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Enhanced sensitivity for peptide mapping with electrospray liquid chromatography–mass spectrometry in the presence of signal suppression due to trifluoroacetic acid-containing mobile phases

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Abstract

A method is described for improving the sensitivity of peptide mapping with electrospray liquid chromatography–mass spectrometry using trifluoroacetic acid (TFA) containing HPLC mobile phases. The signal suppressing effects of TFA are shown to be due to the combined effect of ion-pairing and surface tension modifications. The post-column addition of a propionic acid–2-propanol (75:25, v/v) in a 1:2 proportion with the HPLC mobile phase counteracts the deleterious effects of TFA resulting in 10–100 × improvement of the signal-to-noise ratio. The system described introduces total HPLC flow (plus additive) directly into the electrospray source without splitting. Using 2.1 mm I.D. HPLC columns, minimum detectable quantities are below 40 pmol total protein.

As examples, separations of proteolytic enzyme digests of several proteins are shown using standard HPLC conditions, comparing results with and without the addition of propionic acid. The application of the technique is shown in more depth in the identification of oxidative modification sites in glutamine synthetase. In this application, the enhanced sensitivity allowed location of a modified residue by comparison endoproteinase Lys C digest of native and oxidized forms of the protein without extensive sample preparation or concentration. A third application demonstrates the identification of glycosylation sites in an endoproteinase Arg C digest of single-chain plasminogen activator through the use of in-source collisionally induced dissociation.

1. Introduction

In the last years, electrospray mass spectrometry (ES-MS) has become the method of choice for introduction of liquids into a mass spectrometer. Although there are applications in pharmaceutical, environmental and general chemical analysis areas, the rapid growth and acceptance of the technique in bioscience is largely due to the discovery by Fenn et al. [1] that the multiply-charged ions produced by ES-MS could be deconvoluted to determine the molecular mass of large proteins with masses far in excess of the m/z range of the mass spectrometric analyzer. Using ES-MS, proteins in excess of M_r 500 kDa have been analyzed. For proteins of moderate size ($M_r < 100$ kDa), typically the mass accuracy is <0.02%. Electrospray ion sources have been successfully coupled with most types of mass analyzers including quad-

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rupole, sector [2], ion-trap [3], time-of-flight [4] and Fourier transform mass spectrometry (FTMS) [5] instruments, resulting in a wide range of operational characteristics, cost and ease of use. Currently, the most common configuration consists of an electrospray ion source coupled with a quadrupole (or multiple quadrupole) analyzer.

Early application of liquid chromatography–electrospray mass spectrometry (LC–ES-MS) to "real world" applications suffered from a number of limitations including poor performance with high flow-rates and aqueous mobile phases, inability to accept non-volatile buffer systems and surfactants and signal suppression with strong-acid containing mobile phases such as trifluoro acetic acid (TFA). However, with the rapid development of the technique and the introduction of second and third generation commercial hardware, commonly used mobile phases and flow-rates are routinely accommodated. While non-volatile buffers and surfactants remain problematic, TFA-containing mobile phases can, with the technique described here, be used for a variety of applications.

The use of LC–ES-MS for the characterization of proteolytic enzymatic digests of proteins has greatly increased the ability to identify the resulting digest fragments. Molecular mass data from the mass spectrometer for each of the peaks eluting in a peptide map gives additional qualitative information to the data obtained from the standard UV–Vis detector signal, which can be used to associate a specific peak with a predicted digest fragment. To fulfil this role, it is important that the ES-MS detection system operates with a sensitivity, reproducibility, chromatographic resolution and ease of use comparable to that of the UV–Vis detector. Furthermore, operation of the system should preferably not require modification of established standard HPLC methods. In particular, one should be able to use standard HPLC column-packing materials, column dimensions, flow-rates, and mobile phases.

Trifluoroacetic acid (TFA) is commonly used as an HPLC mobile phase modifier in peptide and protein separations [6]. Its excellent ion pairing and solvating characteristics confer unique chromatographic selectivity on peptide separations, while its low UV cut-off (192 nm) allows detection of the peptide amide bond at 210 nm with minimal background or interferences from mobile phases, even when using a gradient elution mode. Due to its high volatility, TFA can easily be removed from preparative collected fractions by evaporation. Typical concentrations used are 0.1–0.2% (v/v).

Since the earliest application of electrospray LC–MS to peptide mapping, the difficulty of spraying highly aqueous solutions of TFA has been recognized, resulting in spray instability and analyte signal reduction [7–10]. General agreement exists that both spray instability and signal reduction are due to the high conductivity and surface tension of the eluent [8,11,12]. Both high conductivity and high surface tension require, when operating in pure (unassisted) electrospray mode, the onset voltage for generating a spray to be very close to corona discharge conditions. Consequently, either an instable (noisy) spray results from operating too close to discharge conditions or low signal results from operating too far from ideal electrospray formation. Several approaches have been used to overcoming these difficulties. Attempts have been made to overcome the signal suppressing effects by (i) using low flow-rates and capillary HPLC [13,14] which require lower fields for the onset potential, (ii) by using electrosharpened ES needle tips [11] to increase the field gradient at the point at which the electrospray is generated, (iii) through the use of pneumatically-assisted [12] or ultrasonically-assisted electrospray [15], (iv) through the use of surface tension lowering sheath liquids [13] or (v) the use of discharge suppressing sheath gas [16] and heating of the eluent slightly prior to spraying. Another approach has been to improve sensitivity in other ways in order to overcome the loss of sensitivity due to the signal-suppressing effects of TFA without actually addressing these effects. However, these approaches generally compromise the analytical performance in some aspect in exchange for improved sensitivity. For example, applications have been reported with TFA concentrations reduced to below 0.025% [17], re-

sulting in poor chromatographic performance. The signal-to-noise ratio can be improved by electronic filtering or slow acquisition rates but this may result in reduced chromatographic reliability. A common way to gain signal without decreasing chromatographic performance is to reduce the mass spectral resolution; however, this compromises the mass spectral information such as the ability to accurately differentiate charge states in multiply-charged digest fragments based on information contained in the resolution of isotopic peaks. While under unit resolution conditions it is difficult to identify charge states in this way above $[M+3]^{3+}$, many of the peptide fragments encountered in commonly used proteolytic digests appear as $[M+3]^{3+}$ ions.

In this work, it is demonstrated that the signal-suppressing effect of strong acid modifiers, such as TFA, is due not only to the problems introduced during spray generation caused by high conductivity and surface tension, but also to an ion-pairing phenomenon between TFA acid anions and the basic analyte molecules. A method, referred to as the "TFA Fix" which addresses both effects is presented. A number of peptide-mapping applications are demonstrated to characterize the performance of the method.

