

Determination of Residual Trifluoroacetate in Protein Purification Buffers and Peptide Preparations by Reagent-Free™ Ion Chromatography

INTRODUCTION

A Reagent-Free ion chromatography (RFIC) system allows the determination of anions or cations with only the addition of deionized water. For anion analysis, the RFIC system prepares high-purity, carbonate-free potassium hydroxide eluents. After separation on the anion-exchange column, an anion self-regenerating suppressor automatically suppresses the eluent and the sample anions are detected by suppressed conductivity. RFIC allows rapid method development and easy transfer of methods to other labs. In this application note, we used RFIC to determine the concentration of residual trifluoroacetate (TFA) in samples of interest to the pharmaceutical and biotechnology industries.

TFA is commonly used during the purification of pharmaceutical and biotechnology products. For example, TFA is used with an acetonitrile gradient on a preparative reversed-phase HPLC column to purify synthetic peptides. Because TFA is toxic, its removal must be reliably measured in products intended for preclinical or clinical applications. The high-capacity IonPac® AS18 anion-exchange column was used to separate trace TFA from an excess of chloride, phosphate, and other anions in three different pharmaceutical buffers. The method presented in this application note expands on the work presented in Application Note 115, "Determination of Trifluoroacetate (TFA) in Peptides", that described the use of a carbonate/bicarbonate eluent with the IonPac AS14. This new method, based on the use of RFIC with an IonPac AS18 column, improves the sensitivity of TFA determinations and allows more samples to be analyzed directly.

EQUIPMENT

Dionex Ion Chromatography system (ICS-2000 or ICS-2500) consisting of:

GP50 Gradient Pump

CD25A Conductivity Detector

EG50 Eluent Generator with EluGen® EGC II KOH cartridge (P/N 060585)

AS50 Autosampler with thermal compartment

Columns: IonPac AS18 analytical, 4 × 250 mm (P/N 060549)

IonPac AG18 guard, 4 × 50 mm (P/N 060551)

50-μL sample loop (P/N 42950) or 100-μL sample loop (P/N 42951)

Suppressor: ASRS® ULTRA II, 4 mm (P/N 61561)

CR-ATC (Continuously Regenerated Anion Trap Column) (P/N 060477)

Chromeleon® Chromatography Workstation (Release 6.5 and higher)

REAGENTS AND STANDARDS

Deionized water, Type I reagent-grade, 18 MΩ-cm resistance

Sodium trifluoroacetate (trifluoroacetic acid, sodium salt) (Aldrich P/N 13,210-1)

Now sold under the
Thermo Scientific brand

Thermo
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SAMPLES

Phosphate-Buffered Saline, pH 7.4 (Invitrogen Life Technologies catalog number 10010)

- 1.06 mM potassium phosphate, monobasic (KH_2PO_4)
- 155.17 mM sodium chloride
- 2.96 mM sodium phosphate, dibasic 7-hydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)

Protein Purification Buffer #1⁴

- 0.1 M acetic acid, pH 3
- 0.25 M sodium chloride
- 0.01% Tween[®] 20
- 1 mg/mL bovine serum albumin (Fluka P/N 05468)

Protein Purification Buffer #2⁴

- 0.1 M Tris, pH 7.4
- 0.14 M sodium chloride
- 0.01 % Tween 20
- 1 mg/mL bovine serum albumin (Fluka P/N 05468)

Commercial Peptide – Human Angiotensin II
(Sigma-Aldrich P/N A9525)

- Asp – Arg – Val – Tyr – Ile – His – Pro – Phe, Acetate salt

CONDITIONS

Eluent: Potassium hydroxide (EG50 as the source)

Temperature: 30 °C

Eluent Flow Rate: 1.0 mL/min

Detection: Suppressed conductivity, ASRS
ULTRA II, recycle mode

ASRS Current

Setting: See method

Expected Background

Conductivity: <1 μS (22 mM KOH)

Typical System

Backpressure: 14 MPa (2000 psi) to
17.2 MPa (2500 psi)

