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AUTOMATED ON-LINE IDENTIFICATION OF PHENYLTHIOHYDANTOIN-AMINO ACIDS FROM A VAPOR PHASE PROTEIN SEQUENCER

HENRY RODRIGUEZ
Department of Protein Biochemistry, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (U.S.A.)
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SUMMARY

An automatic on-line phenythiohydantoin-amino acid identification system was interfaced to an Applied Biosystems vapor-phase protein sequencer. Two unused solenoids on the sequencer have been utilized for remote start of a high-performance liquid chromatographic (HPLC) controller and for transfer of the phenylthiohydantoin derivatives into an HPLC injection loop. Performance of the sequencer and data interpretation at the subnanomole level have been enhanced by the increased reproducibility afforded by rapid on-line HPLC analysis of the PTH-derivatives. These minor hardware and programming changes to the sequencer have allowed complete automation through the identification process.

INTRODUCTION

Since the introduction of the first automated protein sequencer[1] numerous reports[2–6] have described modifictaions to the spinning cup instrument directed towards obtaining subnanomole sequencer information. At subnanomole sensitivity, it has become apparent that the increased reproducibility afforded by complete automation of the Edman chemistry is an important aspect of such modifications. We have previously demonstrated the feasibility and benefits of on-line phenylthiohydantoin (PTH)-amino acid separation on a Beckman (Palo Alto, CA, U.S.A.) spinning-cup protein sequencer[6].

Hewick *et al.*[7] have developed an automated vapor-phase protein sequencer. This sequencer is commercially available through Applied Biosystems (Foster City, CA, U.S.A.) and is capable of subnanomole primary structure determination of proteins and peptides[7,8]. Presented here is a simple and inexpensive design for automatic on-line high-performance liquid chromatographic (OL-HPLC) separation and identification of the PTH-derivatives from the vapor-phase protein sequencer.

MATERIALS AND METHODS

Sequencer

An Applied Biosystems 470A (Applied Biosystems) vapor-phase protein se-

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quencer equipped with a "mini" conversion flask was used in this study. Early models of the 470A sequencer used a program which required a vacuum pump for drying of the filter cartridge. Current models use a program which substitutes argon gas for drying, thereby eliminating the vacuum pump. Our sequencer had been updated to run the "no vacuum" program, but the EPROM (software) update used with the "no vacuum" program was not used. This allowed utilization of the unused filter cartridge vacuum function (Applied Biosystems, solenoid No. 2,13) and conversion flask vacuum function (Applied Biosystems, solenoid No. 2,9) for automation of the OL-HPLC system (see text). Standard "O2NBGN" and "O2NVAC" programs (*i.e.* "no vacuum" programs), supplied by Applied Biosystems, were used with modifications as noted in the text. A complete program is available from the author upon request. Heptane (S1), ethyl acetate (S2), butyl chloride (S3), 2 mg per 200 ml dithiothreitol in methanol (S4), 5% phenylisothiocyanate (PITC) (R1), 12.5% trimethylamine (R2), trifluoroacetic acid (R3), and 1 *M* hydrogen chloride in methanol (R4) were purchased from Applied Biosystems. Acetonitrile was purchased from Burdick & Jackson (Muskegon, MI, U.S.A.) and mixed (50:50, v/v) with S4 as suggested by Applied Biosystems. Aqueous acetonitrile (40%) was stored in the normally unused R5 bottle and was used to dissolve and transfer the PTH-amino acid into the HPLC injector.