2. Experimental

2.1. HPLC

A schematic of the analytical system is shown in Fig. 1. Primary solvent delivery and separations were performed on a Hewlett-Packard (Palo Alto, CA, USA) HP1090 HPLC equipped with a DR-5 ternary solvent delivery system, autosampler with 250-μl sample capacity, heated column compartment, column switching valve and diode-array detector (DAD) with 1.9 μl high-pressure flow cell. For flow injection analysis (FIA) experiments, the HPLC was connected to the electrospray inlet with approximately 50 cm × 0.025 mm I.D. peek tubing (Upchurch Scientific, Oak Harbor, WA, USA). For peptide mapping, a 250 × 2.1 mm I.D. 5 μm 300A Vydac

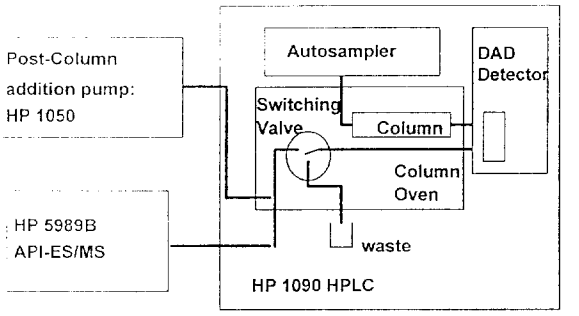

Fig. 1. Schematic of analytical setup for peptide mapping.

Column (The Separations Group, Hesperia, CA, USA) was used. Separations were performed with a linear solvent gradient from 0 to 60% B over a 60-min period at a flow-rate of 0.2 ml/min, unless stated otherwise. Solvent A was 0.2% TFA in water and solvent B was 0.2% TFA in acetonitrile. The column was thermostatted at 40°C.

The "TFA Fix" method consisted of post-column addition of propionic acid–2-propanol (75:25) at a flow-rate of 100 μl/min. As shown in Fig. 1, the TFA Fix was delivered using an HP 1050 HPLC pump and was teed into the column effluent after the DAD detector and after the column-switching valve. The tee was connected to the electrospray needle via approximately 50 cm × 6.35 mm I.D. peek tubing (Upchurch). It was not found necessary to include any additional mixing. The column effluent was diverted from the MS for the first 5 min of the chromatogram, during which excess reagents and unretained components eluted.

2.2. Mass spectrometry

Mass spectrometry was performed on a Hewlett-Packard 5989B quadrupole mass spectrometer equipped with extended mass range, high-energy dynode detector (HED) and a Hewlett-Packard 59987A atmospheric-pressure ionization (API) electrospray source with high-flow nebulizer option. Both the HPLC and MS were controlled by the HP Chemstation software allowing simultaneous instrument control, data acquisition and data analysis. The high-flow

Table 1
Electrospray lens settings

ES parameter	Setting
V_{cap}	−4000 V
V_{end}	−3500 V
V_{ext}	−5500 V
CapEx	100 V
ES_{Skim1}	26.7 V
ES_{Offs}	0.7 V
ES_{Skim2}	6.2 V
ES_{Lens2}	10 V
ES_{Lens3}	−40 V

nebulizer was operated in a standard mode with N_2 as both nebulizing (1.5 l/min) and drying (15 l/min at 300°C) gases. Typical lens settings for the electrospray source are shown in Table 1.

To characterize the effects of the TFA Fix, FIA of selected samples (listed in Table 2) was done at varying flow-rates and mobile phase conditions with data acquisition in the selected-ion monitoring (SIM) mode at a 100 ms dwell-time.

For peptide mapping, MS data was acquired in the scan mode, scanning from 200 u to 1600 u at an acquisition rate of 1.35 Hz and a stepsize of 0.1 Da. Unit resolution was maintained for all experiments. Data was filtered in the mass domain with a 0.03-u gaussian mass filter and in the time domain with a 0.05-min gaussian time filter.

2.3. Chemicals

HPLC grade water was purified in-house (Barnstaed, Dubuque, IA, USA). Acetonitrile and 2-propanol were HPLC grade (Mallincrodt, Paris, TX, USA). Formic acid, acetic acid, hydrochloric acid (HCl) and heptafluorobutyric acid (HFBA) (Sigma, St. Louis, MO, USA), trifluoroacetic acid, butyric acid, valeric acid (Aldrich, Milwaukee, WI, USA) and propionic acid (Mallincrodt) were >99% purity and were not further purified.

Horse heart myoglobin and chicken lysozyme were obtained from Sigma. Native and oxidized glutamine synthetase were obtained by courtesy of Dr. Rodney Levine at National Heart Lung and Blood Institute at the National Institutes of Health. Endoproteinase Arg C digests of single-chain plasminogen activation factor were obtained by courtesy of Erno Pungor, Jr. and Carrie Souders at Berlex Pharmaceuticals. FIA samples (see Table 2) and diphenylthiourea (DPTU) were obtained from Sigma.

Trypsin and endoproteinase Lys C digestion were performed as follows. The samples were

Table 2
Characteristics of FIA samples

Sample	Concentration[a]	M_r	SIM ion monitored
Hydrocortisone	500 pmol/μl	362.4	363 $[M + H]^+$
Sulfamethazine	100 pmol/μl	278.3	279.3 $[M + H]^+$
Lysine	200 pmol/μl	146.2	147.2 $[M + H]^+$
Gly–Tyr	200 pmol/μl	238.2	239.2 $[M + H]^+$
Gramacidin S	50 pmol/μl	1141.48	571.6 $[M + 2H]^{2+}$
Reserpine	50 pmol/μl	608.4	609.4 $[M + H]^+$
Erythromycin	100 pmol/μl	733.95	734.95 $[M + H]^+$
Peptide mixture			
Angiotensin II	50 pmol/μl	1046.21	524 $[M + 2]^{2+}$
ValTyrVal	130 pmol/μl	379.4	380.4 $[M + H]^+$
Met-Enkephalin	90 pmol/μl	555.6	556.6 $[M + H]^+$
Leu-Enkephalin	90 pmol/μl	573.6	574.6 $[M + H]^+$
Biotin	500 pmol/μl	244.31	245.31 $[M + H]^+$

[a] Relatively high sample concentrations were used to allow quantitation of performance under unfavorable conditions.

initially reduced and alkylated by reconstituting 10–30 nmols of lyophilized protein in 100 μl 6 *M* guanidine-HCl. A 100-μl volume of 0.8 mg/ml dithiothreitol in ammonium bicarbonate (pH 7.8) was added and incubated for 30 min at 37°C. A 100-μl volume of 1.1 mg/ml iodoacetic acid in ammonium bicarbonate (pH 7.8) was added and incubated for 30 min at 37°C. To the reduced and alkylated sample, 20 μg proteolytic enzyme [TPCK trypsin or endoproteinase Lys C (Promega Chemical, Maddison, WI, USA)] was added in buffer to a final volume of 650 μl and a final analyte concentration of 1 nmol/25 μl. For modified TPCK trypsin digest, a 50 m*M* ammonium bicarbonate buffer (pH 7.8) was used. For endoproteinase Lys C digest, a 25 m*M* Tris, 1 m*M* EDTA (pH 7.8) buffer was used. For both digests, the samples were incubated at 37°C for 18 h. The digested samples were not further desalted and were stored at <5°C.

