DIONEX

Application Note 115

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Determination of Trifluoroacetic Acid (TFA) in Peptides

INTRODUCTION

Trifluoroacetic acid (TFA) is commonly used in the manufacturing process to release synthesized peptides from solid-phase resins. TFA or acetate is also used during reversed-phase HPLC purification of peptides. TFA is manufactured using acetate and fluoride as precursors, and residual levels of these compounds may be present whenever TFA is used. Residual TFA, fluoride, and, to a much lesser extent, acetate are toxic and undesirable in peptides intended for preclinical and clinical studies. A method for the determination of TFA, acetate, and fluoride must be suitable for peptide formulations and be capable of verifying the removal of these anions during the production process.

TFA has been assayed by gas chromatography[1–5], GC mass spectroscopy[6], reversed-phase HPLC[7], isotachophoresis[8–10], infrared spectrometry[11], titration[12–13], spectrophotometry[14], and ion-exchange chromatography[15–18]. Ion chromatography (IC) is advantageous because it is sensitive, simple, and can be automated.

The separation mechanism of IC is based on an anion-exchange displacement process occurring between the sample ions and eluent ions with the anion-exchange functional groups bonded to the stationary phase. A typical stationary phase consists of a grafted, solvent-compatible, alkyl-based ion-exchange resin. The separation of TFA, acetate, and fluoride illustrated in this application note uses a stationary phase functionalized with alkyl quaternary ammonium groups. Effluent from the analytical column is passed through a suppressor that reduces the total background conductance of the eluent and increases the electrical conductance of the analyte ions. With suppressed conductivity, signal-to-noise ratios are improved approximately 50-fold compared to nonsuppressed conductivity.

This application note describes the analysis of commercially prepared, water-soluble peptides using ion chromatography. This method requires minimal sample preparation, and the analytes, fluoride, acetate, and TFA, are easily separated without significant peptide interference.

EQUIPMENT

Dionex DX 500 system consisting of:

- GP40 Gradient Pump
- CD20 Conductivity Detector or ED40 Electrochemical Detector
- LC30 Oven or LC20 Chromatography Module
- AS3500 Autosampler

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Reagents

Sodium carbonate, 0.5 M (Dionex P/N 37162)

Sodium bicarbonate, 0.5 M (Dionex P/N 37163)

Deionized water, 18 MΩ-cm resistance or higher

Standards

Sodium fluoride, ACS grade (Fisher Scientific, Cat. No. S-299)

Sodium acetate, trihydrate (Sigma Chemical Co., Cat. No. S-8625)

Trifluoroacetic acid, anhydrous, protein sequencing grade (Sigma Chemical Co., Cat. No. T-1647)

Five Anion Standard (fluoride, chloride, nitrate, phosphate, sulfate) (Dionex, P/N 37157)

CONDITIONS

Columns:	IonPac® AS14, Analytical (4 mm) IonPac AG14, Guard (4 mm)
Eluent:	3.5 mM Sodium carbonate/ 0.8 mM Sodium bicarbonate
Flow Rate:	1.2 mL/minute
Inj. Volume:	10 µL
Detection:	Suppressed conductivity, ASRS™ AutoSuppression™ recycle and external water modes
Expected Background Conductivity:	15 µS
Expected System Operating Backpressure:	12.4 MPa (1800 psi)

PREPARATION OF SOLUTIONS AND REAGENTS

3.5 mM Sodium carbonate / 0.8 mM Sodium bicarbonate

Combine 1980 mL of deionized water with 14.0 mL of 0.5 M sodium carbonate and 3.2 mL of 0.5 M sodium bicarbonate. Degas for 20 minutes. Connect the eluent reservoir to the instrument and pressurize with helium.

STOCK STANDARDS

Prepare a 4.5-mg/mL stock analyte standard of fluoride by combining 10.0 mg of sodium fluoride with 1.00 mL of water. Prepare a 7.2-mg/mL acetate standard by combining 10.0 mg of sodium acetate trihydrate with 1.00 mL of water. For a 9.9-mg/mL trifluoroacetate stock solution, mix 10.0 mg of trifluoroacetic acid with 1.00 mL of water. Combine and dilute standard solutions to desired concentrations using the mobile phase eluent as the diluent. Standard solutions should be frozen until needed.

