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A microcapillary trap cartridge-microcapillary high-performance liquid chromatography electrospray ionization emitter device capable of peptide tandem mass spectrometry at the attomole level on an ion trap mass spectrometer with automated routine operation

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Reversed-phase microcapillary chromatography (RP-μLC) combined with electrospray ionization tandem mass spectrometry (ESI-MS/MS) is one of two prevailing techniques in proteomic analysis, the other being matrix-assisted laser desorption/ionization (MALDI). Despite the arguably better dynamic range obtainable with ESI, MALDI is increasingly popular due to ease of use, ruggedness and the ability to decouple separation from ionization. By contrast, in order to take advantage of the sensitivity and dynamic range afforded by the concentration-dependent nature of ESI, it is directly coupled to separations that take place in small i.d. RP-μLC columns. This gain in sensitivity often comes at a loss of ruggedness due to clogging of the small i.d. RP-μLC columns, one result of which is limited sample throughput. Here we describe a combined μpre-column-μLC-ESI device that is sensitive, rugged and modular in design allowing facile construction and troubleshooting. Due to low signal-to-noise as little as 1 attomole of a peptide can be selected by data-dependent methods for collision-induced dissociation. Importantly, the resulting tandem mass spectrum is of high enough quality to identify the peptide sequence by a database search against a complex database using SEQUEST. Finally, the device is demonstrated to be rugged as judged by >60 consecutive reversed-phase μLC separations on complex peptide mixtures before chromatographic resolution is degraded. Copyright © 2003 John Wiley & Sons, Ltd.

Mass spectrometry, in particular the application of electrospray ionization (ESI) coupled on-line with high performance separation techniques such as capillary electrophoresis (CE) and microcapillary HPLC (μLC), has had a dramatic effect on the sensitivity and the speed with which the primary structures of proteins and peptides can be determined.[1] Advances in separation techniques, particularly their implementation in miniaturized formats on-line with high performance mass spectrometers[2–6] and the development of miniaturized sprayers as ESI sources,[6–9] have reduced the amount of peptide required for complete and routine sequence characterization from several picomoles of peptide[10,11] in the mid-1980s to a few femtomoles and below by the mid-1990s.[12–16]

Arguably, much of this gain in sensitivity at the mass spectrometer is due to the combination of concentration dependent[17] type ionization devices like ESI with on-line capillary separation devices of very small internal diameter (i.d.). The increase in sensitivity with small capillary i.d. can be primarily attributed to a reduced mass flow rate of solvents and other background constituents into the ESI source, which allows for greater analyte ionization efficiency.[4,5] For these reasons, exceptional sensitivity in detection of proteins has been achieved even when using single quadrupole mass spectrometers and capillary electrophoresis in 5 μm i.d. fused-silica capillaries.[4] However, for reasons of robustness, most μLC work is done with 75 or 100 μm i.d. capillary columns that clog less frequently than capillary columns of ≤50 μm i.d.

In this paper we describe preparation and testing of a modular, integrated μLC-ESI device that consists of a microcapillary pre-column and a microcapillary analytical column that also serves as the ESI emitter. This type of integrated device, first reported in 1994,[6] has recently gained popularity from work by Yates and coworkers,[18] most notably as a clever biphasic capillary column.[19] One advantage of these devices is the use of a capillary tip, tapered to ~5 μm to hold the C18-derivatized particles in place rather than use of a sintered frit connected to a separate ESI emitter via a union that contains a dead volume that

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produces chromatographic band broadening. An integrated μLC column-μESI emitter minimizes the post-column band broadening due to dead volume that in turn causes a loss of peak capacity and ESI sensitivity.[20]

In this study, we describe an integrated, modular μLC-μESI device that uses a C18 pre-column to increase robustness and allows facile automation. The setup shown in Fig. 1 is similar to one previously described.[21] However, the modular design presented here offers better flexibility by allowing either the pre-column or analytical column to be replaced separately when needed. Additionally, the modular design allows each column, trap and/or analytical, to be custom packed with different stationary phases that, as we present, might minimize loading time for the pre-column and maximize chromatographic resolution in the analytical column. The robustness of the device is demonstrated by the ability to maintain chromatographic resolution for >60 analyses of a complex mixture of isotope coded affinity tag (ICAT) labeled peptides. Using an ion trap mass spectrometer and data-dependent ion selection for CID detection, sensitivity is demonstrated in μLC-ESI-MS/MS mode down to 1 attomole of peptide loaded on-column from an autosampler.