Sample Volume: 5–100 μL

SEPARATION METHOD

Time (min)	EG50 Conc (mM)	SRS Current (mA)
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0.00	22.0	80
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Comments: Load sample loop, 80 mA SRS current setting, Acquisition ON

6.00	22.0	80
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6.01	28.0	80
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Comment: Step to 28 mM KOH

12.00	28.0	80
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12.01	50.0	80
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Comment: Step to 50 mM KOH for cleanup

14.00	50.0	124
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Comment: Step to 124 mA SRS current

15.00	50.0	124
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15.01	22.0	124
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Comment: Step back to 22 mM KOH

17.00	22.0	80
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Comment: Step back to 80 mA SRS current

20.00	22.0	80
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Comment: Acquisition OFF

PREPARATION OF SOLUTIONS AND REAGENTS

TFA Stock Standard Solution 1000 $\mu\text{g/mL}$

Dissolve 0.1203 g of sodium trifluoroacetate in deionized water and dilute to 100 mL in a volumetric flask. Dilute this stock standard solution to the desired concentrations.

SYSTEM PREPARATION AND SETUP

This section describes the procedures for the initial installation and start-up of the ASRS ULTRA II, EGC II KOH EluGen cartridge, and CR-ATC. Prepare the ASRS according to the *Quickstart Instructions for the ASRS ULTRA II* (Document No. 031951). Install the EGC II OH EluGen cartridge according to the instructions in the *Operator's Manual for the EG50 Eluent Generator System* (Document Number 031908). Install the CR-ATC between the EGC II KOH cartridge and the degas module in the EG50 according to the *Operator's Manual for the Continuously Regenerated Anion Trap Column* (Document Number 031910).

Connect the columns and suppressor in the IC system by using the black PEEK 0.010-in. (0.25-mm) tubing. Keep the lengths of connecting PEEK tubing as short as possible to minimize the system void volume and thus ensure efficient chromatographic performance. Carefully use a plastic tubing cutter to ensure the tubing cuts have straight, smooth surfaces. Irregularity on the surface of a tubing end can result in unwanted additional dead volume.

SYSTEM OPERATION

Turn on the gradient pump to begin the flow of eluent through the system. If the system backpressure is below 14 MPa (2000 psi), a length of yellow PEEK 0.003-in. (0.075-mm) tubing should be added between the outlet of the degas assembly in the EG50 and the inlet of the injection valve. A system backpressure of 15.9 MPa (2300 psi) is ideal. Confirm that the chromatographic pathway has no leaks. For more information, see the *Operator's Manual for the EG50 Eluent Generator System* (Document Number 031908).

Using the Chromeleon workstation, turn on the EG50 to deliver the highest eluent concentration required by the method. Allow the AS50 thermal compartment to stabilize at 30 °C. Determine the status of the system by measuring the short-term noise. Baseline noise should be less than 5 nS over a period of 5–10 min when measured in 1-min segments. It may take 12 h or more for the system to equilibrate to a stable background conductivity for trace analysis. When performing trace analysis, we recommend running the system overnight to equilibrate for use the following day.

RESULTS AND DISCUSSION

An anion-exchange column for monitoring residual TFA in high-ionic-strength pharmaceutical buffers should ideally have two characteristics. The column should have a sufficient ion-exchange capacity for the high ionic matrix and should separate TFA from the matrix anions that are present at high concentrations. The IonPac AS18 column has both of these characteristics. The 4-mm AS18 set column has an anion-exchange capacity of 285 µeq and is an excellent match for the target application. TFA is well resolved from the early-eluting anions under optimized conditions. Figure 1 shows the AS18 separation of an anion standard that includes TFA.

