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Research Article

Capillary electrophoresis-mass spectrometry methods for tryptic peptide mapping of therapeutic antibodies

Tryptic peptide mapping is routinely used in the biotech industry to confirm primary sequence, cell line stability, and to analyze posttranslational modifications. Peptide analysis is generally done by reverse phase liquid chromatography with UV or mass spectrometric detection. This method provides excellent resolution and sequence coverage. However, traditional methods are slow, and generally cannot detect small, hydrophilic peptides due to coelution with the column void volume. In this work, complementary CE-MS peptide analysis methods have been developed. The analyses are performed on a traditional CE-MS instrument with a sheath interface, and also on a novel sheathless interface that promises improved resolution and limit of detection. The methods were performed on a tryptic digest of a therapeutic monoclonal antibody for which LC-MS detects 97% sequence coverage. The 3% not covered consists of 11 peptides containing three amino acids or fewer, including two in the critical complementarity binding domain. Without further processing, the same tryptic digest was analyzed by CE-MS. Separation and detection of the 11 small peptides was achieved on CE-MS systems with both interfaces. The sheathless system produced better peak capacity and gave mass spectra with significantly less noise, while the sheath system proved to have better repeatability.

Keywords:
Capillary electrophoresis / Mass spectrometry / Therapeutic antibodies / Tryptic peptide mapping
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1 Introduction

Peptide mapping is a crucial tool for the analysis of therapeutic monoclonal antibodies. This method is routinely utilized to characterize primary proteinstructure for stability and lot release tests during product manufacture [1], and to identify posttranslational modifications [2, 3].

Typically, peptidemapping is accomplished by first digesting the protein with trypsin or another enzyme, and then separating the resultant peptides with liquid chromatography and identifying them by mass spectrometry. This method works quite well for the analysis of antibodies, generally producing baseline resolution of peptides with high (>95%) sequence coverage. However, there are two primary disadvantages of using HPLC for the peptide separation. First, separations using standard HPLC equipment can be quite time consuming, with some analyses taking up to 3 h to perform. A faster option is to use MALDI-TOF for peptide analysis without separation; however, this has the cost of possible ion suppression, difficulty in quantification, and matrix interference that can hide small peptides. LC-MS also does a poor job in detecting small, hydrophilic peptides. In reverse phase chromatography, these short peptides are often not retained by the column, and elute in the void volume with buffer salts that suppress ionization, rendering them unfit for mass spectrometric investigation [4–6]. The loss of these short peptides is of little consequence in identifying an unknown protein, but is crucial when complete sequence coverage is needed for verification of the amino acid sequence of the therapeutic antibody, and/or identification of posttranslational modifications. The importance of these peptides is emphasized further when they occur in the complementarity determining region of a therapeutic antibody, as modifications in this region can affect the ability of the antibody to bind its target. Several strategies can be employed to detect small peptides and improve sequence coverage. High sequence coverage can be obtained without needing to analyze small peptides by using limited proteases to generate only very large peptides [2, 7], or by analyzing additional samples that have been digested with different proteases [2, 4]. Analyzing very large peptides can fail to reveal low level modifications, and performing multiple digests is time consuming, especially if the need for extra sample preparation is not known before beginning

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the initial analysis. Small peptides can be analyzed by careful desalting to eliminate the void volume [8, 9] or by derivatizing peptides to make them more hydrophobic [6, 10, 11], both of which significantly increase sample preparationxbrk time.