3. Results and discussion

3.1. TFA Fix

The signal suppressing effects of strong acids such as trifluoroacetic acid have been well documented and are discussed in the introductory remarks. Fig. 2 shows a comparison of samples run in the FIA mode using 1% acetic acid or 0.2% TFA as a mobile phase. The spectra of the analytes shown in Fig. 2 do not show significant differences between the two acids; this is, however, to be expected since all the analytes, except for gramicidin S and the peptide mixture appear, as singly charged species. The two peptide samples (gramicidin S and the peptide mixture) did not show spectral shifts in the charge states. The chemical equilibria involved in the proposed mechanism are shown in Fig. 3. Equilibrium 1 in the electrospray positive mode generates excess positive charge in the sprayed droplets. If TFA is present in the solvent, the strong acid equilibrium 2 exists. The main ion production mechanism during normal electrospray is shown in equilibrium 3 in which analyte molecules are (multiply-) protonated and can be released into the gas phase by an ion evaporation process. If a weak acid additive, such as propionic acid is also present in solution, the weak acid equilibrium 4 also exists. Equilibrium 5 shows the process primarily responsible for signal suppression by strong acids such as TFA (CF_3COOH): ion-pair formation between analyte ions formed in equilibrium 3 and TFA

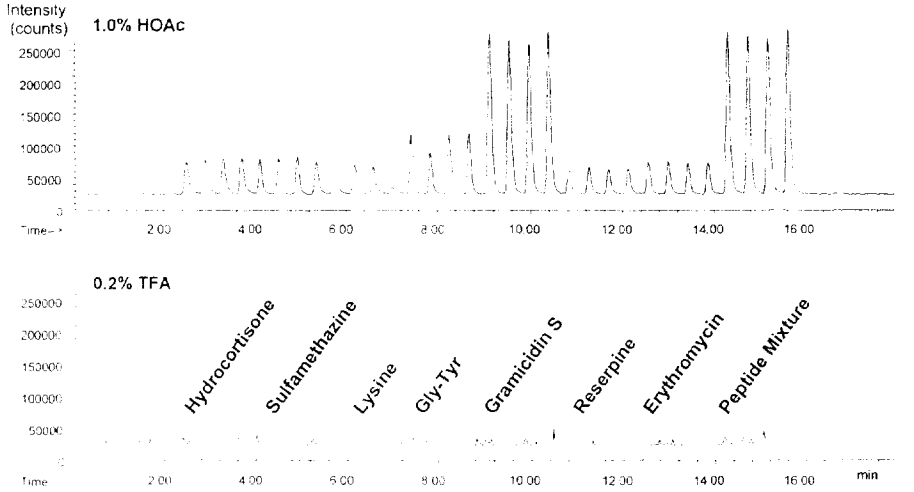

Fig. 2. Signal suppression by TFA containing mobile phases. FIA of probes with 1% acetic acid in 50% acetonitrile (top) or 0.2% TFA in 50% acetonitrile (bottom) at 100 μl/min. See Table 1 for analyte characteristics.

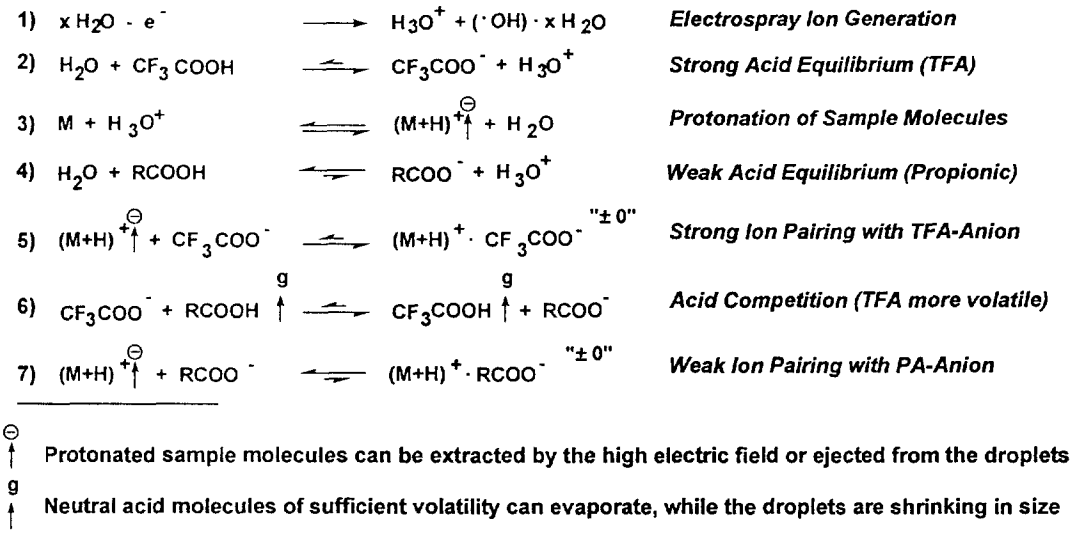

Fig. 3. Proposed mechanism for the TFA signal suppression. See text for discussion.

anions formed in equilibrium 2. Equilibrium 6 shows the basis of TFA Fix; in the presence of high concentrations of a weak acid such as propionic acid (RCOOH), the acid competition between TFA and propionic acid is driven by mass action towards deprotonation of propionic acid. The protonated TFA can be evaporated to some extent from the droplet. Finally, equilibrium 7 shows that the weak ion pairing between the analyte and the weak acid favors the protonated analyte which can be ion evaporated and subsequently focused and mass analyzed.