EXPERIMENTAL

Reagents and materials

Formic acid, HPLC-grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Neurotensin and angiotensin I were from Sigma-Aldrich (St. Louis, MO, USA). Polyimide-coated fused-silica capillaries (50 and 75 μm i.d.) were from Polymicro Technologies (Phoenix, AZ, USA). Magic C18 resins (5 μm, 100 and 200 Å pore) were from Michrom BioResources (Auburn, CA, USA), and a microtee and a microcross from Upchurch Scientific (Oak Harbor, WA, USA).

Preparation of solutions for injection

A stock solution of 1 nmol/μL was prepared by dissolving 1 mg of the acetate salt of neurotensin and angiotensin I in 598 and 943 μL, respectively, of 50% (v/v) acetonitrile/water solvent mixture containing 1% (v/v) acetic acid. The stock solutions were initially diluted to 10 pmol/μL with the same solvent and then serially diluted to 100 amol/μL. The injected solution containing 1 amol/μL of each peptide was prepared by combining 1 μL of 100 amol/μL solutions of each peptide and 8 μL of water containing 0.1% formic acid. The injected solution containing 1 pmol/μL of each peptide was prepared by diluting and combing the 10 pmol/μL solutions. Preparation of ICAT-reagent-labeled peptide mixtures has been described previously.[22]

Instrumentation

The instrumental setup consisted of a binary HP1100 pump from Agilent Technologies (Wilmington, DE, USA), a Famos micro-autosampler from Dionex LC packings (San Francisco, CA, USA), a six-port switching valve integrated on a LCQ Deca ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA), a pre-column (100 μm i.d. × 2.0 cm length) and a μLC analytical column (75 μm × 10 cm) that also served as a μESI emitter.

A schematic overview of the μLC-μESI arrangement is shown in Fig. 1. The device is constructed around a PEEK microcross that prevents formation of specific metal ions present when metal-based microcross unions are used. The microcross serves as the central hub into which the following are connected: (1) the pre-column, constructed as shown in the blowup window of Fig. 1 using a sintered frit to retain C18 resin; (2) a platinum wire through which ESI voltage is supplied; (3) the combined μLC-μESI column/emitter, of which the i.d. of one end is tapered to serve as a frit to retain C18 resin and from which ESI occurs; and (4) the six-port switching valve which directs eluting flow from the pre-column to waste or to the μLC-μESI column.

A slurry packing procedure was employed to prepare the columns. Two C18 stationary phase slurries, one of 100 Å pore for the analytical μLC column that maximized chromatographic resolution and one of 200 Å for the pre-column that minimized back pressure during fast loading, were prepared by placing ~5 mg of each C18 resin in a small vial, and adding 1 mL of methanol and a mini stirring magnet. For the analytical μLC column, one end of a 75 μm i.d. fused-silica capillary was manually pulled in a flame to a fine point estimated to be ~5 μm at the tip. To do this, the capillary was held perpendicular to the flame 10 cm from the cut end and heated. As the silica capillary began to melt the capillary was pulled smoothly and evenly in opposite directions such that a fine point formed. Afterwards, the point was trimmed with a ceramic capillary cutter. For the pre-column, a 100 μm i.d. fused-silica capillary was used and a frit prepared at one end by sintering underivatized silica beads in a microtorch flame. Both columns were packed using a pneumatic pressure cell from Brechbuehler, Inc. (Spring, TX, USA) at constant helium gas pressure of 1500 psi.

Flow paths and valve configurations at sample loading and separation stages are shown in Fig. 2. Sample volumes from 0.1 to 8 μL are loaded onto the pre-column with 5% solvent B (100% acetonitrile) at a flow rate of ~5 μL/min, as shown in Fig. 2(a). During loading of peptides the six-port valve on the LCQ mass spectrometer is in an open position so that eluting flow from the pre-column is directed to waste due to the high backpressure in the analytical μLC column. After 5 min of sample loading and subsequent washing cleanup, the six-port valve is closed (simultaneously opening the waste line of the splitter) and the flow is forced through the analytical μLC column, as shown in Fig. 2(b). The split flow outlet to waste at the splitter is restricted with 30 cm × 50 μm i.d. capillary tubing to generate a desirable column flow rate (~200 nL/min). For standard peptide analyses, a binary solvent composition gradient with solvent A (100% water containing 0.1% formic acid) and solvent B were developed from 5 to 35% B for 40 min, followed by 80% B for 5 min and 5% B for 20 min. For analyses of peptide mixtures labeled with ICAT reagent, the binary gradient from 5 to 35% was developed for 90 min. The above procedures, including sample loading, valve-switching, and solvent composition gradient elution, are fully automated and controlled by the software of the Thermofinnigan DECA ion trap mass spectrometer.