Capillary electrophoresis provides fast, high-resolution separations of biological samples, and thus has frequently been applied to the analysis of peptides [12–14]. A disadvantage for peptide mapping by CE has been difficulty in interfacing with MS, a process that can limit buffer selection and reduce resolution and reproducibility. These issues have been addressed through the development of more robust interfaces and buffer systems, and the last decade has seen a wide application of CE-MS to peptide mapping [15]. Multiple CE modes, capillary coatings, and interfaces have been used. The most common mode is CZE due to problems caused by MS incompatible buffers in other modes. For peptide analysis, CZE is run in acidic buffer so that all peptides elute in positive mode, and coated capillaries are used to improve resolution and prevent absorption of large peptides. The sheath liquid interface is the current standard on commercial CE-MS instruments. It facilitates a consistent ESI spray by diluting the CE eluent in an MS compatible buffer flowed around the capillary at a constant rate [16, 17]. CE-MS employing sheath liquid interfaces has been successfully used for the analysis of a range of biomolecules, including peptides and their posttranslational modifications, intact proteins, and oligonucleotides [18]. We previously reported a method for the identification of small peptides by isolating and analyzing the void volume using CE-MS using a sheath liquid interface [19]; the method was successful, but at the cost of requiring an additional analysis for the larger peptides.

A unique sheathless CE-MS interface was developed by Moini and Whitt [20, 21]. This sheathless interface relies on etching a short section of the terminal part of the capillary until it becomes porous to small ions, which allows electrical grounding to occur. The capillary tip is then placed at the entrance to the mass spectrometer, the ESI voltage is applied, and the tip becomes the ESI sprayer. This interface design has several advantages over the more common sheath design. It does not introduce a dead volume, which can degrade separation, and does not dilute the sample with large amounts of buffer, which can decrease sensitivity [21]. A possible concern with this interface type is consistency of etching from capillary to capillary. Beckman Coulter (Brea, CA, USA) recently developed a line of sheathless sprayers that offer a commercially manufactured source for this interface option, and the usefulness of this sprayer for CE-MS analysis of peptides has been demonstrated [22].

In the present work, a CE-MS method was developed to determine the presence of tryptic peptides that are missed by traditional LC-MS analysis. A therapeutic monoclonal antibody was digested, then analyzed by LC-MS and CE-MS. The antibody studied is produced in Chinese hamster ovary (CHO) cells and consists of two heavy chains (449 amino acid residues each) and two light chains (214 amino acid residues each). Three CE-MS methods were utilized: a traditional sheath interface with uncoated and electroosmotic flow reducing PVA-coated capillaries, and a Beckman-Coulter sheathless interface using an uncoated capillary. The CE-MS methods had several advantages for the analysis of small peptides: a sample prepared for LC analysis requires no additional sample processing or cleaning, the methods can be preformed on small volumes, and they are fast and reproducible.

2 Materials and methods

2.1 Chemicals

All chemicals were used as received. Sample preparation reagents were of reagent grade, and CE-MS buffer reagents were of LC-MS grade. Therapeutic recombinant monoclonal antibody was produced by Genentech (South San Francisco, CA, USA). Trypsin was of mass spectrometry grade (Promega, Madison, WI, USA).

2.2 Sample preparation

The therapeutic protein was diluted to 10 mg/mL with formulation buffer. A total of 100 μL of this protein was added to 900 μL of denaturing and sulfitolysis reagent (6 M guanidine hydrochloride, 360 mM TRIS, 2 mM EDTA, 130 mM sodium sulfite, 26 mM sodium tetrathionate, pH 8.6) and heated at 37°C for 20 min. The protein was then buffer exchanged into 2 mL of 10 mM Tris and 0.1 mM calcium chloride at pH 8.3. Twenty microliters of 10% octyl β-glucoside and 20 μL of trypsin were added to 1 mL of the buffer-exchanged protein. Digestion was carried out at 37°C for 5 h. The reaction was quenched by the addition of 25 μL of 10% TFA.

2.3 LC-MS

The peptides were analyzed by RP-HPLC using a Zorbax 300SB C8 column (4.6 mm × 150 mm, Agilent Technologies, Santa Clara, CA, USA) on an Agilent Technologies 1100 HPLC. A total of 100 μL of peptide digest were separated after a 5-min hold at initial conditions with a linear gradient from 0% to 17% solvent B in 57 min, to 32% solvent B at 149 min, to 45% solvent B at 162 min, and to 95% solvent B at 173 min. Solvent A consisted of 0.1% TFA in water and solvent B consisted of 0.08% TFA in ACN. The column was maintained at 35°C and eluted at a flow rate of 0.5 mL/min. The elution profile was monitored at 214 nm and 280 nm. Masses of the separated peptides were determined using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