In optimizing the TFA Fix method, a range of weak organic acids at varying concentrations were evaluated. Fig. 4 shows the effect of adding a series of *n*-alkyl carboxylic acids: formic, acetic, propionic, butyric and valeric acid at concentrations ranging from 0 to 20%. The data shown in this figure represent a plot of the mean effect based on a factorial experiment for a number of FIA probes (see Experimental section) in 0.2% TFA, varying the additive acid and additive concentrations. In a factorial experiment, all combinations of a several variables are evaluated with respect to one or more responses. The effect due to the individual variables can be statistically extracted from this data. Interactions between the variables can also be assessed. For the factorial experiments described in Fig. 4, the variables were the analyte, organic acid and acid concentrations; the response was the signal intensity. The mean effect shows the statistically calculated effect averaged over different samples caused by a change in acid species and the change in acid concentration. With the exception of formic acid, all these additives gave enhanced performance. The effect increased in the order acetic, propionic and butyric acid and decreased with valeric acid. We speculate that this is due to an optimal volatility. If the acid is too volatile,

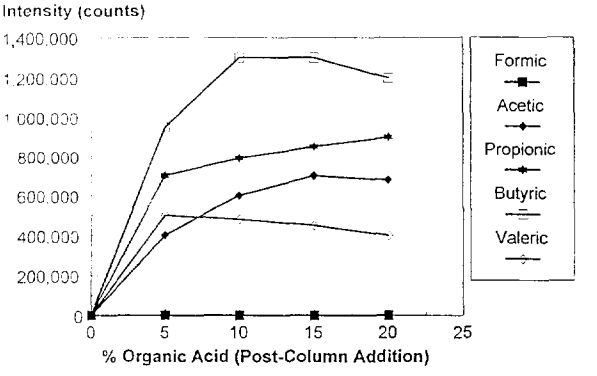

Fig. 4. Effect of different organic acid additives. Signal abundance based on mean main effect for a mixture of peptides (see Table 2). Post-column additive in 2-propanol added at 100 μl/min for mobile phase flow of 0.2% TFA in 50% acetonitrile at 200 μl/min.

e.g. acetic acid, the mass action driving the acid equilibrium (in Fig. 3) towards deprotonation of the weak acid will not be as effective and some signal suppression will still occur. On the other hand, if the acid is not volatile enough, as is the case with valeric acid, addition of large amounts of the acid to the droplets prevents rapid desolvation, interfering with the ion evaporation process. For routine work, propionic acid was chosen because the slight performance advantage of butyric acid did not balance its obnoxious odor.

In addition to the weak organic acid, an organic carrier solvent is also added in the TFA Fix method. The role of this solvent is primarily that of a carrier, but the surface tension reducing effects may also play a role in generating a stable enhanced signal. A number of different solvents were evaluated including 2-propanol, acetonitrile, methanol, ethanol, butanol–acetonitrile, 2-methoxyethanol, 2-methoxyethanol–2-propanol and 2-methoxyethanol–butanol. The results are shown in Fig. 5. The data in this plot represent a factorial experiment for a number of FIA probes in 0.2% TFA with varying additives composed of various organic solvents with a constant 15% acetic acid as the weak organic acid additive. In addition, the same samples were run without any additive with and without TFA present. As can be seen from the graph, 2-propanol yielded the best results at approximately 105% of the signal

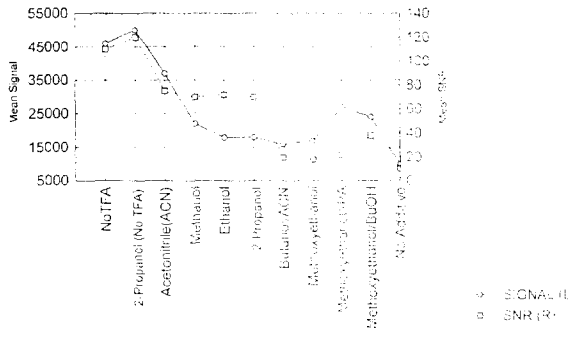

Fig. 5. Effect of organic carrier solvent. Signal abundances and SNRs based on mean main effect for a series of analytes (see Table 1). Post-column additive include 15% acetic acid in organic solvent added at 100 μl/min for mobile phase flow of 0.2% TFA in 50% acetonitrile at 200 μl/min.

obtained in the absence of TFA. This represents a 10-fold improvement relative to the same samples run in 0.2% TFA in the absence of any additive. With acetonitrile a signal of about 70% is found, with mixtures of 2-methoxyethanol and butanol of ca. 40%, while with 2-methoxyethanol alone a signal of only 20% is found. Experiments were also conducted to evaluate the mutual interaction between organic acid and organic carrier solvent. However, no significant interactions were found. For routine work, 2-propanol was used as the carrier solvent.

Using an additive of propionic acid–2-propanol, the proportions of the two components and the flow relative to the mobile-phase flow-rate were evaluated. In general, it was found that a maximal signal and signal-to-noise ratio were obtained through post-column addition of a solution of propionic acid–2-propanol (75:25) at a flow-rate approximately half that of the mobile phase.

As mentioned in the introduction, it had been thought previously that the signal suppression was primarily due to conductivity and surface tension effects. If this were the case, then the addition of 2-propanol alone would result in reduction of both the conductivity (by dilution) and the surface tension, and consequently would result in increased performance. However, as can be seen in Fig. 6a, this experiment results in only a 1.8-fold improvement compared to the 30-fold increase in the optimized TFA-Fix experiment (Fig. 6c). The FIA sample shown in Figs. 6a,c consisted of a mixture of ValTyrVal, biotin, reserpine and gramicidin s, each at the concentrations shown in Table 2. Evaluation of the individual extracted ion chromatograms showed improvements similar to those of the total-ion chromatogram of the mixture. The ion-pairing mechanism is further corroborated by the observation that analysis of a weakly basic, "almost neutral" compound such as diphenylthiourea actually shows a 2-fold increase in performance in a 0.2% TFA containing mobile phase relative to a 1% acetic acid containing mobile phase (Fig. 6b). In this case, the strong acid does not ion pair, but does enhance the protonation of the analyte.