HPLC system

The PTH-amino acids were resolved on a Microsorb 5-μm C_8 HPLC column, 25 cm × 4.6 mm, (Rainin Instrument, Woburn, MA, U.S.A.). Two Waters Model 510 pumps, a Model 680 gradient controller, and a Model 440 absorbance detector (monitoring at 254 nm) were employed (Waters Assoc., Milford, MA, U.S.A.). A Nelson Analytical Model 6000 (Nelson Analytical, Cupertino, CA, U.S.A.) data aquisition system was interfaced to the detector. The solvent system was a modification of the system described by Waterfield *et al.*[9]. Separation of all twenty PTH-amino acids was accomplished with a gradient from 100% A [8 m*M* sodium phosphate pH 4.5, in water–acetonitrile (80:20)] to 100% B [water–acetonitrile (30:70)] in 22 min at a flow-rate of 1.5 ml/min. The column was heated in a waterbath to 43°C. The injector was a Valco Model AC 10U (Valco Instrument, Houston, TX, U.S.A.) ten-port air-actuated valve, with two injection loops. The 100-μl loop was for sequencer samples and the 25-μl loop was for a PTH-amino acid standard mixture (25 pmoles of each PTH-amino acid in 25 μl). The HPLC injector was air-actuated with two stainless steel Skinner 12V DC (New Britain, CT, U.S.A.) 3-way solenoid valves powered and controlled by the "external events" switches programmed in the "timed events" program of the Waters 680 controller.

Sample preparation

PTH-amino acid standards were purchased from Pierce Chemical Company and esterified with 1 *M* hydrogen chloride in methanol for 25 min at 50°C. Human factor VIII was purified as previously described[10]. A factor Xa proteolytic fragment (mol.wt. 67 000) of human factor VIII was purified using sodium dodecyl sulfate polyaxcrylamide gel electrophoresis (SDS–PAGE) and isolated by electro-elution (11). The cartridge filter was precycled once with 1.5 mg of polybrene (Pierce Chemical) prior to the application of the sample. Two sample wash cycles were used prior

to sequencing to remove UV absorbing contaminants on the filter from the gel elution procedure. The wash cycle is a modified "O2NVAC" program which omits PITC delivery. The factor VIII fragment was kindly provided by Dr. Dan Eaton, Genentech.

RESULTS

Presented in Fig. 1A is a schematic of the on-line PTH-amino acid identification system interfaced to the Applied Biosystems reagent-solvent delivery system. The OL-HPLC system required three hardware modifications to the sequencer. First, the "bubbler" line from the conversion flask, which is connected to the "B" valve