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2.4 CE-MS

2.4.1 Sheath-liquid interface

Traditional sheath interface analyses were performed on an Agilent 7100 CE. Conditions were identical using either a 55-cm PVA-coated capillary with a 50 μm inner diameter (Agilent Technologies), or a 55-cm uncoated capillary with 50 μm inner diameter (Beckman Coulter, Brea, CA, USA). The polyimide coating was removed from the terminal ~1 cm of the capillaries by shaving (neutral capillary) or burning (uncoated). Running buffer was 10% acetic acid in water, pH 2.3 [22]. Acetic acid at this pH gives a positive charge to all peptides, as calculated by GPMAW (Lighthouse data, Odense, Denmark), guaranteeing all peptides will migrate in a neutral capillary, and providing the charge necessary for size/charge-based separation of CZE. Capillaries were rinsed with buffer for 4 min between runs. Samples were injected by applying 50 mbar of pressure for 5 s, resulting in an 8.5 nL injection volume. Separations were driven by the application of 30 kV. CE-MS coupling was accomplished with a sheath flow interface (Agilent Technologies) using a sheath buffer of 0.1% acetic acid flowing at 3 μL/min from an Agilent 1200 LC pump adapted with a 99:1 splitter. A nitrogen sheath gas was applied at 6 psi. Mass spectrometry was performed with an Agilent 6100 TOF running in an enhanced resolution low mass range (100–1700 *m/z*) state. Sample drying was with 5 L/min of N2 heated to 200°C. CE and pump control were via ChemStation software (Agilent Technologies), MS control was through MassHunter Data Acquisition (Agilent Technologies).

2.4.2 Sheathless interface

The 50 cm long, 30 μm inner diameter, sheathless uncoated capillary sprayer cartridges were obtained from Beckman-Coulter and used in a PA800 plus CE instrument. Running buffer was 10% acetic acid; capillaries were rinsed with buffer for 4 min between runs. Samples were injected by applying 50 mbar of pressure for 5 s, resulting in a 1.1 nL injection volume. Separations were driven by the application of 30 kV. Mass spectrometry was performed with a Bruker MicrOTOF (Bruker Daltonics, Billerica, MA, USA), with CE triggering through the Hystar V3.2 software (Bruker Daltonik, Bremen, Germany). An ESI potential of 800 V was applied, and the capillary tip was positioned to produce an ESI current of ~30nA.

2.5 Data analysis

Mass spectrometry data were analyzed using MassHunter Qualitative Analysis B.03.01 (Agilent Technologies) for the Agilent mass spectrometer with sheath system. For the sheathless system with the Bruker mass spectrometer, DataAnalysis 3.4 (Bruker Daltonics) was used. Base peak chromatograms (BPC) were background subtracted. Prediction of peptide masses was performed with GPMAW 8.00 (Lighthouse data, Odense, Denmark). Chromatograms were extracted for a width of 10 ppm around expected peptide *m/z* for the MH+ through M4H+ species within the instruments 100–1700 *m/z* range. The charge state that produced the most intense peak was chosen to calculate mass accuracy and reproducibility data.