SAMPLE PREPARATION

Commercial Peptides

Tyr-[Trp^{2}]-MSH Release Inhibiting Factor;
Tyr-Pro-Trp-Gly-NH_2
Trifluoroacetate salt, abbreviated here as MSH-RIF.

[Sar^{1}, Thr^{8}]-Angiotensin II;
Sar-Arg-Val-Tyr-Ile-His-His-Pro-Thr
Acetate salt, abbreviated here as AT-II.

Ala-D-Isoglutaminyl-Lys-D-Ala-D-Lys
Acetate salt, abbreviated here as IGA.

[Gln^{4}]-Neurotensin; pGlu-Leu-Tyr-Gln-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
No counterion specified, abbreviated here as NT.

FMRF Amide Related Peptide;
Asn-Arg-Asn-Phe-Leu-Arg-Phe amide
Trifluoroacetate salt, abbreviated here as FMRF.

Peptide Samples from In-Process Manufacturing

Eleven Amino Acid Crude Peptide; Ac-D-Ala-Gly-Arg-His-Tyr-Ala-Arg-Val-Ala-Leu-Arg-amide
No purification, abbreviated as "Crude Peptide".

Eleven Amino Acid >70% Pure Peptide; Ac-D-Ala-Gly-Arg-His-Tyr-Ala-Arg-Val-Ala-Leu-Arg-amide
Purified by gel permeation chromatography, abbreviated as "GPC Pure Peptide".

Commercial peptide samples are dissolved in eluent to peptide concentrations of 1 mg/mL. Peptide samples from in-process manufacturing are dissolved in eluent to dry weight concentrations of 1 mg/mL. Both the commercial and in-process peptide solutions are further diluted with eluent to concentrations of 40 and 400 µg/mL. Peptide solutions are also diluted with standard solutions to evaluate the spike recovery by the method of standard addition.

DISCUSSION AND RESULTS

Figure 1 shows the separation of fluoride, acetate, chloride, nitrate, phosphate, sulfate, and TFA using the 3.5 mM sodium carbonate/0.8 mM sodium bicarbonate eluent.

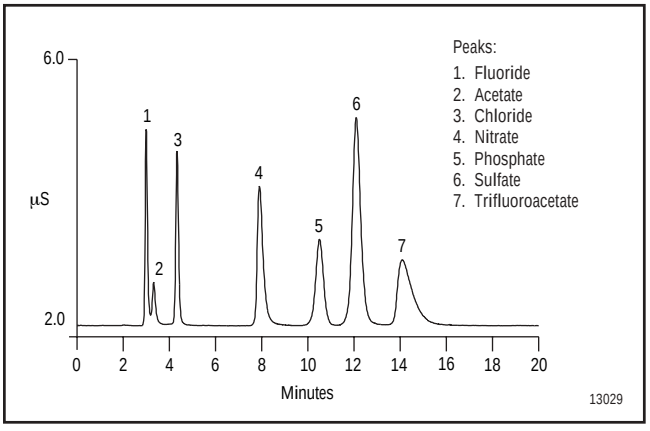

Figure 1 *Seven common anions found in peptide samples.*

Method Detection Limits

The method detection limits (MDL) for a 10-µL injection of fluoride, acetate, and trifluoroacetate are given in Table 1. The MDL is defined as the minimum concentration required to produce a signal-to-noise ratio of 3. Two modes of suppression were compared for their effect on MDL. The recycle mode, which used the postdetector eluent as feed through the regenerant chamber to achieve suppression, was compared to the external water mode, which used deionized water from a separate, pressurized bottle as regenerant feed. Suppression in the external water mode compared to the recycle mode produces lower background noise, thus a lower MDL. The MDL can be further decreased by increasing the injection volume above the 10 µL used in this Application Note.

Table 1 Detection limits

Sample	Recycle Mode (ng)	Recycle Mode (ng/mL)	External Water Mode (ng)	External Water Mode (ng/mL)
Fluoride	0.3	30	0.1	10
Acetate	2	200	1	100
Trifluoroacetate	6	600	3	300

Linearity

Fluoride standards of 0.023, 0.045, 0.23, 0.45, 2.3, 4.5, 23, 45, 230, 450 µg/mL were injected (n = 6 per concentration) for this study. The method was found to be linear for fluoride over the range tested (r^2 = 1.000). Acetate and TFA were also linear (r^2 = 0.999 in each case), using 0.36, 0.72, 3.6, 7.2, 36, 72, 360 µg/mL acetate standards and 0.50, 1.0, 5.0, 100, 50, 100, 500 µg/mL trifluoroacetate standards (n = 6). For all three analytes, linearity was demonstrated over at least three orders of magnitude.