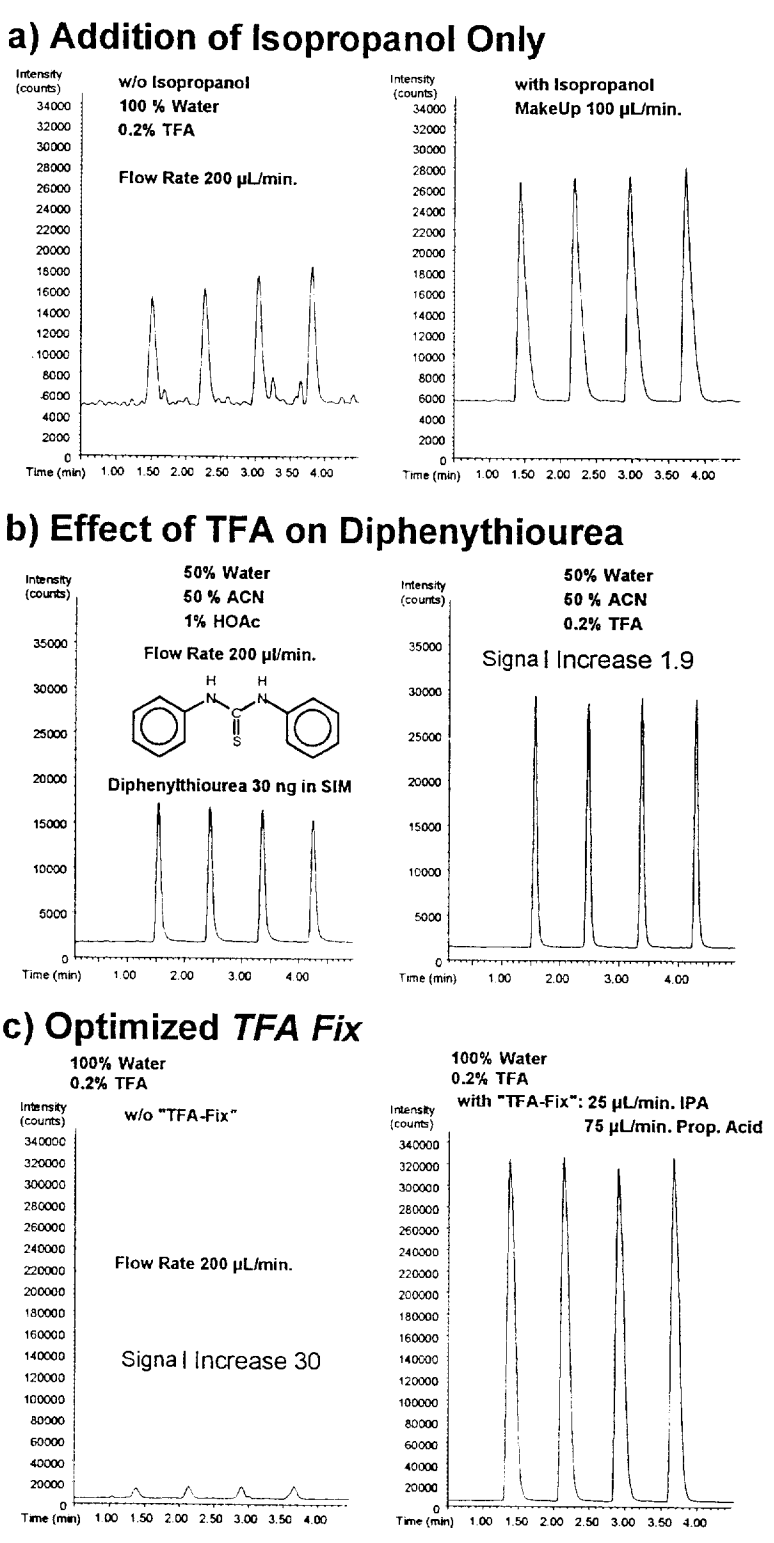

Fig. 6. Mechanism of TFA Fix. See text for discussion.

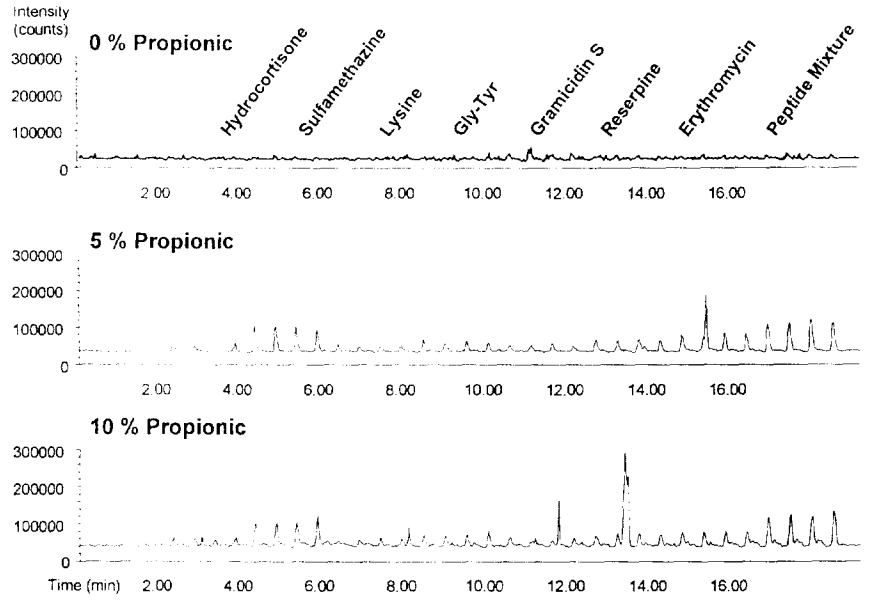

Fig. 7. Effect of TFA Fix on HCl. FIA of probes with 1% HCl in 50% acetonitrile at 100 μl/min. See Table 1 for analyte characteristics. Post-column additive in 2-propanol added at 100 μl/min for mobile phase flow of 200 μl/min.

Although most of the optimization studies and applications presented here were aimed at mobile phases containing trifluoroacetic acid, the TFA Fix also works for other strong acids that may be used as mobile phase additives. Figs. 7 and 8 show the effect of adding 0, 5 and 10%

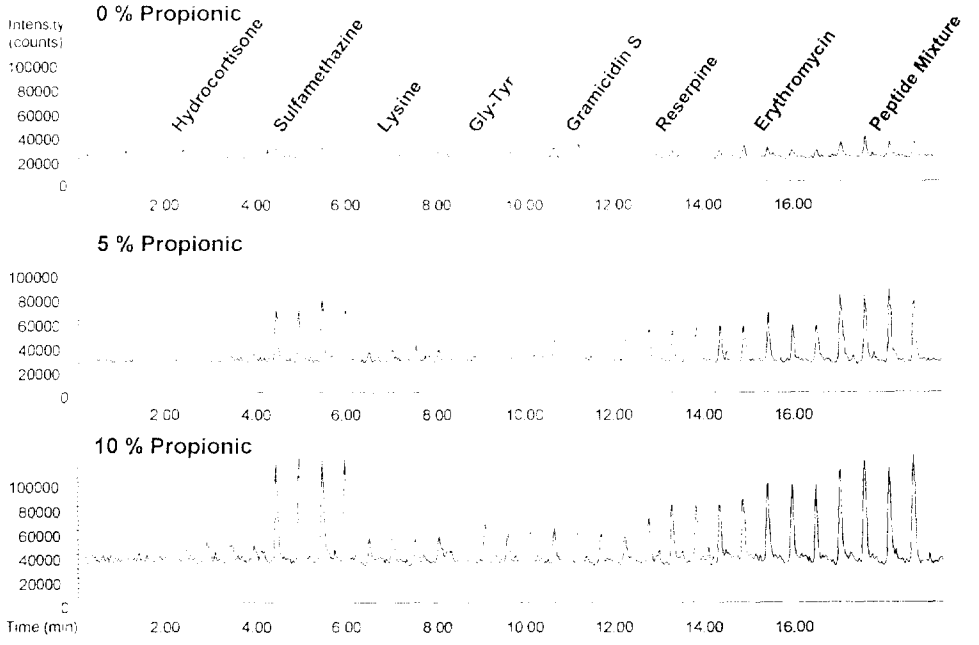

Fig. 8. Effect of TFA Fix on HFBA. FIA of probes with 1% HFBA in 50% acetonitrile at 100 μl/min. See Table 1 for analyte characteristics. Post-column additive in 2-propanol added at 100 μl/min for mobile phase flow of 200 μl/min.

