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High-throughput analysis using gated multi-inlet mass spectrometry

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Gated multi-inlet mass spectrometry is introduced for high-throughput chemical analysis. In this design, multiple high-pressure liquid chromatography (HPLC) columns or capillary electrophoresis (CE) capillaries are attached to multiple electrosprayers (one for each column or capillary) that spray toward a gated multi-inlet time-of-flight mass spectrometer (TOF-MS). Although all of the sprayers are spraying continuously, only one inlet is exposed at any given time for a specific duration set by the MS data system. The gated multi-sprayer, multi-inlet design significantly enhances the performance of the multi-ESI, multi-inlet TOF-MS with minimal cost and reduces analysis time. The gated multi-sprayer, multi-inlet design was applied to the investigation of column-to-column reproducibility of multiple HPLCs using a peptide mixture and to the simultaneous analysis of four protein digests. In addition, it was applied to the analysis of peptide mixtures using eight CE capillaries. The gated multi-inlet MS has several advantages compared to our previous non-gated multi-inlet MS. For example, because only one inlet is open at one time, the original manufacturer's inlet inner diameter and pumping system can be used, which enhances the sensitivity of detection for each inlet and minimizes the manufacturing cost. In addition, the number of inlets can be increased as desired. The maximum number of liquid streams that can be concurrently analyzed is limited by: (1) the number of inlets, (2) the chromatographic (electrophoretic) peak width, and (3) how fast the gate can move from one position to the next. **Copyright © 2011 John Wiley & Sons, Ltd.**

High-throughput analysis of complex mixtures has been the need of industries such as pharmaceutical and biotechnology, as well as combinatorial chemistry, where a large number of reaction by-products must be analyzed. In recent years, the effort to increase throughput using separation in conjunction with mass spectrometry has been focused on reducing the separation time. For example, ultra-high-pressure liquid chromatography (UHPLC) is a rapidly growing technique that greatly increases sample throughput. UHPLC takes advantage of short HPLC columns packed with $<2\,\mu m$ particles, at elevated pressures, to significantly reduce analytical run times.[1] In capillary electrophoresis (CE), shorter analysis time is also achieved by using ultrafast CE (UFCE) by reducing capillary inner diameter (i.d.) ($<10\,\mu m$), and capillary length ($<30\,cm$), while increasing separation voltage ($>1000\,V/cm$).[2] To increase throughput beyond these techniques, most researchers are forced to use several HPLC/MS or CE/MS systems in parallel. Increase in the cost of high-performance mass spectrometers, however, put this option outside the budget range of many facilities. To remedy this cost issue, in the past decade several techniques were introduced in which several parallel liquid streams were analyzed by one mass spectrometer at the same time: (1) The effluents of several columns are attached to several electrosprayers (one for each column) and a sampling device is attached to the MS inlet, which samples each sprayer for a specific period of time.[3] (2) The effluents of several HPLC columns enter a switch box, where the effluent of only one column at a time is allowed to enter the electrosprayer.[4,5] (3) The effluents of several columns are attached to several electrosprayers (one for each column), each spraying to a dedicated inlet (one inlet for each sprayer).[6–8] In method 3, the MS atmospheric pressure sampling inlet (nozzle, sampling orifice, or heated capillary) is modified by replacing its single inlet with multiple atmospheric pressure inlets. This allowed for the parallel introduction of multiple liquid streams into the MS using one electrosprayer for each inlet so that the chemical contents of all liquid streams are analyzed concurrently using a single MS. Recently, Kelly *et al.* used muti-inlet mass spectrometry in conjunction with nanoelectrospray ionization multiemitters and electrodynamic ion funnels for efficient ion transmission through the first vacuum stage.[9]