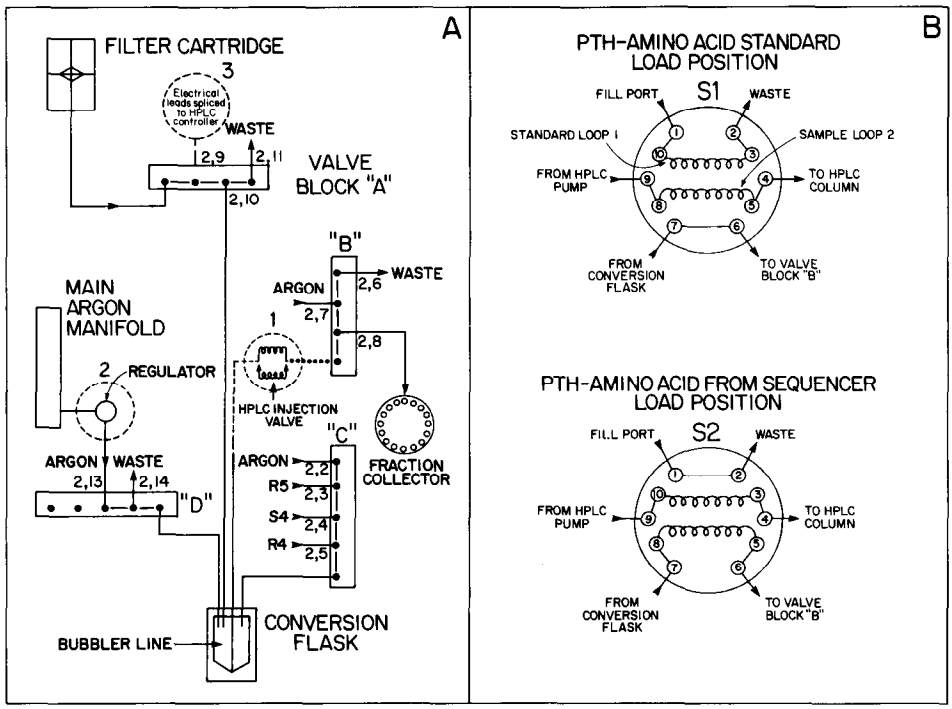

Fig. 1. Schematic of the OL-HPLC and the reagent/solvent delivery system on the Applied Biosystems sequencer. (A) Diagram of the filter cartridge, conversion flask and four reagent/solvent valve blocks (A, B, C, D). Three hardware changes (dashed circles 1, 2 and 3) were made to the sequencer: 1, an HPLC injection valve was spliced (---,···) into the line between the conversion flask and the "B" valve block; 2, the unused flask vacuum solenoid 2,13 was converted to supply argon via its own regulator; 3, the electrical leads controlling the unused cartridge vacuum solenoid 2,9 were used for remote start of the HPLC controller which operates the mobile phase gradient and HPLC injector. (B) Details of the HPLC injector. The ten-port HPLC injection valve contains two injection loops (standard loop 1 and sample loop 2). In the S1 position the PTH-amino acid standard mixture can be manually loaded through the fill port into loop 1. The HPLC controller automatically switches the injector to the S2 position which injects the standard mixture. Immediately after injection of the standard the conversion flask is programmed to "empty" so that loop 2 is cleared of the HPLC mobile phase. In the S2 position transfer of the PTH-derivative from the conversion flask into loop 2 and its subsequent injection is controlled automatically by the sequencer and the HPLC controller, respectively.

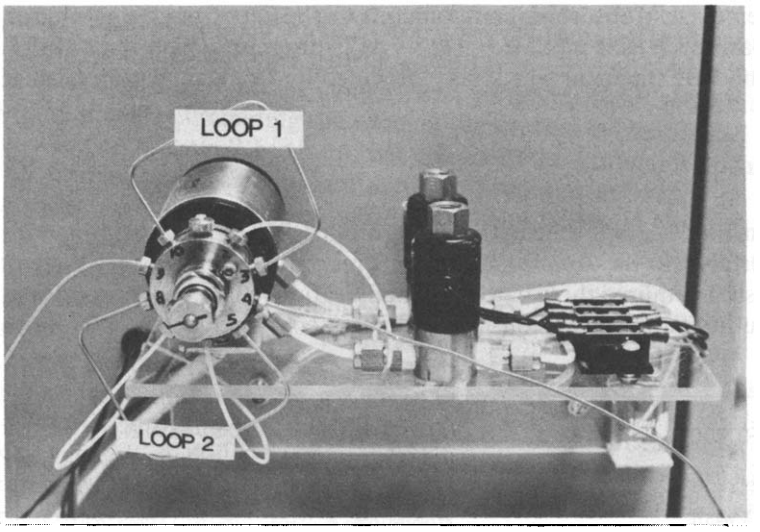

Fig. 2. Photograph of the OL-HPLC system interfaced to the sequencer. The photograph displays the ten-port HPLC injection valve (note labeled loop 1 and 2) and three-way solenoid valves. The components are mounted on an acrylic sheet and bolted to the left side of the sequencer. The extended "bubbler" line and electrical leads of solenoid 2,9 were passed through a hole made in the left side of the sequencer.

block, was extended (----) with a flare to flare PTFE coupler and connected to an HPLC injector with a short piece (approx. 15 cm) of PTFE tubing (0.5 mm I.D.); this allowed mounting of the injection valve externally on the left side of the sequencer, adjacent to the fraction collector (Fig. 2). Another PTFE line (····) (0.8 mm I.D.) connected the other end of the injection valve to the "B" valve block (Fig. 1B).