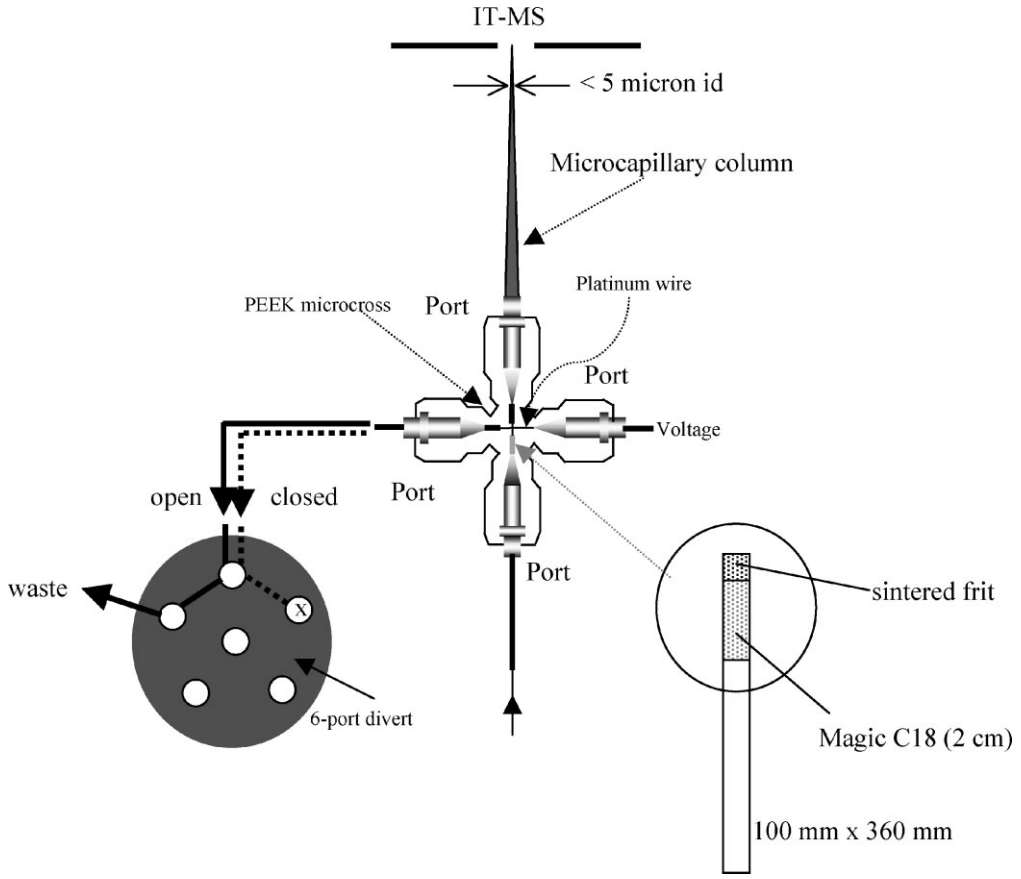

Figure 1. Schematic representation of the μLC-μESI column system.

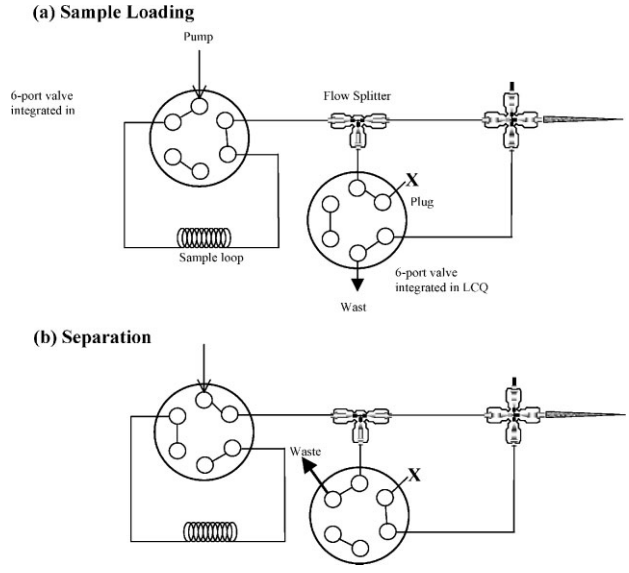

Figure 2. Flow paths and valve configurations at (a) sample loading and (b) separation stages.