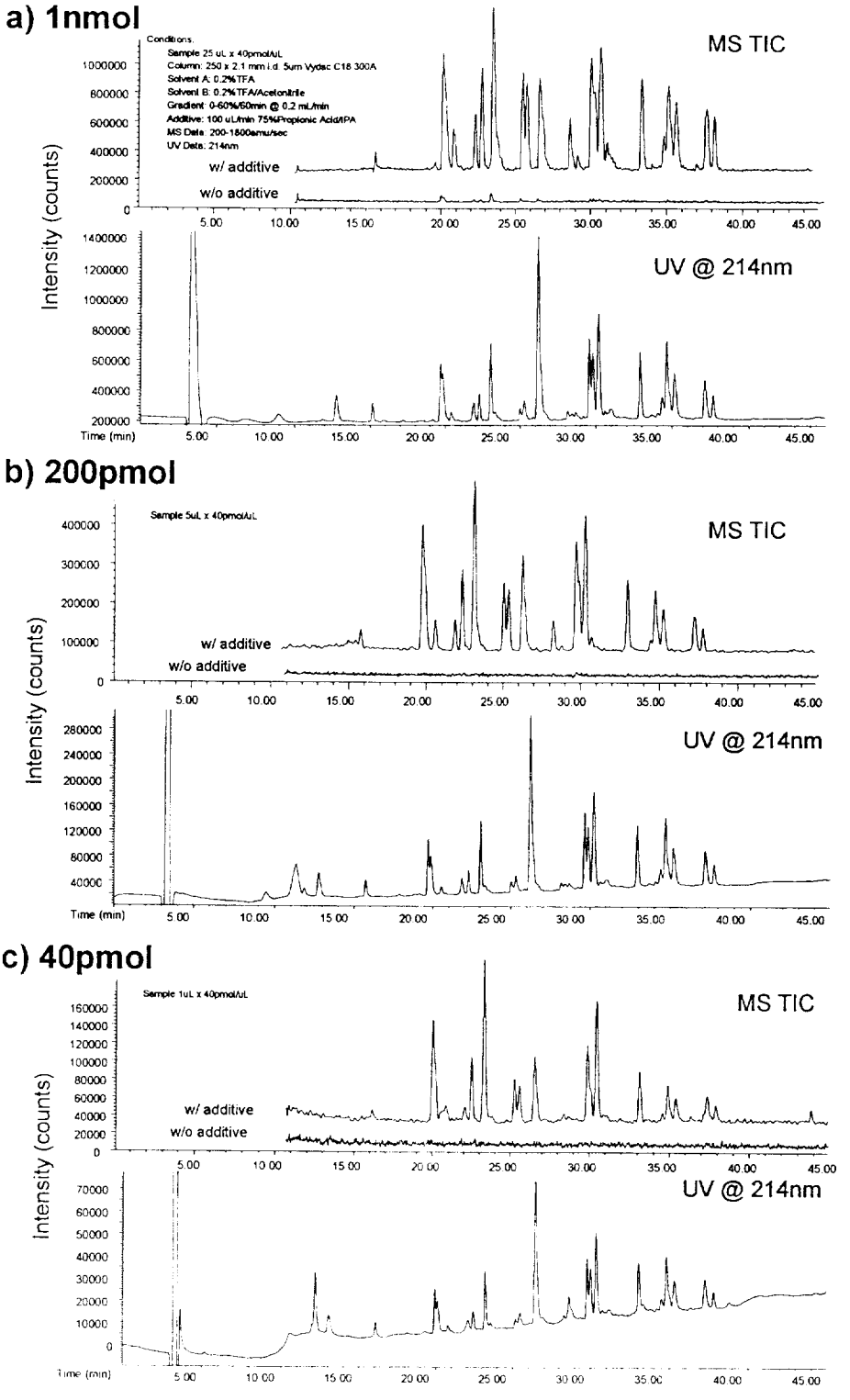

Fig. 9. Peptide maps of tryptic digest of lysozyme.

propionic acid (in 2-propanol) to mobile phases containing HCl and HFBA for a series of FIA probes. The results observed are similar to those obtained in the TFA experiments.

Experiments have shown that TFA Fix works well for peptides and small proteins. However, the effect decreases rapidly as the molecular mass increases: the magnitude of the TFA signal suppression as well as the degree of signal recovery by the TFA Fix is more dependent on the concentration of the sample and the TFA concentration than for smaller molecules such as peptides. For example, for a 1 pmol/μl solution of cytochrome c, infused at 50 μl/min, 0.2% TFA will result in a 250-fold decrease in signal intensity relative to the same sample in 1% acetic acid. Adding the TFA Fix results in an approximately 60-fold signal recovery. For larger proteins, it is not possible to break up enough ion pairs with TFA anions to efficiently recover the signal. We speculate that this effect is due to the greater number of protonation sites.

3.2. Applications: peptide mapping

As examples of the practical use of the TFA Fix, the following demonstrates a number of peptide mapping applications. For routine operations, the instrumental setup shown in Fig. 1 was used. In this system, the column effluent passes through the diode-array detector and the switching valve before the post-column additive is introduced. This allows UV–Vis signal and spectra to be acquired independent of the spectral characteristics of the additive. The switching valve allows the initial 5 min of the separation to be shunted to waste preventing excess reagent and salts from entering the electrospray chamber. Monitoring the UV signal ensures that no useful peaks are missed during this initial elution period.

Fig. 9 shows the analysis of a tryptic digest of lysozyme. The sample was separated under standard conditions on a 2.1 mm I.D. column at a flow-rate of 200 μl/min with a 60-min gradient from 0 to 60% acetonitrile with a constant 0.2% TFA. The amount of total protein was 1 nmol, 200 pmol and 40 pmol in Fig. 9a, b and c, respectively. At the 1 nmol level, essentially all components that can be seen in the UV (214 nm) signal are present in the MS total-ion chromatogram (TIC). Furthermore, in the absence of the TFA Fix, the signal is markedly reduced, although some peaks can be seen. For the 200 pmol and 40 pmol samples, there are essentially no peaks in the MS TIC trace for the sample run without the TFA Fix, while the sample run with the TFA Fix still shows most peaks seen in the UV signal. At the 40 pmol level, the UV signal shows some increase in baseline which is not present in the MS TIC trace. It should be noted that both solvent A and B in the gradient system contained 0.2% TFA. Carefully balancing the TFA content of the solvents could reduce the increase in baseline in the UV trace.