The second hardware change was the installation of a miniature low pressure regulator, set at 15 p.s.i., in the unused port on the main argon manifold of the sequencer. The outlet from this regulator was connected to the conversion flask vacuum solenoid 2,13 (Applied Biosystems solenoid identification number) in place of the vacuum line. This change provided a programmable sequencer function for transfer of the PTH-derivatives. The separate gas regulator allowed independent pressure regulation of the conversion flask for positioning of the PTH-derivative within the HPLC injection loop as previously described[6]; pressurization of the conversion flask moves the dissolved PTH-amino acid into the HPLC injection loop. The pressure required to move the sample into the injection loop is directly proportional to the volume of the tubing from the end of the "bubbler" line in the conversion flask to the "B" valve block. The slightly larger I.D. tubing used between the injector and the "B" valve block (·····) was necessary to keep the length of tubing and pressure required for on-line injection to a minimum. If the pressure is too high the sample will not move smoothly into the injection loop and air bubbles will be introduced into the sample. This results in sample injections which are not reproducible in volume. If tubing of less than 0.8 mm I.D. is used, then longer lines are necessary which may restrict the flow of liquid and cause air locks.

The third hardware change involved modification of the filter cartridge vacuum solenoid 2,9. This change provided a programmable sequencer function which could

start the HPLC controller and data system. The electrical leads to the solenoid were disconnected and spliced to a 12V DC relay to start the HPLC gradient controller and data system which required a contact closure for remote start. The HPLC controller could then independently run a gradient program and control the injection valve while the data system provided identification and quantitation.

The OL-HPLC system was automated in the following fashion: the extracted anilinothiazolinone (ATZ)-derivatives are dried in the conversion flask and converted to PTH-derivatives with 1 *M* hydrogen chloride in methanol (R4) in the usual manner. The additional tubing required to splice in the injection valve causes R4 (containing the dissolved ATZ-derivative) to migrate up the flask "bubbler" line due to capillary action. Because of inadequate heating, conversion to the PTH-derivative was not complete. To circumvent this problem, the volume of R4 delivered to the conversion flask was increased from approximately 70 μl to 200 μl. The increased volume minimized the percentage of R4 that migrated up the "bubbler" line relative to that remaining in the conversion flask. The conversion flask program was also modified to insure that all of the ATZ-derivative was converted. This was accomplished by dividing the conversion time (25 min, 50°C) so that 2 s of argon gas could be delivered through the "bubbler" line to the flask every 125 s for the duration of the conversion reaction. The Applied Biosystems conversion flask function "argon dry" was used for this purpose. The "argon dry" steps reintroduced any sample which had migrated up the "bubbler" line back down into the conversion flask and mixed it with the flask contents. Upon completion of the conversion reaction the PTH-derivative was dried and 50 μl of S4 (methanol–acetonitrile) was delivered; followed by 150 μl of water–acetonitrile (60:40) (contained in the R5 bottle). To facilitate dissolution of the PTH-derivative, six 2 s "argon dry" steps (approximately 100 s apart over a period of 10 min) were added to the conversion flask program prior to injection. The sample volume and concentration of acetonitrile in the injected sample was lowered with two additional (approx. 20 s total) "argon dry" steps. The reduction in volume provided better control of the desired injection volume (usually 100 μl out of 125 μl) and also made the sample compatible with the HPLC solvent system.

On-line separation of the dissolved and concentrated PTH-derivative proceeds as follows: solenoid 2,13 (formerly the conversion flask "vacuum" function) pressurizes the conversion flask with argon such that the PTH-derivative is transferred up the "bubbler" line to a reproducible point in the injection loop. A programmed remote start signal from solenoid 2,9 (formerly the filter cartridge "vacuum" function) of the sequencer simultaneously activates the HPLC controller and the data system. The HPLC controller independently controls the HPLC "gradient program" and the "timed events" program. The "gradient program" develops the gradient, equilibrates HPLC column, and reduces the flow-rate to 100 μl/min until reactivated by the sequencer. The "timed events" program controls the injection valve and also resets the injection valve for the next sample (Fig. 1B). Any uninjected sample is washed and collected into the sequencer's fraction collector. Even though the injection loop is a fixed volume, any portion of the sample can be injected by adjusting the volume of R5 delivered to the flask. The remaining sample can be collected in the fraction collector.