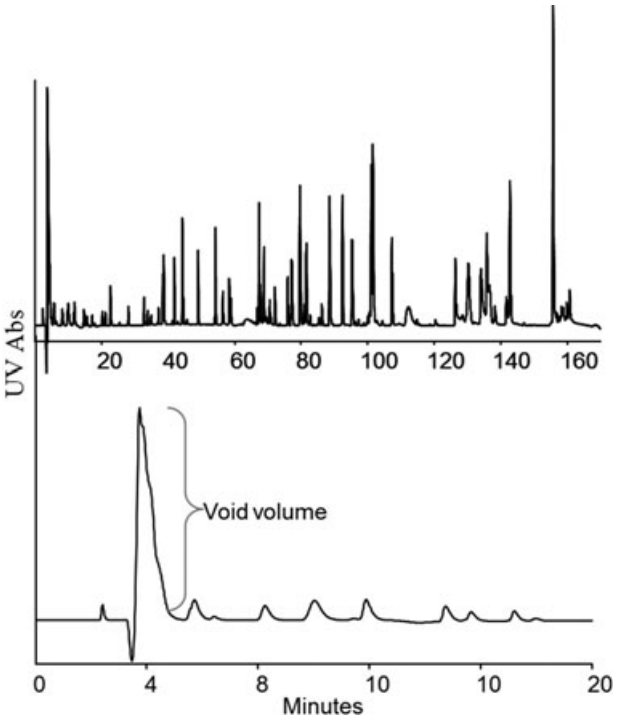


Figure 1. LC chromatogram of tryptic peptide digest. Bottom is enlarged to emphasize void volume that is not analyzed.

3. Results and discussion

3.1 LC analysis

Figure 1 shows the LC peptide map of the monoclonal antibody. Table 1 summarizes the theoretical peptides obtained by trypsin cleavage. Predicted peptides are listed in order of increasing mass, and named with a three character code: the first letter (T) indicates the enzyme used in the digest (trypsin), the middle number (1–41) signifies the peptide number beginning with the amino terminus, and the final letter (H or L) indicates whether the peptide is located on the heavy (H) or light (L) chain. Forty-nine of 61 (80%) of the predicted peptides were observed by LC, with all of the missed peptides being four or fewer amino acids in length. These short peptides elute in the solvent front, which was not analyzed because it contains digest buffer components that interfere with the MS analysis. A number of the short peptides could be detected as part of incompletely digested fragments; for example, peptide T20L (GEC) was not found, but the uncleaved peptide T19L-T20L (SFNRGEC) was. Including

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Table 1. Table of peptides expected from tryptic digest of a monoclonal antibody, ordered by increasing mass

Peptide	Sequence	Detected by	Peptide	Sequence	Detected by
T16H	K	All CE–MS	T29H	ALPAPIEK	All
T9L	R	All CE–MS	T1920L	SFNRGEC	All
T31H	AK	All CE–MS	T5L	LLIY**SASYR**	All
T6H	GR	All CE–MS	T35H	NQVSLTCLVK	All
T8H	SK	All CE–MS	T12H	GPSVFPLAPSSK	All
T39H	SR	All CE–MS	T33H	EPQVYTLPPSR	All
T17L	HK	All CE–MS	T10H	AEDTAVYYCAR	All
T5H	**FK**	All CE–MS	T13H	STSGGTAALGCLVK	All
T4L	APK	All	T9H	NTLYLQMNSLR	All
T27H	CK	All CE–MS	T15L	DSTYSLSSTLTLSK	All
T12L	EAK	All CE–MS	T22H	FNWYDGVEVHNAK	All
T15H	VDK	All	T25H	VVSVLTVLHDQWLNGK	All
T20L	GEC	All CE–MS	T11L	SGTASVVCLLNNFYPR	All
T26H	EYK	All	T37H	TTPPVLDSDGSFFLYSK	All
T28H	VSNK	All	T1L	DIQMTQSPSSLSASVGDR	All
T30H	TISK	All	T1H	EVQLVESGGGLVQPGGSLR	All
T32H	GQPR	All	T18L	VYACEVTHQGLSSPVTK	All
T17H	VEPK	All	T10L	TVYACEVTHQGLSSPVTK	All
T8L	VEIK	All	T3L	**ASQDVSIGVA**WYQQKPGK	All
T3H	QAPGK	All	T14L	VDNAJQSGNSQESVTEQDSK	All
T23H	TKPR	All	T21H	TPEVTCVVVDVSHEDPEVK	All
T19L	SFNR	All	T4H	GLEWVA**DVNPNSGGSIYNQR**	All
T18H	SCDK	All CE–MS	TL1+VHS	VHSDIQMTQSPSSLSASVGDR	All
T13L	VQWK	All	T36H	GFYPSDIAVEWESNGQPENNYK	All
T38H	LTVDK	All	T2H	LSCAAS**GFTFTDYTMD**WVR	All
17H	KVEPK	All	T24H(G0)	EEQYNSTYR	All
T16L	ADYEK	All	T11H	**NLGPSFYFDY**WGQGTLVTVSSASTK	All
T34H	EEMTK	All	T40H	WQQGNVFSCSVMHEALHNHYTQK	All
T41H	SLSLSPG	All	T19H	THTCPPCPAPELLGGPSVFLFPPKPK	All
T2L	VTITC**K**	All	T7L	FSGSGSGTDFTLTISSLQPEDFATYYC **QQYYIYPYT**FCQGTK	LC, sheathless CE-MS
T6L	**YT**GNSR	All			
T20H	DTLMISR	All	T14H	DYPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVSSSLGTQTYICNVNHKPSNTK	All
T7H	FTLSVDR	All			