Peptides eluting into the ion trap mass spectrometer were selected for collision-induced dissociation (CID) using a procedure that alternated between a MS survey scan over the *m/z* range 400–1800 with 5 microscans and a MS/MS scan in which the most abundant peptide ion was subjected to CID and then dynamically excluded from re-selection for 3 min. Each MS-MS/MS scan cycle lasted an average of ~1.6 s. A voltage of 1.8 kV was applied for stable ESI operation.

RESULTS AND DISCUSSION

In reversed-phase microcapillary chromatography coupled on-line to ESI-MS, a sample trapping pre-column prior to the analytical separation column allows decreased loading time, sample de-salting and facilitates automation. Despite these advantages, the dead volume between the pre-column and the microcapillary analytical column can be a limiting factor due to introduction of band broadening that will decrease ESI sensitivity for those analytes present at low relative stoichiometry. To overcome this limitation, attempts to integrate a pre-column and a microcapillary analytical column as one piece have been described.[20,21] In this study, we separate the two columns with a small dead volume (~50 nL geometrical volume), making it more practical to assemble and replace either column as necessary, but without introducing undue band broadening in part because the analytical column separation of peptides occurs after this dead volume. Also, the dimensions of the pre-column may be easily increased or decreased by changing the i.d. of the pre-column capillary tubing and/or the eluting flow rate from the pre-column without any influence on the C18 packing requirements of the microcapillary column.[23] To evaluate the separation efficiency of our device (Fig. 1), we applied a mixture of the peptides neurotensin and angiotensin I to the μLC/MS system. Figure 3 shows the base peak chromatogram obtained by injecting 100 fmol of each peptide. Baseline separation of the two peptides was accomplished with narrow peak widths, ~20 s at 50% peak height.

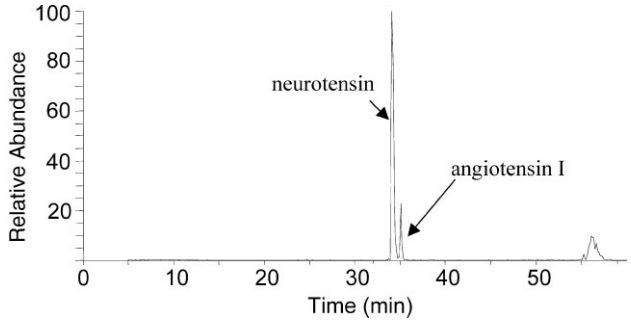

Figure 3. Base peak chromatogram of a peptide mixture of 100 fmol of neurotensin and angiotensin I.

The long-term stability and reproducibility of the pre-column and the μLC column were demonstrated by consecutive analyses of ion-exchange chromatographic fractions of a ICAT-labeled peptide mixture.[22] For each μLC/MS analysis of ICAT-labeled peptide mixtures, ~2 μg of total peptide were loaded onto the pre-column. The standard peptide mixture of neurotensin and angiotensin I was injected periodically to monitor the separation efficiency, as shown in Fig. 4. Comparable peak resolution, retention time, and signal intensity were observed for the first 60 runs. Although retention times changed over time and chromatographic peak resolution degraded slowly, signal intensities were consistent until ~60 analyses had been performed which was an indication that peptide recovery off of the column was maintained. After 67 analyses a decrease in signal intensity became apparent and the pre-column was replaced allowing the microcapillary analytical column to be used for >100 separations (data not shown) before it also needed to be replaced.

The use of PEEK-only components at the four-way union where the ESI voltage was supplied produced a much more stable ESI signal and better signal-to-noise (S/N) ratio due to decreased chemical noise (data not shown) than when a stainless steel four-way union was used. To evaluate the on-column peptide detection sensitivity of the integrated pre-column and analytical column, a dilution experiment was performed using the standard sample mixture mentioned above. Blank analyses were performed between each injection of standards to ensure that there was no carry-over between injections of peptide solutions of increasingly lower concentration. Figure 5 shows the results of one μLC-ESI/MS analysis after on-column injection of 1 amol of the standard peptide mixture. Figures 5(b) and 5(c) show MS survey scans where angiotensin I and neurotensin eluted, respectively. In the MS survey scans, ions corresponding to three charge states (singly, doubly, and triply charged) were clearly detected, with a maximum S/N ratio of 10 for triply charged neurotensin. For the triply charged neurotensin ion, a high quality tandem mass spectrum was obtained (Fig. 5(d)) that, when submitted to database search analysis using SEQUEST, identified the correct peptide sequence. This indicated that, under ideal conditions, our automated device allowed detection of precursors and subsequent tandem MS down to a few attomoles injected on-column. Due to increased competition for ionization and randomness of ion selection we would not necessarily expect an ion observed at such a low S/N ratio to be selected from a sample with diverse analyte composition such as is often the case in biological samples.[24]