The sensitivity shown here is not the ultimate that could be obtained. In these experiments, care was taken to use a "standard" HPLC method. Specifically, a 2.1 mm I.D. column was used at conventional flow-rates. It has been shown [18], that electrospray operates as a concentration-, rather than as a mass-sensitive detector. This causes reduction of the HPLC column diameter to result in increased peak concentration for a given amount of sample. In principle, the TFA Fix could be used with miniaturized HPLC systems using 320 μm I.D. packed capillaries (or smaller), to take advantage of the concentration sensitivity inherent to all electrospray systems. However, such systems are generally less routinely used and require somewhat more expertise. It should also be noted that the mass spectrometric data acquisition was performed under strict standards. Acquisition rates for all experiments were at least 1 Hz in order to preserve chromatographic resolution. Slower acquisition rates might be used to reduce the noise levels through averaging, but this would result in compromising the chromatographic separation. Additionally, in these experiments, unit resolution was maintained across the mass range of the mass spectrometer. Using lower resolution may result in enhanced sensitivity at the price of spectral resolution. Using a unit resolution is useful in determining charge states of ions in the peptide mass spectra. In

typical digests, common charge states are +5 and less. Unit resolution allows explicit discrimination between singly- and doubly-charged ions. For doubly-charged ions, the isotopic peaks are not completely resolved, but two separate peaks can clearly be seen. For charge states higher than +3, isotopes are generally not resolved, but the unresolved peak width differentiates these ions from singly- and doubly-charged ions.

As a second application, Fig. 10 shows the identification of sites of oxidative modification of glutamine synthetase. Glutamine synthetase is known to undergo oxidative modification in vivo, resulting in reduced enzymatic activity [19]. The enzyme can be oxidized in solution using an iron–ascorbate system [20]. Glutamine synthetase is known to have three sites of oxidation. In this applications, endoproteinase Lys C digests of both native and oxidized glutamine synthetase were run under identical conditions. Examination of the MS TIC shows clear differences in the signal at two specific areas. The first, at approximately 47 min shows a molecular mass of 2770. From the previously determined cDNA-based sequence of glutamine synthetase a Lys C fragment would be predicted with molecular mass of 2784 (L13 + L14). One possible explanation of the 14 Da discrepancy is a DNA point mutation, or more probably, a single point error in the original sequence determination. The fragment at 47 min was collected and sequenced and found to have an Ala instead of a Gly at residue 267. In the oxidized digest, the L13 + L14 fragment disappears and a peak at a slightly later retention time appears with a molecular mass of 2786 Da. The 16 Da difference between the two suggests an oxidation. Collection and sequencing of this fragment in the digest of the oxidized form of glutamine synthetase, the expected His[269] disappeared and a new unidentified peak appeared between Asp and Ser. Subsequent electrospray mass spectral analysis of modified amino acid collected from Edman sequencing indicates a structure consistent with 2-oxo-histidine based

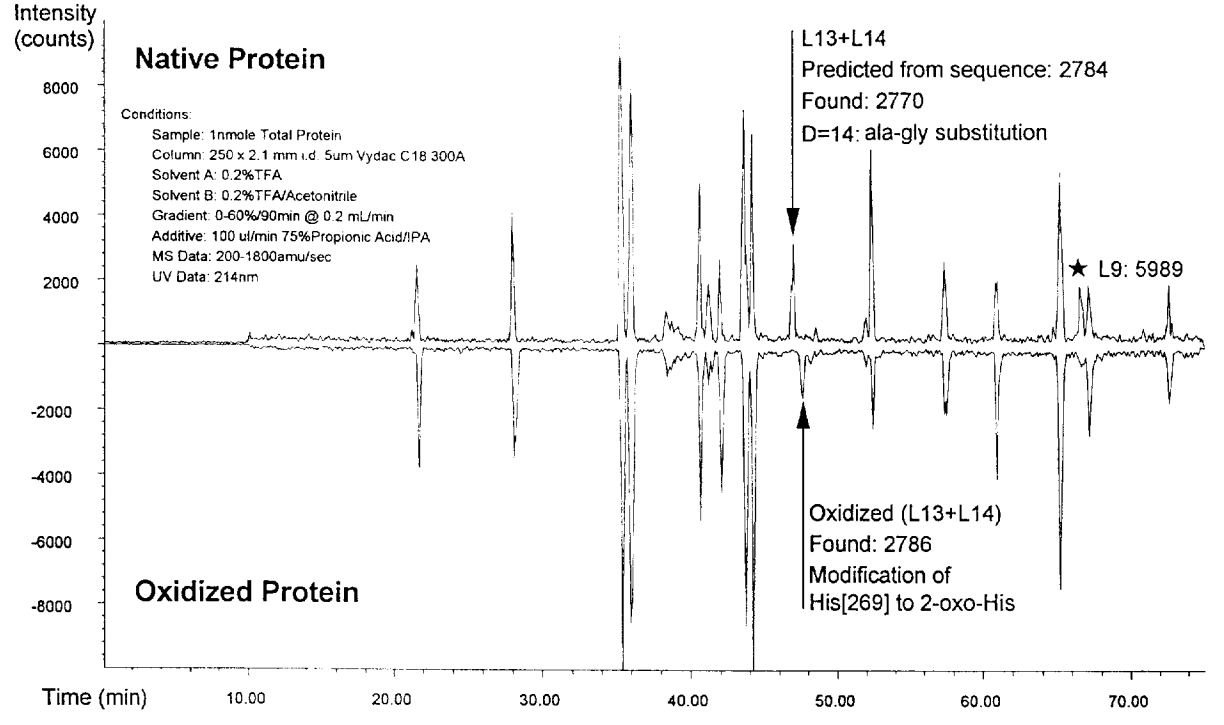

Fig. 10. Identification of oxidative modifications in endoproteinase Lys C digests of glutamine synthetase.

on molecular mass and possible fragmentation.

The second site, at a retention time of 68 min, shows a diminished signal in the oxidized digest, although no corresponding new peak appears. The molecular mass of the component in the native digest is 5989 Da which is consistent with the predicted Lys C digest fragment L9. This fragment, indeed, contains the HHH sequence at residue 177–230 which is also believed to undergo oxidative modification. Similar experiments with trypsin digest (not shown) yielded similar results.