In our original multi-sprayer, multi-inlet design,[6–8] all inlets were open to the atmosphere at all times and the ESI sprayers either sprayed continuously or were turned on one at a time using a high-voltage switch. Since all of the inlets were open to the atmosphere at all times, the pressure in the first stage of the vacuum system was too high for the original pumping system to evacuate when multiple inlets were used. To maintain operating pressure within the MS, the inlet i.d. was reduced and multiple pumps were used. Even under these conditions, the pressure problem was exacerbated when the number of inlets exceeded three. For example, in a four-inlet system, the introduction of two additional pumps

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to the original pumping system of the MS did nothing to improve the sensitivity of detection (per inlet) compared to that of a standard single-inlet design. In order to maintain the original inlet i.d. and pumping system while still maintaining the original sensitivity (per inlet), a gated multi-sprayer, multi-inlet design was developed. Here, as a proof of concept we introduce a gated multi-sprayer, multi-inlet design and demonstrate its application to high-throughput analysis using multiple HPLC columns or CE capillaries.

EXPERIMENTAL

Reagents

Other than the HPLC-grade water (EM Science; Gibbstown, NJ), all chemicals used were purchased from Sigma. A commercially available peptide standard containing Gly-Tyr (MW 238), Val-Tyr-Val (MW 379), leucine-enkaphalin (MW 555), methionine-enkaphalin (MW 573), and angiotensin II (MW 1046) was diluted to give a final concentration of 5 μg/mL (except for Gly-Tyr, which was at a concentration of 1.25 μg/mL).

Protein digest preparation

Four different protein solutions (coccidioides immitis chitinase, cytochrome c, hemoglobin, and casein; all at a concentration of 24 pmol/μL) were digested with trypsin using the previously reported procedure.[10]

Gated multi-sprayer, multi-inlet design

The gated multi-sprayer, multi-inlet design introduced here (Fig. 1) contains eight sprayers positioned in front of eight corresponding inlets. This was achieved by replacing the single inlet (0.4 mm i.d., 19.8 mm long) of the MS with an in-house fabricated eight-inlet (each 0.4 mm i.d., 28.5 mm long). The outlet of each inlet points to the instrument's first skimmer. In this design, a rotating disk containing a single opening is interfaced to the in-house fabricated eight-inlet. The rotating disk, which is mounted on a stepping motor, rotates about a specific angle (45, 90, or 180°) when signaled (using the instrument contact closure output) to do so by the operating system of the MS. The rotating disk then stops over the hole and the disk temporarily exposes a single inlet to the atmosphere while the MS acquires data from the liquid stream that is spraying into that inlet. The process continues until another contact closure signal prompts the disk to rotate and expose other inlets in a sequential manner.

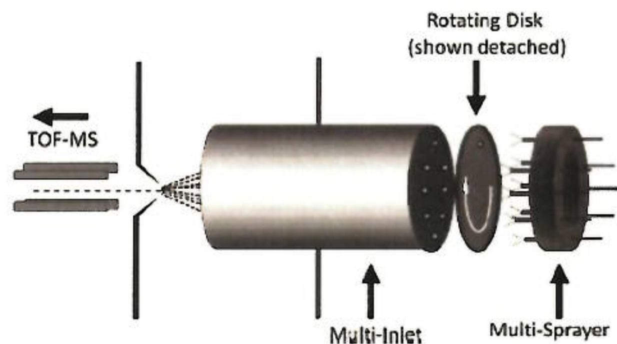

Figure 1. Schematic of the gated eight-sprayer, eight-inlet TOF-MS.

Under this design, only one of the eight inlets is exposed to the atmosphere at any given time while all other inlets are vacuum-sealed by the disk. Using the eight-inlet design, the rotating disk can be programmed to rotate either 0, 45, 90, or 180° (upon receiving the contact closure signal from the MS operating system) and can, therefore, be programmed to concurrently analyze the liquid streams coming from either one, two, four, or eight sprayers.