To assess the performance of the device for analysis of complex samples containing thousands of peptides, a mixture of ICAT-reagent-labeled peptides was analyzed

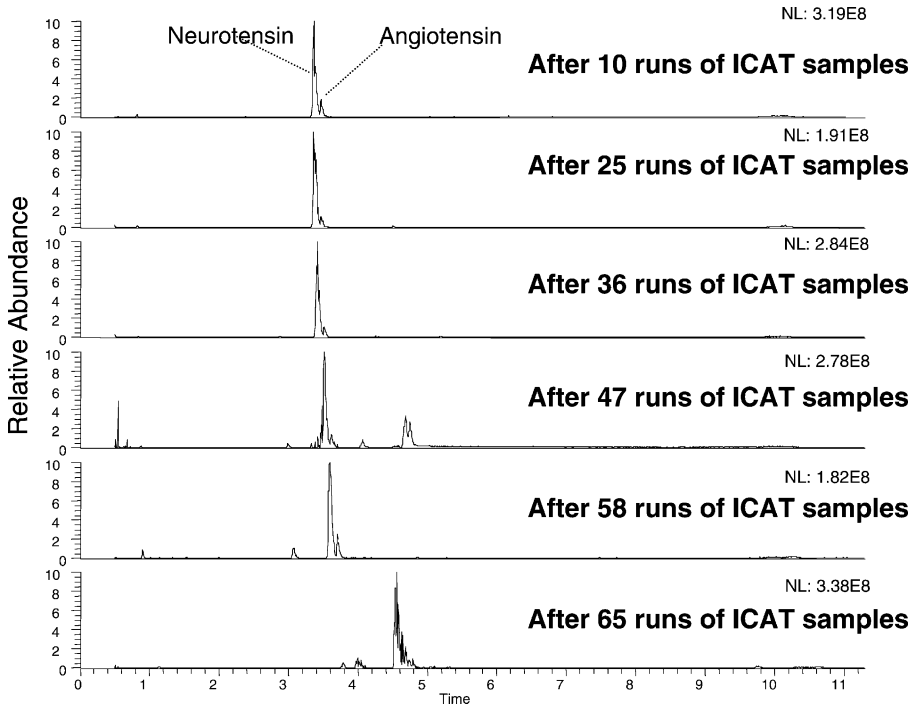

Figure 4. Monitoring of μLC performance over multiple runs by injecting a standard peptide mixture of 100 fmol of neurotensin and angiotensin I. After 67 analyses a decrease in signal intensity became apparent and the pre-column was replaced.

