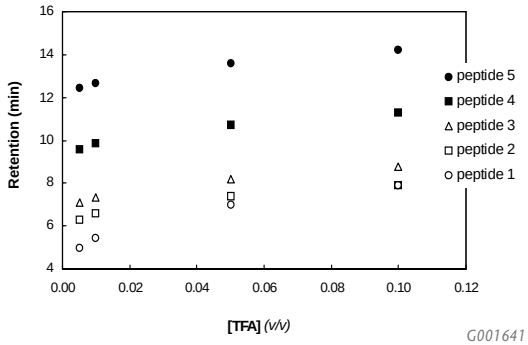
Figure B. Effect of TFA Concentration on Retention

Column: Discovery BIO Wide Pore C18, 15cm x 2.1mm, 3µm; **Cat. No.:** 567202-U; **Mobile Phase A:** x% TFA in water; **Mobile Phase B:** 50:50 (x% TFA in water) : (x% TFA in MeCN); (x = 0.005, 0.01, 0.05, 0.10); **Flow Rate:** 0.208mL/min; **Det.:** 215nm; **Temp.:** 35° C; **Inj.:** 0.2µL (~0.2µg ea peptide)

Gradient:

Min	%B
0	20
20	60

Peptide 1: RGAGGLGLGK-amide
Peptide 2: ac-RGGGGLGLGK-amide
Peptide 3: ac-RGAGGLGLGK-amide
Peptide 4: ac-RGVGGLGLGK-amide
Peptide 5: ac-RGVVGLGLGK-amide



sigma-aldrich.com/supelco

Order/Customer Service 800-247-6628, 800-325-3010 ● Fax 800-325-5052 ● E-mail supelco@sial.com
Technical Service 800-359-3041, 814-359-3041 ● Fax 800-359-3044, 814-359-5468 ● E-mail techservice@sial.com
SUPELCO ● Supelco Park, 595 North Harrison Road, Bellefonte, PA 16823-0048 ● 814-359-3441

ISO 9001 REGISTERED

We are committed to the success of our Customers, Employees and Shareholders through leadership in ***Life Science, High Technology*** *and* ***Service.***

The SIGMA-ALDRICH Family SIGMA ALDRICH Fluka Riedel-de Haën SUPELCO

T202005
ENS