Stability

Standards of fluoride (4.5 µg/mL), acetate (7.2 µg/mL), and TFA (10 µg/mL) were injected over 48 hours using an equilibrated system. Sample vials were at ambient temperature. Peak areas (Figure 2) and retention times (Figure 3) were reasonably stable throughout this period.

Precision

Precision is affected by concentration; RSD values increase as the concentrations approach the MDL. The peak area RSD values for fluoride (45 ng/injection), acetate (72 ng/injection), and TFA (99 ng/injection) were 0.5, 2.3, and 3.8% respectively for 12 injections.

Retention time precision (RSD) values were 0.4, 0.4, and 0.3% for fluoride, acetate, and TFA, respectively.

Recovery from Peptide Matrix

Table 2 shows the recovery of fluoride, acetate, and TFA from commercial peptides. Anions were spiked into the peptide solutions by the method of standard addition. Recovery for fluoride ranged from 93 to 100%, acetate from 79 to 106%, and TFA from 91 to 106%. Only

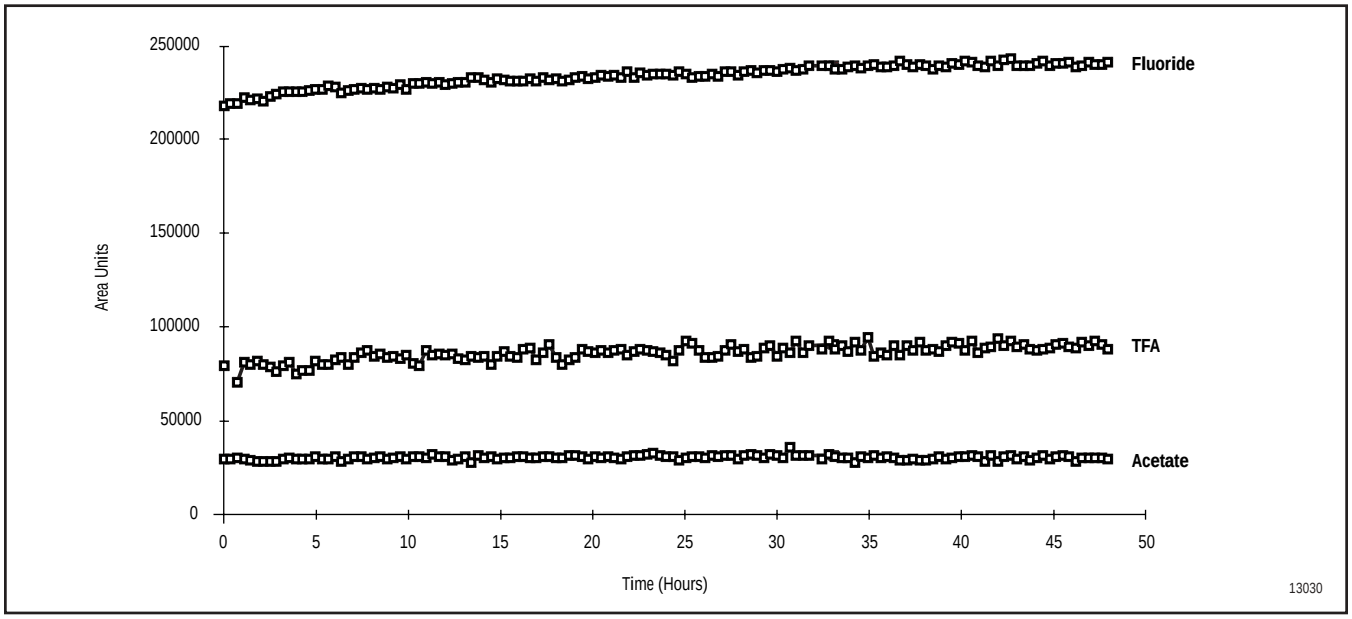

Figure 2 *Peak area stability over 48 hours.*

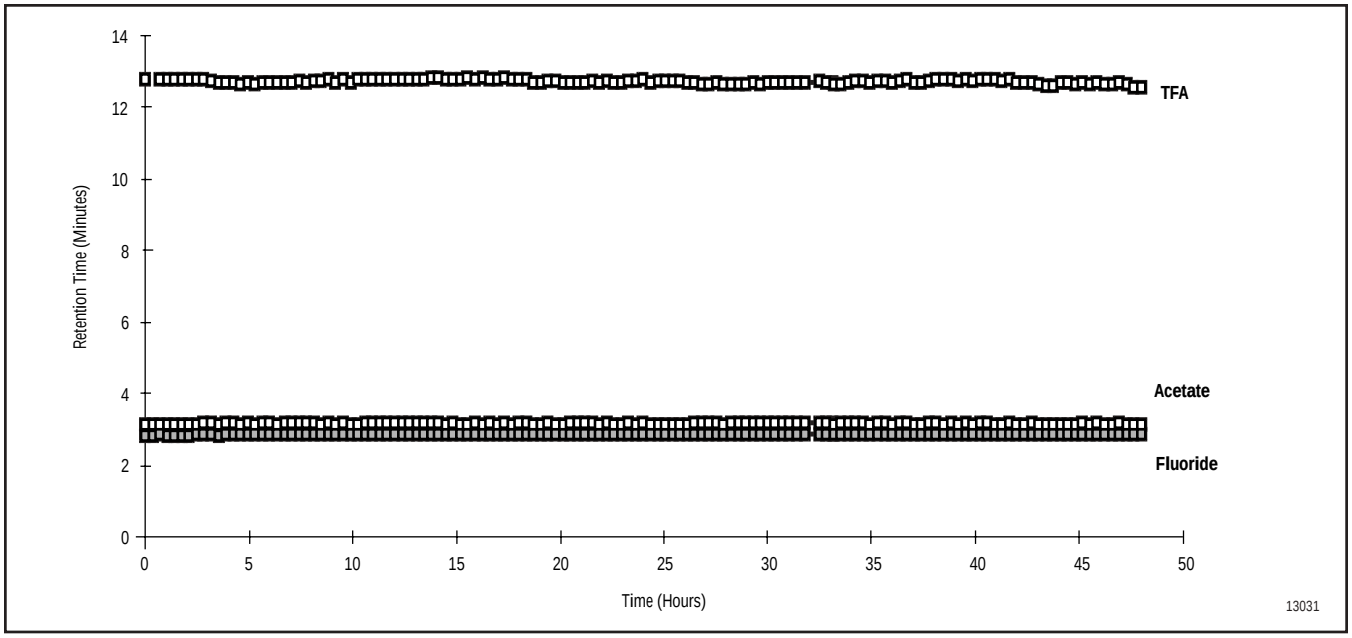

Figure 3 *Retention time stability over 48 hours.*

Table 2 Recovery of fluoride, acetate, and trifluoroacetate from peptides

Anion	Percent Recovery (Mean)					
	Spiked (μg/mL)	MSH-RIF	AT-II	IGA	NT	FMRF
Fluoride	1.8	93.3	93.7	96.7	95.4	93.6
	3.6	94.8	95.2	98.4	96.9	96.4
	5.4	96.4	97.4	99.4	98.0	99.9
Acetate	2.8	97.9	79.2	96.4	95.2	101
	5.7	102	99.6	99.9	98.2	101
	8.6	98.3	99.4	97.7	100	106