Sperm whale apomyoglobin is routinely used to verify the performance of the

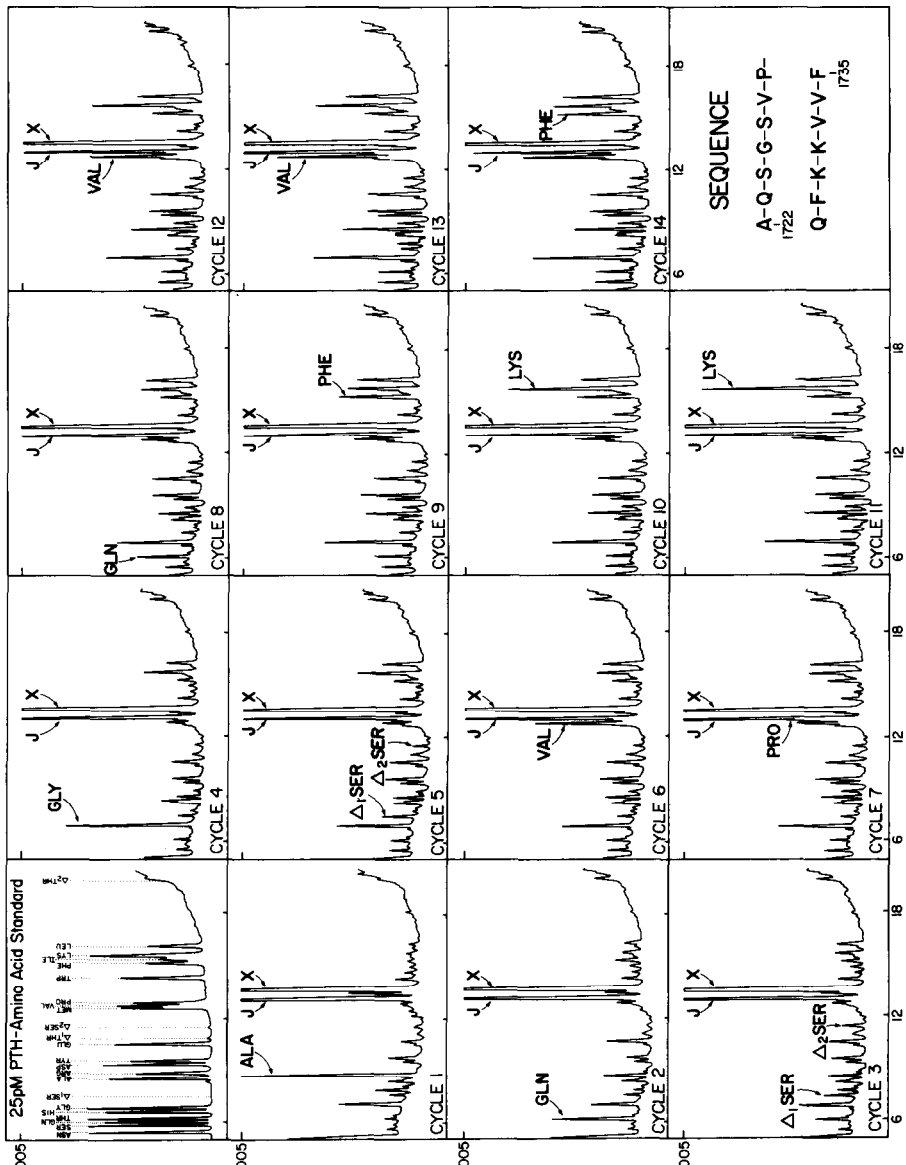

Fig. 3. On-line HPLC sequence analysis on 50 pmol of a proteolytic fragment of human factor VIII. The sequence of the polypeptide corresponds to residues 1722 through 1736 of the intact molecule[10]. The residue at each cycle and the sequence identified are shown. The peak labeled "J" is an artifact from the gel elution procedure. The diphenylthiourea peak ("X") is a by-product of the Edman chemistry. The PTH-amino acid standard (25 pmol) was used to quantitate and calibrate the data system. The elution positions for the PTH-serine (Δ_1Ser, Δ_2Ser) and PTH-threonine (Δ_1THR, Δ_2THR) by-products are shown in the standard trace. The HPLC gradient is taken to 70% acetonitrile in order to elute the Δ_2PTH-threonine by-product.