The MS method used for the eight-sprayer, eight-inlet system includes one segment with 16 events. The first event and each subsequent event were used to rotate the disk between the inlets. These events were programmed so that a contact closure signal was sent out at the beginning of each event to the motor, causing it to rotate by a specific angle (45° for the eight-inlet), thereby exposing the next inlet. With the motor (TMG, Mountain View, CA, USA) used in these experiments, the travel time between inlets is ~100 ms for both 45 and 90° rotations. The other eight events were used for data acquisition. The disk does not move for these events, allowing the MS to sample from the electrosprayer spraying towards the open inlet. With the MS used for this work, the minimum acquisition time that could be used for each acquisition event while still allowing the data system to control the MS, acquire data, and reproducibly execute all 16 events was 0.5 s. The operating system of the MS continued cycling through these 16 events until the end of the analysis time. The total turn-around time for all 16 events was ~4.8 s. Even though all the data was collected in one file, the Mariner data system kept track of each acquisition event, allowing data collected from each inlet to be displayed in separate, individual chromatograms or electropherograms. This enabled concurrent data analysis on multiple liquid streams.

MS

All electrospray ionization experiments were carried out using a Mariner time-of-flight mass spectrometer (ABI, Framingham, MA, USA). In order to increase the transmission efficiency of ions coming out of the inlets, the orifice of the first skimmer was enlarged from 0.4 mm i.d. to 0.6 mm i.d. An additional mechanical pump (Edwards High Vacuum International, Crawley, Sussex, UK) with a pumping capacity of 10 L/s was then added to the original pumping system of the Mariner, which includes a mechanical pump (Varian Vacuum Products, Lexington, MA, USA) and a turbo pump (Pfeiffer Vacuum Technology Inc., Nashua, NH, USA) with respective pumping capacities of 7.5 L/s and 210 L/s, to maintain an analyzer pressure of $<5 \times 10^{-6}$ Torr. This additional pump was connected to the original mechanical pump using a Tee. These two mechanical pumps together evacuate the first stage of the vacuum housing (between the inlet and the first skimmer) and also pump the output of the turbo pump. Under these conditions, the analyzer's pressure was $\sim 4.0 \times 10^{-6}$ Torr.

For all HPLC experiments, the ESI sprayers were at the same voltage (4 kV). An external high-voltage power supply

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(Power Designs Pacific Inc., Palo Alto, CA, USA) had to be used since the instrument's power supply was incapable of providing the current needed for multi-sprayer operation. The inlet temperature was maintained at 140°C. The instrument was tuned to a resolution of 5000–6000 (FWHM) using the original inlet. The scan range of the MS was set to m/z 375–600 for the analysis of the peptide mixture and m/z 500–2000 for the analysis of the protein digest. The MS was operated at a rate of 10 000 acquisitions/s. In this study, 5000 single shot spectra were averaged to generate one averaged spectrum every 0.5 s.

HPLC

A Michrom (Magic 2002; Michrom BioResources, Inc., Auburn, CA, USA) HPLC system with a dual pump was used as the solvent delivery system in all HPLC/MS experiments. The output of the HPLC mobile phase (after the mixer) was connected to an eight-way flow splitter (Valco Instruments, Houston, TX, USA). Each outlet of the eight-way flow splitter was connected to an injector (Rheodyne, Rohnert Park, CA, USA), each equipped with a 20 μL loop volume. Nitrogen, at a flow rate of 0.5 L/min, was used as the nebulizing gas. In the first set of HPLC experiments (column-to-column reproducibility), the output of each injector was connected to a 10 cm long C18 reversed-phase column (Agilent, Wilmington, DE, USA). The HPLC mobile phase flow rate was 100 μL/min (~13 μL/min for each column) and the mobile phase gradient was from 95% A (0.1% trifluoroacetic acid (TFA) in 99% water + 1% acetonitrile) to 80% B (0.1% TFA in 90% acetonitrile + 10% water) in 30 min. In these experiments, ~5 μL of the peptide standard were injected into each of the eight injectors. In the second set of HPLC/MS experiments (analysis of the protein digest solutions), the outputs of four of the injectors (every other injector) were connected to four SGE columns (SGE, Austin, TX, USA) while the outputs of the other four injectors were plugged. The HPLC mobile phase flow rate was 50 μL/min and the gradient was from 95% A (0.1% TFA in 99.9% water) to 65% B (0.1% TFA in 99.9% acetonitrile) in 15 min. In these experiments, each of the four SGE columns ran a different protein digest solution.