TFA	4.0	105	98.1	91.1	104	101
	8.0	105	102	94.4	103	101
	12.0	98.3	100	99.7	106	103

fluoride showed a slightly higher recovery with increasing levels of standard. The amount of peptide (FMRF) injected had no effect on recovery (see Table 3).

Table 3 Effect of peptide (FMRF) concentration on recovery of fluoride, acetate, and trifluoroacetate

Peptide Inj. (ng)	Percent Recovery (Mean)		
	Fluoride	Acetate	TFA
140	97.1	115	99.3
420	96.4	101	101
1010	97.7	102	120

Residual Anions in Commercial Peptides

Commercially available peptides contained counterions that were quantifiable by ion chromatography. Table 4 lists the fluoride, acetate, and trifluoroacetate measured in the commercial peptides. All peptides labeled by the manufacturer as trifluoroacetate salts (MSH-RIF and FMRF) contained TFA at approximately the same concentrations (193–202 mg of TFA per gram of peptide). All peptides labeled as acetate salts (AT-II and IGA) contained acetate at measurable levels. The peptide NT was labeled as containing salts, but did not indicate which salts. By this method, NT was determined to contain TFA at levels similar to those found in MSH-RIF and FMRF. Furthermore, IGA was labeled as containing acetate as the counterion, but additionally contained fluoride. All the peptides investigated in this Application Note also contained other anions, such as chloride, sulfate, phosphate, and nitrate, which can be effectively resolved by this method. Other anions were detected but not identified.