"Detected by" lists the instruments on which each peptide was successfully observed.

the two peptides found in incompletely digested fragments, the total peptides identified in the antibody tryptic peptide map comprised 208 of the 214 light chain amino acids and 433 of the 449 heavy chain amino acids. The remaining 11 undetected peptides contain 22 amino acids, including two peptides that contain three amino acids located in the complementarity determining region.

As shown in Fig. 1, peak resolution is excellent. This method is able to separate over 50 peptides in 165 min. The inset shows the void volume that is not analyzed but contains the small peptides. Sequence coverage was 97% in this 3 h run.

3.2 CE-MS analyses

3.2.1 Comparison to LC-MS

CE-MS was explored as an alternative to the traditional LC-MS peptide map. CE analyses do not have the same issue with high salts eluting in the void volume, and small peptides are separated based on size-to-charge ratio, rather than hydrophobicity. The same tryptic digest sample used for LC-MS was examined by CE-MS. Using a buffer below pH 3, all peptides are expected to contain a positive charge and migrate toward the detector when operated in normal polarity. Figure 2 shows base peak electropherograms and extracted peptide electropherograms for both coated and uncoated capillaries with the sheath system, and an uncoated capillary with the sheathless system. Both systems utilized a background electrolyte of 10% acetic acid (pH 2.3), with MS data collected in the positive ion mode. In all three figures, the extracted ion electropherograms demonstrate that each peptide produces a Gaussian peak. Figure 2 also show a significant advantage of CE-MS for peptide mapping: reduced time of analysis. CE-MS runs were completed in 17-35 min, depending on mode; which is a significant time savings over the 3 h LC-MS run. Finally, Fig. 2 illustrates a disadvantage of this CE-MS method compared to LC-MS: the peptides are not

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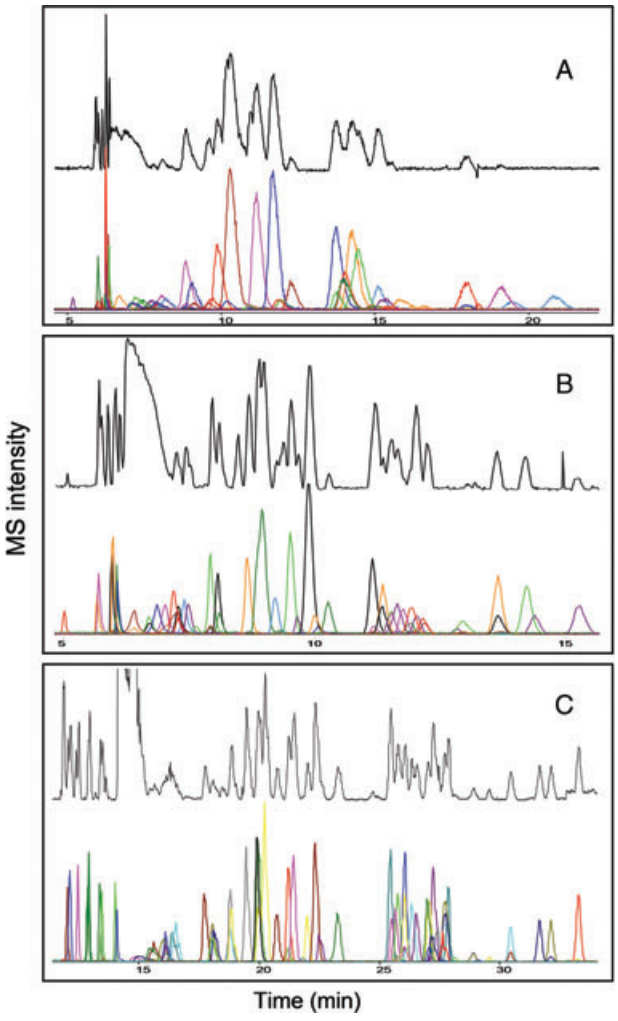