CE

For multi-CE, multi-inlet TOF-MS analysis, an in-house fabricated CE instrument capable of handling eight samples and eight buffer vials was used (Fig. 2). The CE instrument was capable of pressure programming during a voltage separation. Pressure and voltage were supplied by a Pace System 2100 (Beckman Coulters, Fullerton, CA, USA). Eight 50 μm i.d., 150 μm o.d. CE capillaries, ranging between 50–80 cm in length, were interfaced to the multi-inlet MS using a sheathless CE/ESI-MS interface.[11] The capillaries were APS derivatized according to the previously reported procedure.[12] Approximately 5 nL of the peptide standard were injected into each capillary using the pressure injection mode. The CE was operated in reverse polarity mode (–15 kV applied at the CE inlet) and a 0.1% acetic acid solution was used as the BGE.

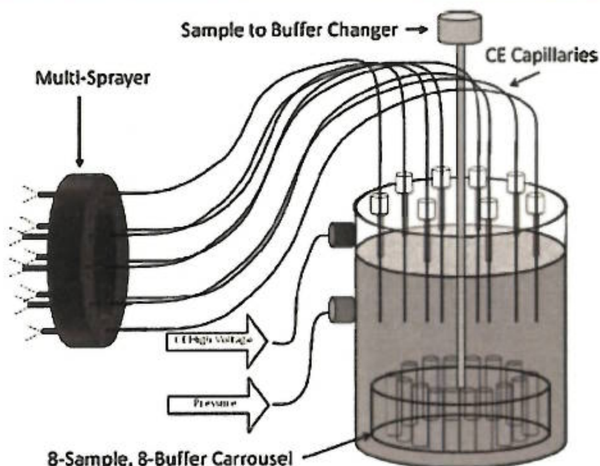

Figure 2. Schematic of the eight-capillary CE instrument capable of providing pressure programming under voltage separation.

RESULTS AND DISCUSSION

Performance of the gated multi-sprayer, multi-inlet design

The performance of the gated multi-sprayer, multi-inlet design depends on two major factors. The first factor is the ability of the rotating disk apparatus (the gate) to rapidly and reproducibly switch between inlets. Under the current design, the minimum travel time between inlets is ~100 ms. Thus, the gate can reproducibly complete a full rotation in 0.8 s (100 ms/inlet) when using eight inlets. The second factor that dictates the performance of the gated multi-sprayer, multi-inlet design is the efficiency of the MS operating system in simultaneously operating the MS, outputting contact closure signals, and acquiring and storing data. While the Mariner's operating system was acquiring data and controlling the instrument, a minimum of 0.5 s of acquisition time between output signals was required in order to reproduce output contact closure signals to the gate. Therefore, the Mariner requires 4.8 s to reproducibly complete a full rotation of the gate when using eight inlets (4.0 s acquisition time + 0.8 s travel time). If the acquisition time was less than 0.5 s, the output contact closure signal was not reproducible, causing a mix-up of the events and of the information obtained from each inlet. Thus, in these experiments, the MS operating system was the limiting factor in the number of data points that could be acquired on each chromatographic or electrophoretic peak. In multi-capillary CE/MS analysis, therefore, the separation was performed at –15 kV to obtain multiple data points across the CE electrophoretic peaks.