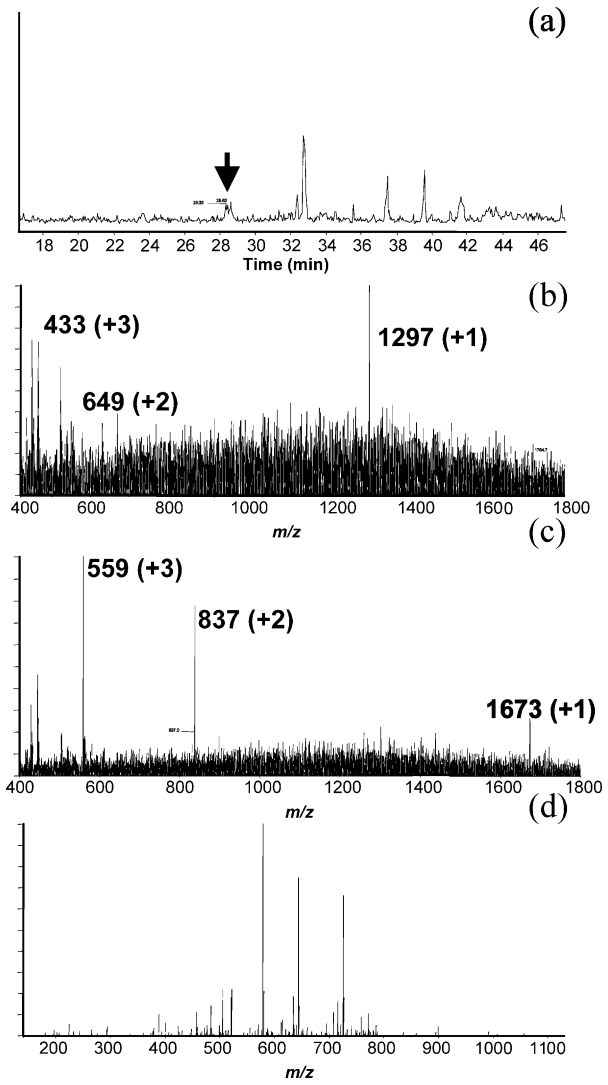

Figure 5. LC/MS analysis of 1 amol of neurotensin and angiotensin I: (a) base peak chromatogram; (b) MS spectrum of angiotensin I; (c) MS spectrum of neurotensin; and (d) MS/MS spectrum of triply charged neurotensin.

using a 90 min gradient over 5–35% B solvent composition range. The resulting base peak chromatogram is shown in Fig. 6. Ion chromatograms of four selected peptides obtained from XPRESS software[23] are shown in separate panels. Each ion chromatogram shows that the eluting peaks are highly resolved, with 30 s average peak widths through the entire separation period. Thus the device maintained high resolving power even when very complex mixtures were analyzed. This device is now routinely used in our high-throughput laboratory for analysis of ICAT-reagent-labeled peptides on both ion trap and quadrupole-time-of-flight mass spectrometers.

CONCLUSIONS

We have described a device for automated analysis of attomole levels of peptides that is analytically rugged. As expected, use of a μpre-column for sample loading and desalting preserved the life of the analytical μLC column which could typically be used for >100 analyses. Practically speaking this means that we can routinely change the columns after ~50 analyses and almost never lose a precious sample due to column clogging or a deterioration of performance. Like other combination μLC-μESI devices, band broadening is minimized by use of a tapered ESI tip that serves as the frit to retain C18 resin rather than using a pre-column junction to couple an analytical column to a separate ESI emitter. Unlike other devices the pre-column and analytical column are prepared separately so that they may be designed with specific purposes in mind like fast sample loading times or high peptide binding capacity or many other possibilities.[21] The modular assembly of the pre-column and analytical column enables rapid changes of the trap column when it fails without the need to change the analytical separation column. The robustness of our device was demonstrated by performing >100 consecutive analyses of complex ICAT-reagent-labeled peptide mixtures before changing the analytical column, and on-column peptide sensitivity down to 1 attomole of a standard peptide mixture.

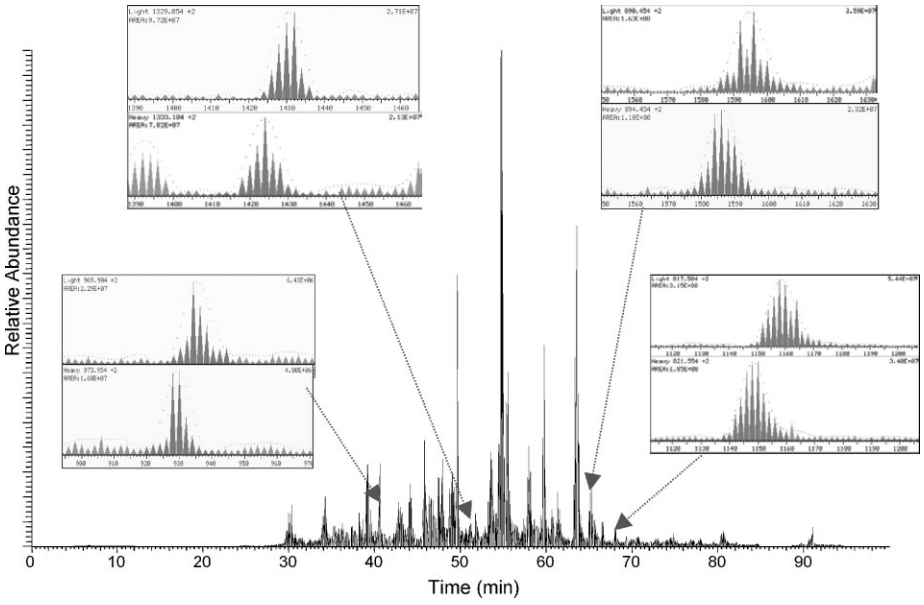

Figure 6. Base peak chromatogram of the ICAT-reagent-labeled peptide mixture.

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