Figure 2. Comparison of CE-MS base peak electropherogram (top) and overlaid expected peptide extracted ion electropherograms (bottom) for different CE-MS interfaces and capillaries. (A) Sheath interface with PVA coated capillary. (B) Sheath interface with uncoated capillary. (C) Sheathless system with uncoated capillary.

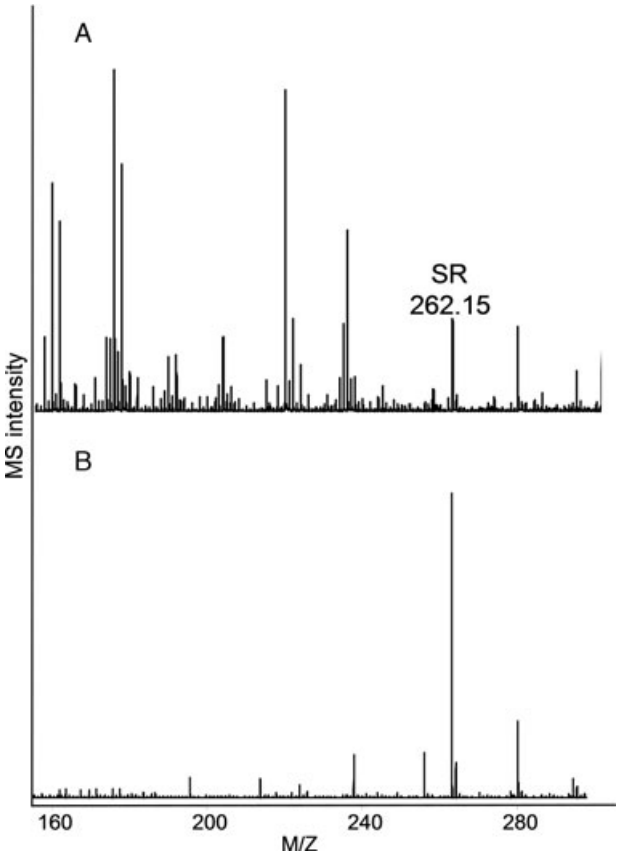


Figure 3. Comparison of low mass noise in sheath versus sheath less system. Mass spectra were averaged over the width of the EIE peak of peptide SR (mass 262.15). (A) Sheath interface. (B) Sheathless interface.

baseline resolved. This peak overlap results in messy BPC that could hinder the recognition of unexpected peaks. For this reason, CE-MS is considered complementary to LC-MS for peptide separations, but is not recommended as a sole replacement.

3.2.2 Sheath-liquid CE-MS analyses

On the system utilizing a sheath-liquid interface, peptides are reliably separated with modest resolution and sensitivity. Most importantly, using an uncoated or neutrally coated capillary, the small peptides that are of greatest interest to the current work are all detected. The sheath system gives fast separations; the small peptides elute within 7 min, and all peptides elute within 25 min (Fig. 2A and B). The speed is enhanced by the nebulizing gas that induces laminar flow in the capillary; this flow also degrades resolution. The force from the nebulizing gas could be countered by applying an appropriate vacuum on the inlet buffer vial, or by carefully lowering the inlet buffer vial relative to the capillary terminus, thereby achieving a gain in resolution at a cost of speed.