Multi-HPLC column, multi-inlet MS

In HPLC analysis, column-to-column reproducibility is important for both identification and quantitation purposes. While run-to-run reproducibility is a parameter that is evaluated by the manufacturer of the HPLC column, column-to-column reproducibility is not. As the role of peptide retention times in protein identification becomes

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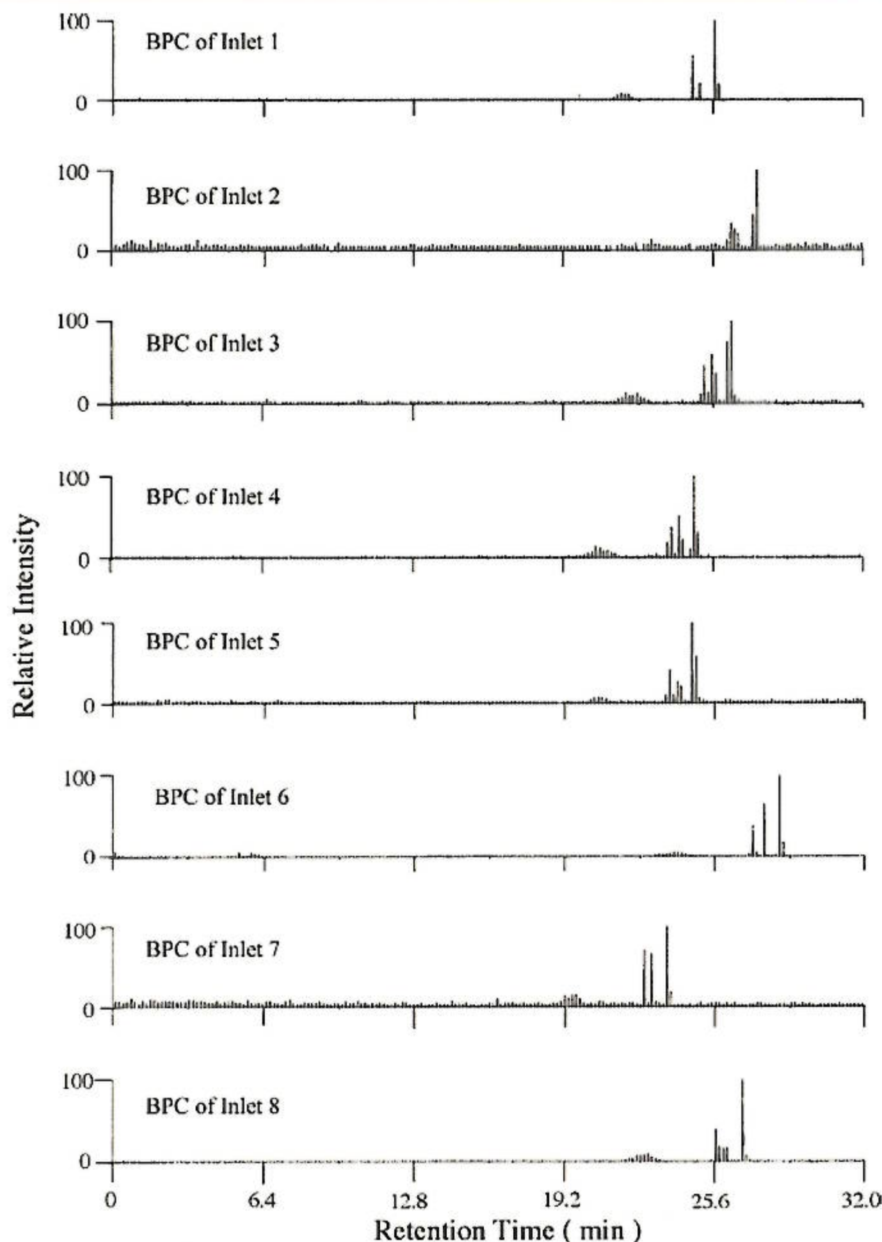

Figure 3. Separation of the peptide mixture using eight HPLC columns in conjunction with gated eight-sprayer and eight-inlet system. Base peak chromatograms (BPC) of each inlet are shown in separate panels.