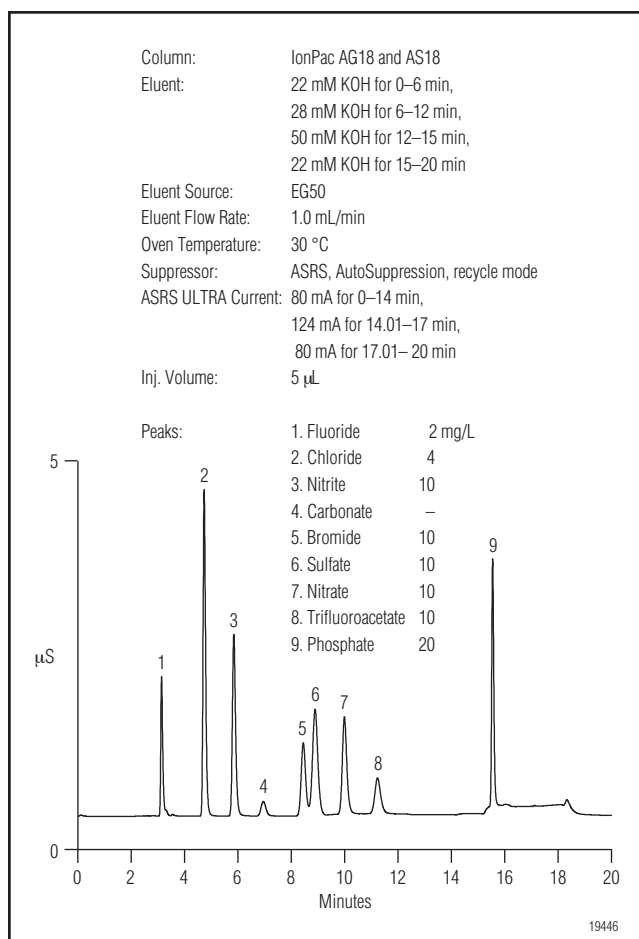


Figure 1. Analysis of anion standard with TFA.

The separation of TFA from the anions in the samples was achieved with a series of eluent concentration step changes. The method begins with an initial eluent concentration of 22 mM KOH to elute weakly retained ions such as fluoride, acetate, and formate. An eluent step change to 28 mM KOH at 6 min separates trifluoroacetate from the other matrix anions such as sulfate and nitrate. The SRS current setting at the time of separation of TFA is set at 80 mA. This current provides the optimum suppression with the least baseline noise. After TFA has eluted, the eluent is step changed to 50 mM KOH at 12 min to clean the column of any highly retained matrix anions such as phosphate. Afterward, the eluent is stepped back to 22 mM KOH to reequilibrate the column for the next injection.

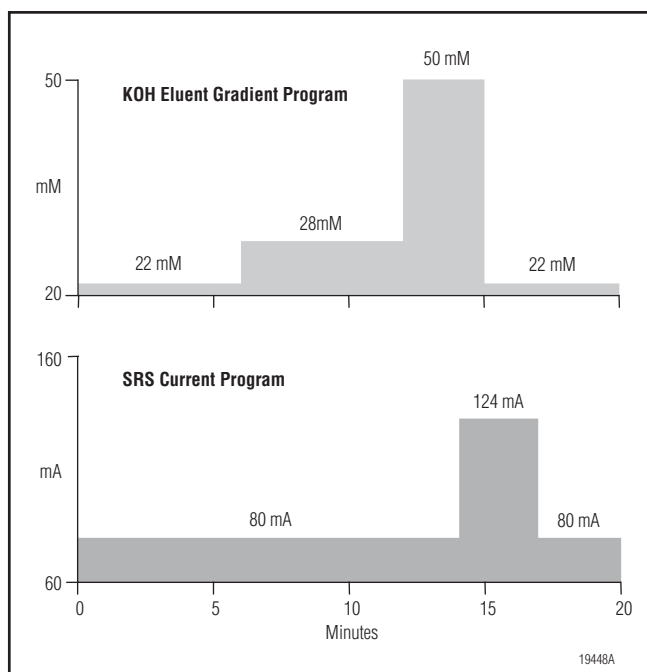


Figure 2. IC method for determination of TFA using the IonPac AS18 and RFIC.

This optimized eluent step change method was quickly developed using the RFIC system. By simply programming the EG50 Eluent Generator, a number of isocratic methods and a variety of step-gradient eluent methods were quickly evaluated. This evaluation did not require the preparation of different eluents to achieve the ideal proportioning for proper method evaluation. A higher ASRS current setting of 125 mA is applied at 14 min to account for the higher eluent concentration used. A delay of approximately 2 min occurs for the higher eluent concentration to reach the suppressor after the eluent concentration change command is given to the EG50. This delay is mainly due to the column void volume. In contrast, the change in current setting to the ASRS shows an immediate response. By applying the optimal current to the suppressor, it is possible to achieve low noise at every point of the separation. Separations are performed at 30 °C to provide the best retention time reproducibility. Figure 2 illustrates the eluent gradient program and ASRS current program for the method.