As a final example, the electrospray LC–MS was used with the TFA Fix for the identification of sites of post-translational glycosylation in an endoproteinase Arg C digest of single-chain plasminogen activation factor. In this application, in-source collisionally induced dissociation (CID) was used to generate fragments which can be used as markers for glycosylation. The technique, based on the method described by Carr and co-workers [21,22], tracks glycoforms by looking for fragments characterizing fucose (m/z 147), HexNAc (m/z 204), sialic acid (m/z 292) and HexNAc + Hex (m/z 366). Fig. 11 shows the results of two separate acquisitions. The top TIC shows a full-scan acquisition with CID energy set relatively low (CapEx voltage, 100 V). Under these conditions, there is very little fragmentation and most digest fragments are detected as singly- or multiply-charged ions with molecular masses corresponding to the nonfragmented peptide. The four lower chromatograms were acquired in a SIM acquisition in which only the ions listed above corresponding to glycosylation markers were recorded. For this acquisition, the set CID potential was higher (CapEx voltage, 200 V) resulting significant structural fragmentation. Although these ion chromatograms could be extracted from full-scan acquisition data, using SIM enhanced the detection limits for the fragmentation. As can be seen from the chromatograms, the glycosylation patterns are relatively complex. Note the relatively broad peaks in the glyco-marker SIM traces compared to the scan TIC. This is due to the microheterogeneity of the glycoforms resulting in broad, un-resolved groups at individually lower levels

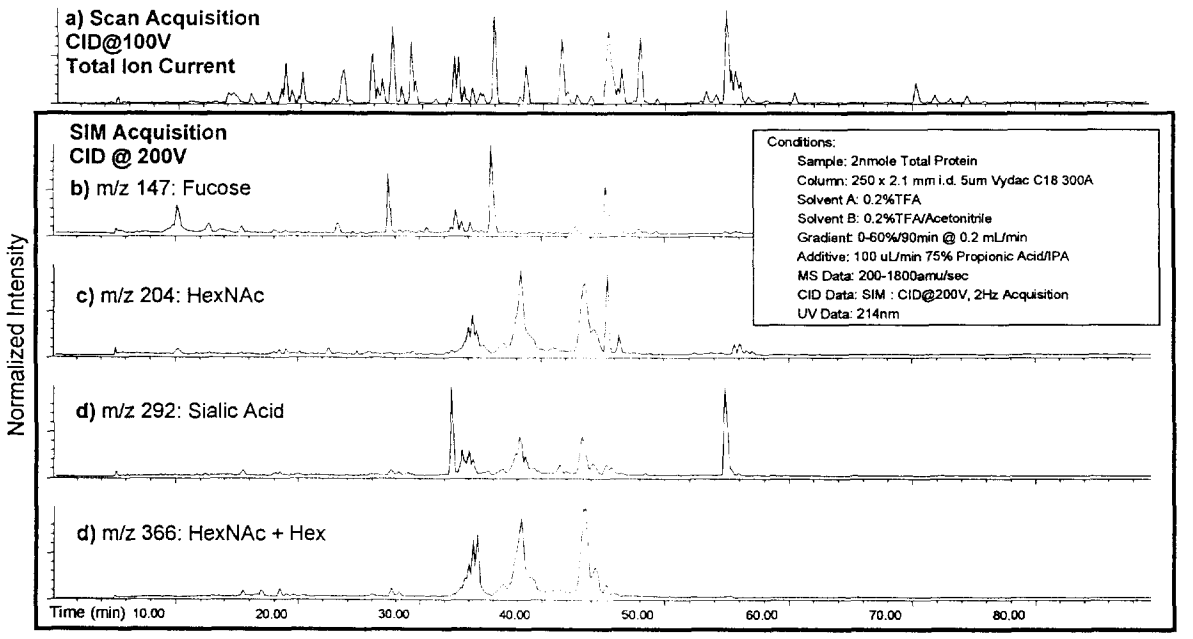

Fig. 11. Detection of glycosylation in endoproteinase Arg C digest of single-chain plasminogen activation factor. (a) Scan acquisition with low CID energy (CapEx, 100 V). (b)–(d) SIM acquisition with high CID energy (CapEx, 200 V).

which are not detected by the scan acquisition. This demonstrates a rapid and easy method to screen samples for glycosylation.

4. Conclusions

In conclusion, the method presented here is able to counteract the signal suppression effects of trifluoroacetic acid containing mobile phases in electrospray LC–MS. The method, termed TFA Fix consists of post-column addition of propionic acid–2-propanol (75:25) at a flow-rate half that of the mobile phase flow-rate. The TFA Fix results in 10–50 fold improvement in signal. The proposed mechanism for both TFA signal suppression and signal recovery by the TFA Fix is based on ion pair formation between analyte and the TFA anion. The TFA Fix acts by competitively interfering with this equilibrium. The method was shown to be useful for a number of different applications of peptide mapping.

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References

[1] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong and C.M. Whitehouse, Science, 246 (1989) 64–71.
[2] M.H. Allen, J.A.S. Lewis, Rapid Commun. Mass Spectrom., 3 (1989) 255–258.
[3] G.J. Van Berkel, G.L. Glish and S.A. McLuckey, Anal. Chem., 62 (1990) 1284–1295.
[4] O.A. Mirgorodskaya, A.A. Shevchenko, I.V. Chernushevich, A.F. Dodonov and A.I. Miroshnlkov, Anal. Chem., 66 (1994) 99–107.
[5] K.D. Henry, E.R. Williams, B.H. Wang, F.W. McLafferty, J. Shabanowitz and D.F. Hunt, Proc. Natl. Acad. Sci. USA, 86 (1989) 9075–9078.
[6] G. Winkler, P. Wolschann, P. Briza, F.X. Heinz and C.J. Kunz, J. Chromatogr., 347 (1985) 83–88.
[7] S.K. Chowdhury and B.T. Chait, Anal. Chem., 63 (1991) 1660–1664.
[8] J. Eshraghi and S.K. Chowdhury, Anal. Chem., 65 (1993) 3528–3533.
[9] R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barinaga and H.R. Udseth, Anal. Chem., 62 (1990) 882–899.
[10] U.A. Mirza and B.T. Chait, Anal. Chem., 66 (1994) 2898–2904.
[11] S.K. Chowdhury and B.T. Chait, Anal. Chem., 63 (1991) 1660–1664.
[12] A.P. Bruins, T.R. Covey and J.D. Henion, Anal. Chem., 59 (1987) 2642–2646.
[13] P.R. Griffin, J.A. Coffman, L.E. Hood and J.R. Yates, Int. J. Mass Spectrom. Ion Processes, 111 (1991) 131
[14] E.C. Huang and J.D. Henion, Anal. Chem., 63 (1991) 732–739.
[15] J.F. Banks, S. Shen, C.M. Whitehouse and J.B. Fenn, Anal. Chem., 66 (1994) 406–414.
[16] M.G. Ikonomou, A.T. Blades and P. Kebarle, J. Am. Soc. Mass Spectrom., 2 (1991) 497–505.
[17] G. Neubauer and R.J. Anderegg, Anal. Chem., 66 (1994) 1056–1061.
[18] A.P. Bruins, Mass Spectrom. Rev., 10 (1991) 53–77.
[19] K. Nakamura and E.R. Stadtman, Proc. Natl. Acad. Sci. USA, 81 (1984) 2011–2015.
[20] R. Levine, J. Biol. Chem., 258 (1983) 11828–11833.
[21] M.J. Huddleston, M.F. Bean and S.A. Carr, Anal. Chem., 65 (1993) 877–884.
[22] S.A. Carr, M.J. Huddleston and M.F. Bean, Prot. Sci., 2 (1993) 183–196.