more important, column-to-column retention time reproducibility also becomes more important. Column-to-column reproducibility is also important in quantitative mass spectrometry using ESI because a major factor that affects retention time reproducibility is the variation of solvent/buffer flow rates in each column for HPLC/MS. When one HPLC system is used to deliver solvent to multiple HPLC columns, each column receives identical head pressure and mobile phase composition; however, the mobile phase flow rate in each column depends on the packing characteristics of each column. Under these conditions, the flow rate through each column depends on the uniformity of the packing material and packing conditions in each column. The mobile phase flow rate through the column affects retention time and separation efficiency. In addition, because ESI is a concentration-sensitive technique, any change in the flow rate directly affects the sensitivity of detection and, therefore, quantitation. To examine column-to-column reproducibility, eight HPLC columns were tested using the peptide standard as a test mixture. As is shown in Fig. 3, the retention times of the peptides for all eight columns were close but not identical. This problem, however, can be remedied through software manipulation by normalizing the time scales using an internal standard (not studied). In this experiment, an eight-inlet, eight-sprayer system was used. As is shown (Fig. 3), there is no memory effect between the inlets (the chromatographic peak of one column does not show up on the chromatogram of other columns). This is a big advantage

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of multi-inlet mass spectrometry in which each stream of liquid has its own ion path into the MS and there is minimal interaction between the streams.

With the gated multi-sprayer, multi-inlet design it is not necessary to use all of the inlets at all times. An eight-inlet system can be used for the analysis of up to eight liquid streams. For example, the rotating disk can be programmed to analyze the liquid streams of four sprayers by rotating 90° each time (as opposed to 45°), therefore exposing every other inlet. For example, Fig. 4 shows the simultaneous analysis of the tryptic digests of four proteins (hemoglobin, cytochrome c, casein, and chitinase [coccidioides immitis]) using the four columns. For these proteins, 12, 9, 7, and 5 peaks were detected, respectively. The results clearly show that even with a low number of data points across each peak, high-throughput proteomics can be achieved with the gated inlet design.

High-throughput analysis of peptide mixtures using multi-CE capillary, multi-inlet TOF-MS

Capillary electrophoresis (CE) has a number of practical advantages over conventional scale analytical separation methods including high separation efficiency, high speed, and the ability to handle small sample sizes. In practice, CE is used either as an alternative separation method to HPLC, capable of handling a smaller sample volume while providing faster analysis times with higher efficiency, or as a complementary technique to HPLC. Therefore, when multiple CE capillaries are combined with a multi-sprayer, multi-inlet mass spectrometer, they allow for even faster compound identification by analyzing multiple samples, such as several protein digests, simultaneously. Recent developments in interfacing CE to MS[11,13,14] have allowed us to apply CE/MS to the analysis of a variety of complex biological mixtures, ranging from amino acids to protein complexes and the chemical analysis of intact cells.[15–23] In this study, the interfacing of eight CE capillaries to our gated multi-inlet TOF-MS is investigated.

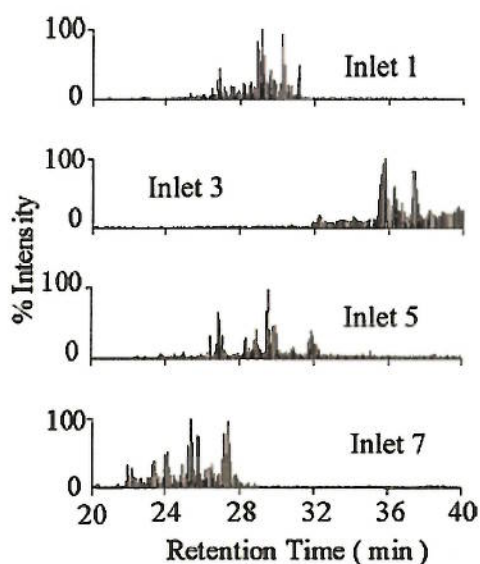

Figure 4. Separation of four-protein digests using four HPLC columns. The eight-inlet system was used but the rotating disk was programmed to bypass every other inlet.