This method was applied to the determination of TFA in the following samples: a phosphate-buffered saline (PBS),¹ two buffers used in a recombinant protein recovery process,⁴ and a commercial peptide.^{2,3} Figure 3 shows the determination of 300 ng/mL of TFA in PBS

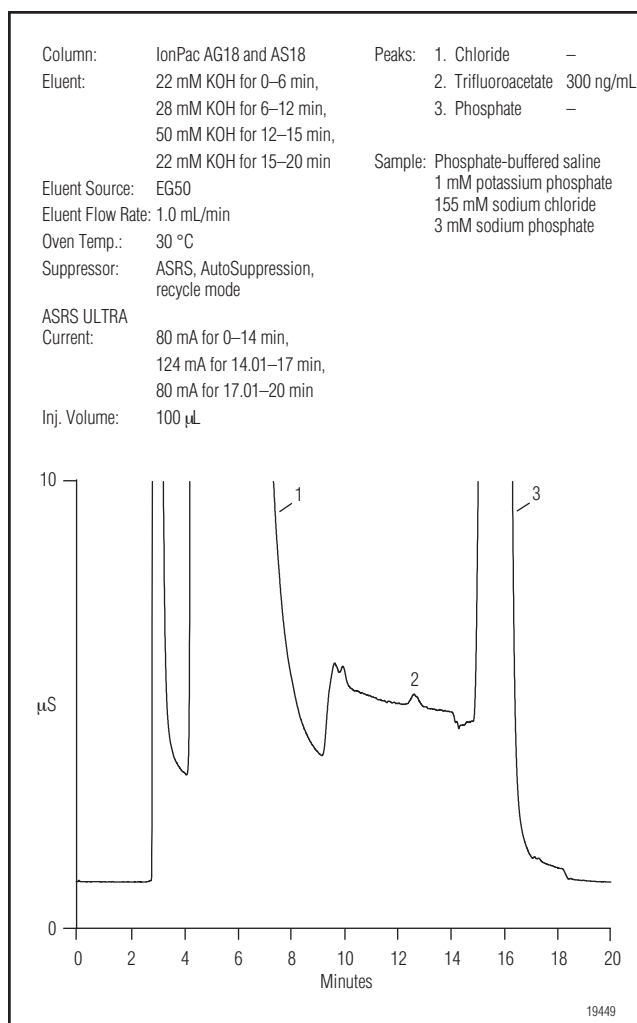


Figure 3. Determination of trace TFA in phosphate-buffered saline using the IonPac AS18 and RFIC.

using a 100-µL injection. This separation is challenging because of the excess presence of matrix components that elute before and after the detection of a trace amount of TFA. We recommend using the detection parameter in Chromeleon software called “Inhibit Integration” to ensure the accurate determination of TFA. Setting the “Inhibit Integration” command to “On” at 0.00 min stops the integration from the beginning of the run. It remains off until the command to turn the “Inhibit Integration” command “Off” is set at 11.0 min. This setting starts integration until “Inhibit Integration” is turned “On” again at 13.5 min. Integration will be inhibited from this point until the end of the run. Because TFA is the only analyte of interest, this detection parameter greatly simplifies detection and data processing. The integration window for stopping and starting detection should be modified according to the specific matrix of interest.