Table 4 Fluoride, acetate, and trifluoroacetate in commercial peptides			
	mg anion/g peptide		
Peptide	**Fluoride**	**Acetate**	**Trifluoroacetate**
MSH-RIF	0.26	0.90	202
AT-II	0.18	93	<0.3
IGA	24	80	<0.3
NT	0.02	0.13	184
FMRF	0.02	0.15	193

Residual Anions During In-Process Peptide Manufacturing

This IC method can be used to evaluate the effectiveness of purification during manufacturing. A crude synthetic peptide sample (prior to purification) was determined to contain 19.6% TFA by dry weight. Gel permeation chromatography (GPC) was used as a primary purification step after synthesis, producing a peptide sample containing 16.7% TFA. Figures 4A and 4B show chromatograms of these peptide solutions. These results suggest that TFA was not effectively removed by GPC. Appreciable levels of chloride and sulfate were measured in the crude peptide (0.02 and 1.0%, respectively). After GPC, much higher levels were observed (0.83% chloride and 2.6% sulfate). The GPC-purified peptide sample also contained fluoride and nitrate at 0.026% and 0.014% respectively. Knowing the amount and type of these counterions in peptide preparations is important to the ultimate safety and effectiveness of the product. Ion chromatography is therefore an effective in-process quality control method. This ion chromatography method can also assist with defining the mass balance of the peptide preparations.

CONCLUSION

This isocratic IC method using the IonPac AS14 column with suppressed conductivity detection can be used to evaluate peptides for residual TFA, fluoride, and acetate. The method also resolves other common anions such as chloride, sulfate, nitrate, and phosphate. TFA, fluoride, and acetate can be detected at the mg/L level. The recovery of fluoride, acetate, and TFA from peptide matrices is normally greater than 90%.

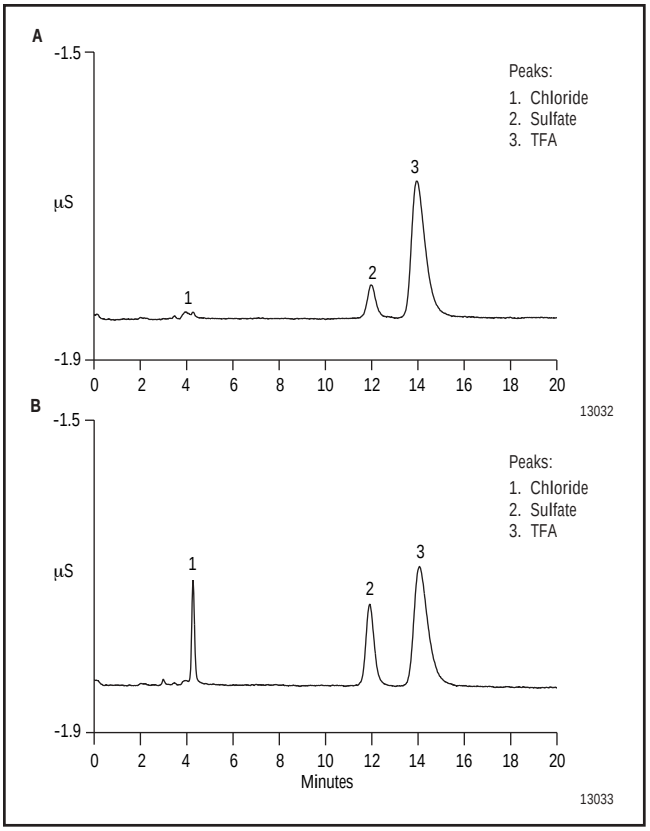

Figure 4 *A: Crude in-process peptide (10 μL of 40 μg/mL). B: GPC-purified in-process peptide (10 μL of 40 μg/mL).*

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