A disadvantage of the sheath system is that the sheath liquid inevitably brings some contamination, and the relatively enormity of the sheath volume relative to the CE flow means that minor contaminants can hide a CE analyte signal. This is especially true in the low m/z range, where numerous contaminants are present [23]. Interfering low molecular weight species are demonstrated in Fig. 3A, which shows average MS data for the peak corresponding to peptide serine-arginine (SR). Despite the use of MS grade solvents, contaminants are present at a level similar to that of this analyte. Subtracting an average background spectrum allows SR to easily be detected, but this level of background would pose a serious difficulty if a low abundance analyte has a mass-to-charge ratio similar to that of a major contaminant.

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3.2.3 Sheathless CE-MS analyses

On the sheathless system, peptides are separated with greater resolution and with less noise than on the sheath system. However, the resolution still does not approach that of reverse phase LC. The one and two amino acid peptides elute within 12 min, and all peptides elute within 40 min.

The sheathless system clearly produced better resolved peaks; this can be quantified by calculating the resolution of peptides T10H and T13H in Fig. 2. These peptides have MH+ masses of 1341.51 and 1344.61. The resolution was calculated using the FWHM (full width at half maximum) equation, assuming Gaussian peak shapes:

$$Rs = 2(t_2 - t_1)/(1.7(FWHM_1 - FWHM_2))$$

The sheath system with an uncoated capillary produces a resolution of 0.51 for these peaks; with a neutrally coated capillary, the resolution of T10H and T13H is 0.40. With the sheathless system, their resolution is 0.98.

Enhanced sensitivity is expected with the sheathless design, and our sheathless system does produce a much better signal-to-noise ratio. The signal-to-noise improvement is partly due to a significant decrease in background signal, and therefore background noise. This is evident in the individual peptides' extracted ion electropherograms; the sheathless extracted ion electropherograms (EIEs) generally have a baseline intensity of zero, whereas the sheath system shows some baseline noise for all extracted peptide masses. Again, the absence of baseline noise with the sheathless system is likely due to the lack of sheath liquid contaminants. Figure 3B shows the average mass spectra calculated over the peak of peptide SR with the sheathless system; it is clear that the low *m/z* contaminants are present at a much lower level than in the sheath data shown in Fig. 3A.

3.3 Sequence coverage and mass accuracy

Table 1 reveals the utility of CE-MS as a complementary peptide mapping method. CE-MS excelled at revealing low mass peptides: the smallest eight peptides (all one or two amino acids in length) were seen only with CE. Both capillaries used with sheath-liquid CE-MS were able to detect 60 of 61 peptides overall (98%). However, the single peptide not detected was quite large (4812 Da, due to poor ionization), reducing the sheath CE-MS sequence coverage to 94%. This compares to an LC-MS sequence coverage of 97% (including missed cleavages), despite that method detecting only 51 of 61 peptides (84%). With the sheathless CE-MS system, all peptides were observed, giving 100% sequence coverage. Mass accuracy for CE-MS was excellent, with all peptide masses detected within 20 ppm.