A calibration curve was obtained using TFA standards at 100, 300, and 1000 ng/mL prepared in PBS. Three replicate injections were performed at each concentration level. Results showed that TFA yielded a linear response with a coefficient of determination (r^2) of 0.9979. The method detection limit (MDL) was estimated to be 100 ng/mL TFA in PBS by measuring a TFA peak three times higher than the background noise ($S/N = 3$). An MDL was calculated using the standard deviation for seven replicate injections of 100 ng/mL TFA in the PBS.⁵ TFA was spiked at 100 ng/mL to be in the same concentration range as the estimated MDL and multiplied by the Student's t value for the 99.5% confidence limit. The standard deviation was multiplied by the Student's t value for the 99.5% confidence limit. A method detection limit for TFA was calculated to be 86 ng/mL in the PBS matrix under these conditions. Recovery of TFA for a 300-ng/mL spike in PBS was 98.7% for (6) replicate injections (293 ± 2.7 ng/mL). The retention time of TFA was 11.7 ± 0.011 min with an RSD of 0.09%. These results compare favorably with the work by Fernando and coworkers using the IonPac AS11-HC column.¹ They reported an MDL of 10 ng/mL for TFA in PBS after reduction of the matrix chloride concentration using an OnGuard® Ag pretreatment cartridge. We report an MDL of 86 ng/mL for TFA in PBS without a sample preparation step.

This method is also applicable to monitoring TFA in the buffers used in a recombinant protein recovery process. Buffers were prepared according to the specification of the manufacturer (see the "Samples" section) with the addition of 1 mg/mL bovine serum albumin to simulate the presence of protein. The method was optimized using a 50- μ L injection to give the best sensitivity for the determination of TFA in these two buffers. Recovery of TFA for a 300-ng/mL spike in this buffer was 98% for five replicate injections (296 ± 19 ng/mL) based on a calibration curve prepared in the matrix. The retention time of TFA was 12.7 ± 0.021 min with an RSD of 0.17%. Kabakoff and coworkers reported an MDL of 300 ng/mL for TFA in this sample with a 10- μ L injection using a 4×250 mm IonPac AS14 column (65 μ eq/column capacity) with a carbonate-based

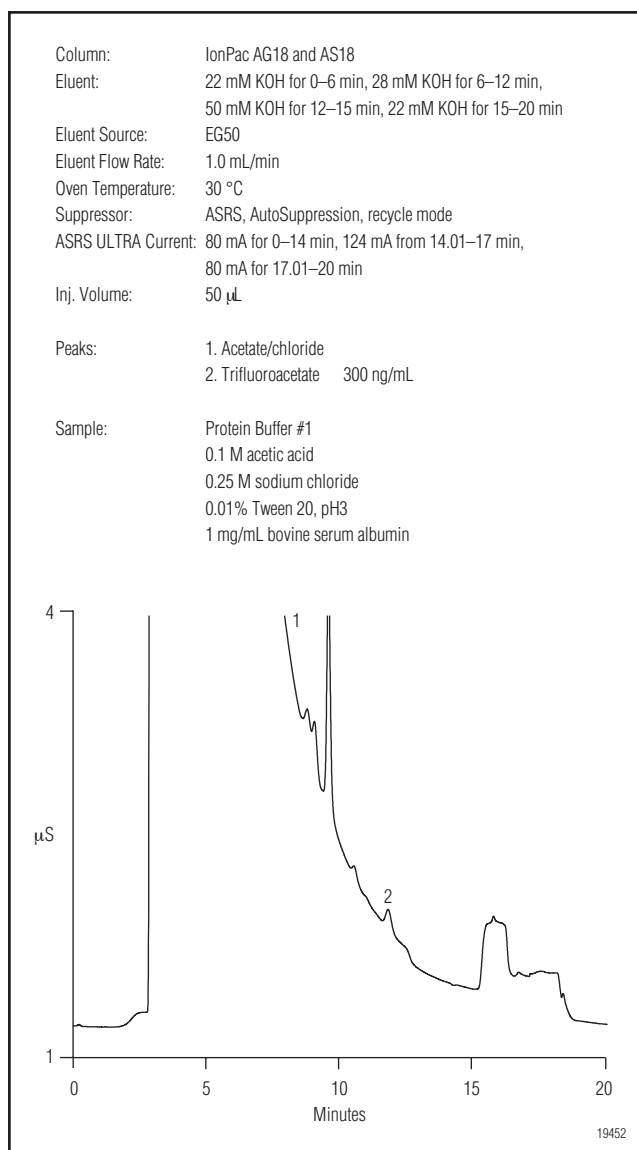


Figure 4. Determination of trace TFA in protein buffer #1.

eluent.⁴ By using a higher injection volume (50 μ L) with the higher-capacity IonPac AS18 column, we were able to achieve an MDL of 36 ng/mL for TFA. Figures 4 and 5 show chromatograms for a 300 ng/mL TFA spike in both of these buffers. Linearity for TFA in these two buffers yielded coefficients of determination (r^2) greater than 0.999. Recovery of TFA for a 50 ppb spike in Protein Buffer #2 was 115% for seven replicate injections.