Figure 5(top) shows the total ion electropherogram of the analysis of the peptide standard using eight CE capillaries. The in-house fabricated eight-capillary CE instrument (Fig. 2) was utilized for this study. This device allows simultaneous injection and separation of up to eight CE capillaries. Moreover, it allows application of pressure during separation. Figure 5(bottom) shows the reconstructed total ion electropherogram for one of the capillaries. The low resolution achieved in this electropherogram was because of the wider i.d. (50 μm) capillary in conjunction with low separation voltage (~15 kV) used in this experiment. The use of a wider capillary and lower separation voltage was necessary to obtain peak widths that were compatible with the acquisition time (~4.8 s turn-around time) of our current multi-inlet instrument.

Future directions include reducing the turn-around time of the gated multi-inlet by using a faster TOF MS, which can easily handle large amounts of data and control external devices, as well as a faster gated inlet that can switch between the inlets quickly. This combination will allow the application of a gated multi-inlet MS to modern separation techniques without compromising their high separation efficiency.

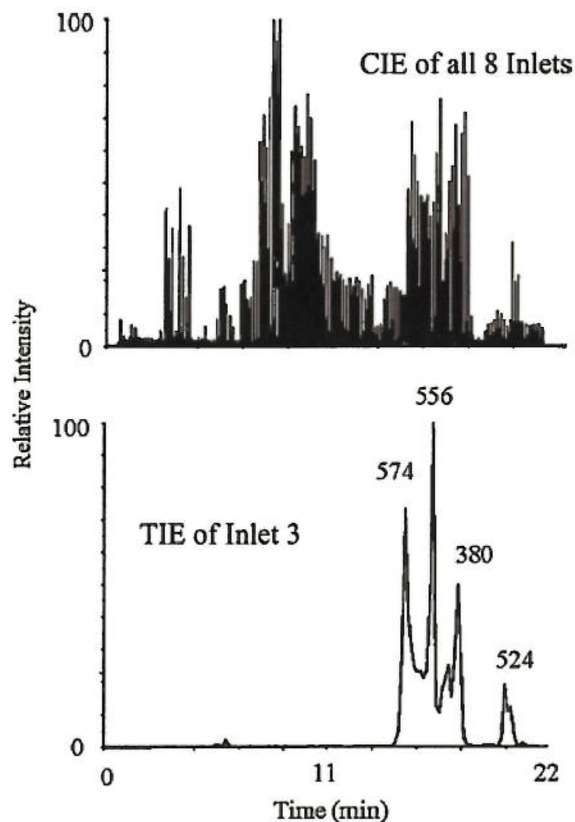

Figure 5. Combined ion electropherogram (CIE) of the eight-CE capillary system (top). The same peptide standard was injected in each capillary. Extracted total ion electropherogram (TIE) of one capillary/inlet is shown in the bottom panel. The m/z 574 (1+), 556 (1+), 380 (1+), and 524 (2+) are, respectively, Met enkephalin, Leu enkephalin, Val-Tyr-Val, and angiotensin II.

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CONCLUSIONS

Gated multi-inlet mass spectrometry is a viable technique for high-throughput analysis, where multiple streams of liquids from several HPLC and/or CE columns can be analyzed using a single mass spectrometer. Up to eight peptide mixtures from up to eight HPLC or CE columns were analyzed in about 30 min. The number of peptide fragments observed of each protein digest from each column was adequate to identify the protein using online protein databases. At this time, the minimum acquisition time for each inlet is 0.5 s, with an additional 0.1 s for travel time between inlets. Therefore, the turn-around time for the eight-inlet system is 4.8 s. This turn-around time was dictated by the 15-year-old Mariner TOF MS data acquisition system, which was the main limiting factor for faster analysis times and higher resolution. However, the proof of concept presented here demonstrates the usefulness of the gated multi-inlet system for high-throughput analysis.

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