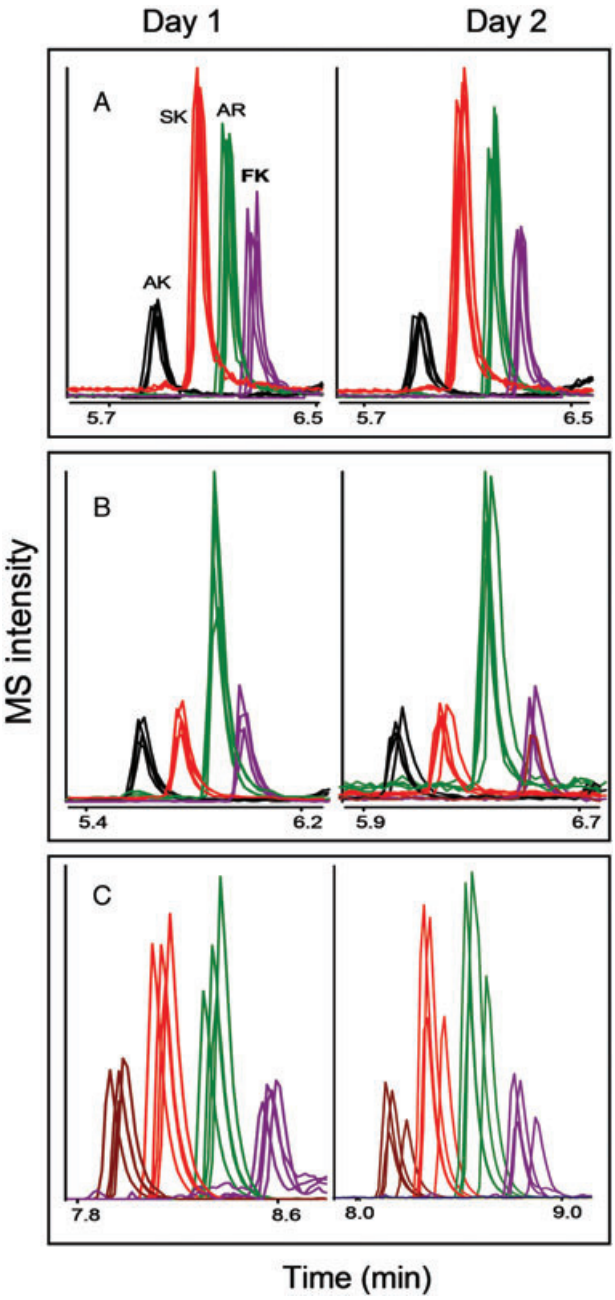


Figure 4. Extracted ion chromatograms demonstrating reproducibility of two amino acid peptides not seen in LC-MS, $n = 4$ consecutive separations on two consecutive days. (A) Sheath interface with PVA coated capillary. (B) Sheath interface with uncoated capillary. (C) Sheathless system with uncoated capillary.

3.4 Reproducibility

For a method to be useful in the routine analysis of pharmaceuticals, it must provide reproducible results. Figure 4 demonstrates intraday repeatability and interday reproducibility for all CE-MS analyses. On day 1, four consecutive injections of peptide digest were analyzed. For each of these injections, extracted-ion electropherograms for selected small peptides are overlaid (Fig. 4A). These runs were

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very repeatable; this was quantified by calculating the relative standard deviation (RSD) in peak height and migration time for the complementarity determining regions (CDR) peptide phenylalanine-lysine (FK). Using the sheath system with a neutral capillary, FK produced migration times with an RSD of 0.3%, and peak heights with an RSD of 4.4%. With the uncoated capillary in the sheath system, FK's migration time and peak height RSDs were 0.1% and 29%, respectively. For the sheathless system, the values were 0.3% and 7.6%, respectively: the variation in the figure appears to be greater because the peaks have longer migration times. The following day, the same sample was analyzed with an additional four consecutive injections using the same capillary and buffers. These injections had similar repeatability, but there was a small drift in migration time for the uncoated capillaries in both systems.

4 Concluding remarks

The goal of this work was to develop a fast, simple, reproducible CE-MS method to confirm the presence of small, hydrophilic tryptic peptides contained within monoclonal antibodies that are not detected by typical reverse phase LC-MS peptide maps. Using LC-MS, a very high percentage of the antibody amino acid sequence was covered, with the non-covered sequence consisting of small peptides. The same sample was analyzed by CE-MS without further preparation on a traditional sheath liquid interface with uncoated and neutrally coated capillaries, and the missing peptides were observed, as well as 60 of 61 overall. Critically, all 14 peptides of three amino acids or fewer were recognized. The peptides were identified with a mass accuracy of <20 ppm, and the map completed within 25 min. Using a CE-MS system with Beckman Coulter's sheathless interface, all peptides were detected, and resolution was enhanced. The peptide separation had high intra- and interday reproducibility. CE-MS is an effective tool for quickly confirming the presence of small, hydrophilic peptides, and is especially powerful when used in conjunction with LC-MS that does not reveal these peptides to achieve 100% sequence coverage.

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The authors have declared no conflict of interest.

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