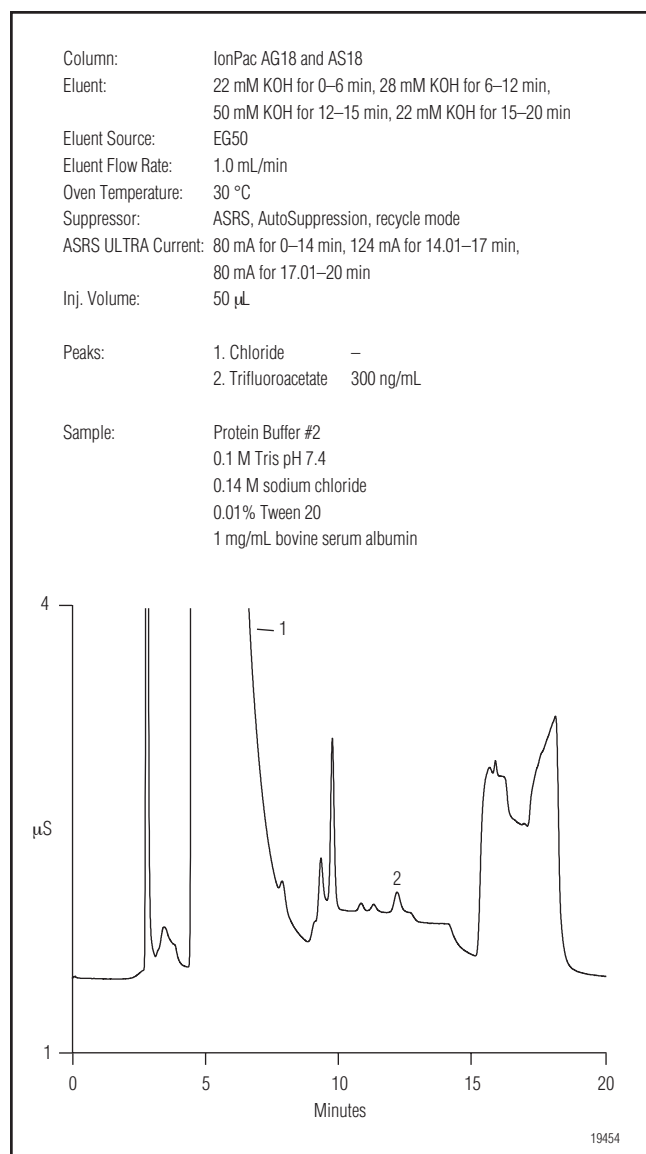


Figure 5. Determination of trace TFA in protein buffer #2.

This method was also applied to the determination of TFA in a commercial peptide. A solution of human angiotensin II protein was prepared at 40 µg/mL in deionized water with and without a spike of 100 ng/mL of TFA. Both solutions were analyzed using the method developed in this study. No TFA was detected in the

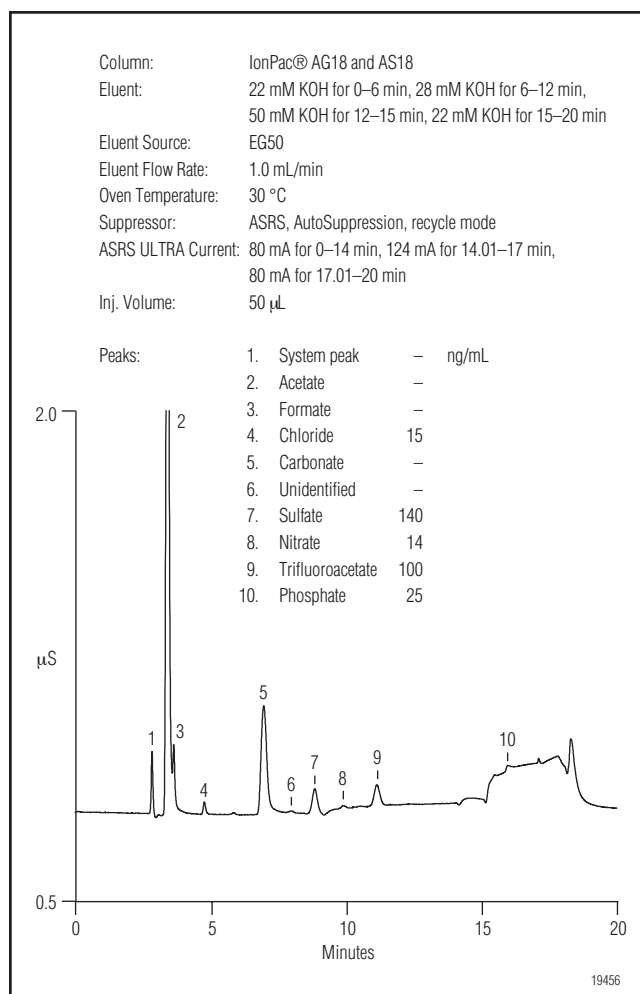


Figure 6. Determination of trace TFA in a commercial peptide: 40 µg/mL angiotensin II spiked with 100 ng/mL TFA.

peptide preparation. Figure 6 shows the peptide solution spiked with 100 ng/mL of TFA. Because this peptide was prepared as an acetate salt, a large acetate peak appears. Trace amounts of chloride and sulfate were also detected. A 50 ng/mL spike of TFA was completely recovered (101% for $n = 7$), demonstrating that the method is valid for determining TFA in this sample. Table 1 summarizes the calibration results and calculated method detection limits in the human angiotensin II protein solution, including the three different buffers.

Table 1. Calibration Results and Calculated MDLs in the Human Angiotensin II Protein Solution Including the Three Different Buffers

Matrix	Data points**	r ²	Dynamic Range (ng/mL)	Method Detection Limit (MDL)* (ng/mL)	Standard Used to Calculate MDL (ng/mL)
Phosphate Buffered Saline	9	0.9979	100–1000	86	100
Protein Buffer #1	9	0.9997	100–1000	36	100
Protein Buffer #2	9	0.9986	100–1000	15	30
Commercial Peptide	9	0.9997	30–300	4	10

* MDL = (S.D.) × (*t*_s) 99.5%, where (*t*_s) is for a 99.5% single-sided Student's *t* test distribution for n = 7

** Three concentrations injected in triplicate

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5. Glaser, J.; Foerst, G.; McKee, G.; Quave, S.; Budde. *W. Environ. Sci. Technol.* **1981**, *15*(12) 1426.

SUPPLIERS

Fisher Scientific, 2000 Park Lane, Pittsburgh, PA 15275-1126 USA, Tel: 800-766-7000, www.fishersci.com.

Fluka Chemika-BioChemika, Fluka Chemie AG, Industriestrasse 25, CH-9471, Buchs, Switzerland, Tel: +81 755 25 11, www.sigma-aldrich.com.

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Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

Dionex Corporation
Salt Lake City Technical Center
1515 West 2200 South, Suite A
Salt Lake City, UT
84119-1484
(801) 972-9292

Dionex U.S. Regional Offices
Sunnyvale, CA (408) 737-8522
Westmont, IL (630) 789-3660
Houston, TX (281) 847-5652
Atlanta, GA (770) 432-8100
Marlton, NJ (856) 596-0609

Dionex International Subsidiaries
Australia 61 (2) 9420 5233 *Austria* (01) 616 51 25 *Belgium* (03) 353 42 94 *Canada* (905) 844-9650 *China* (852) 2428 3282
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The Netherlands (0161) 43 43 03 *Switzerland* (062) 205 99 66 *United Kingdom* (01276) 691722
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