Applied Biosystems sequencer. The initial yield (50–80%) and repetitive yield (>92% at 100 pmol, >94% at 500 pmol) obtained on the sequencer with and without OL-HPLC analysis are the same (data not shown). Fig. 3 shows a 50-pmol NH_2-terminal sequence run on a SDS–PAGE electro-eluted[10] fragment (mol. wt. 67 000) of human factor VIII. Prior to the incorporation of OL-HPLC analysis of the PTH-amino acids there was a high degree of uncertainty in the assignment of serine and threonine residues below 200 pmol (unpublished results). The yield of PTH-serine and PTH-threonine is generally poor (<20%), which makes assignment of their respective amino acids unreliable at the picomole level. It is doubtful that the assignment of serine at cycle 3 and 5 (Fig. 3) would have been possible if the on-line system was not present. OL-HPLC analysis has enabled the recovery of two by-products of PTH-serine (sum of products equals approximately 40% yield) and two for PTH-threonine. Another benefit of on-line analysis is apparent in the cycle to cycle baseline reproducibility in the HPLC trace (Fig. 3). This reproducibility in the background PTH-amino acids (derived from the sample, filter, decomposition, etc.) is so consistent that 1–2 pmol of a PTH-amino acid derived from the protein can be determined with a high degree of confidence with the HPLC system described.

DISCUSSION

The Applied Biosystems sequencer programs are composed of "function numbers" (sequencer functions preprogrammed as subroutines) which control the filter cartridge and conversion flask. The "function numbers" control all the solenoid valves required for any given process. For example, delivery of R2 (Applied Biosystems function number 4) requires activation of three solenoid valves. It is not possible to program the individual solenoid valves which control the sequencer functions. If it were possible, it would have been easier just to use solenoid number 2,2 (Fig. 1A) to pressurize the conversion flask rather than modifying solenoid 2,13; however, there is no "function number" which can independently control solenoid 2,2. Therefore, solenoid 2,2 cannot be independently controlled by the sequencer program. It was fortuitous that Applied Biosystems developed the "no vacuum" program because it allowed use of the filter cartridge (solenoid 2,9) and conversion flask (solenoid 2,13) vacuum functions for the purpose of automation. Otherwise, automation would have been more difficult. These unused vacuum functions are not accessable on the currently available sequencer. However, there are functions in the current sequencer which can be used for the purpose of automation.

The standard Applied Biosystems sequencer program is composed of one "begin cycle" (O2NBGN) followed by as many standard cycles (O2NVAC) as desired. The "begin cycle" program differs from the standard cycle program by double PITC coupling the first residue. To completely automate the identification process, a quantitated PTH-amino acid standard mixture is manually loaded into loop 1 of the injector (Fig. 1B) prior to the start of a sequencer run. By adding 2 lines to the "begin cycle" program, a PTH-amino acid standard is automatically injected and calibrated by the data system prior to the first round of Edman degradation. The first line of the program simultaneously activates the HPLC Controller and the data system via the modified filter cartridge "vacuum" function (solenoid 2,13). After the injector is reset by the HPLC controller, the second line in the sequencer program

empties the injector (loop 2) of the HPLC mobile phase using the conversion flask "empty" function (Fig. 1B). The Nelson data system provides automatic quantitation based on a PTH-amino acid standard mixture injected by the modified "O2NBGN" program. The subsequent PTH-derivatives from each cycle of the Edman chemistry are automatically injected and analyzed.

There are two independent processes occurring simultaneously at any given step in the Applied Biosystems sequencer program (*i.e.* cartridge and flask functions). Therefore, care must be taken to conserve the status of one function when adding or deleting steps to the other so that sequencer performance is not compromised. For example, the conversion flask "argon dry" steps which were added to the program during conversion occur during the delivery of R2 to the cartridge. In order to maintain a constant flow of R2 during the additional "argon dry" steps to the conversion flask required adding R2 delivery to the filter cartridge at the same step. The total time for R2 delivery must then be adjusted to compensate for the additional time added by the "argon dry" steps.

The number of steps in the "O2NVAC" program was increased from 48 to 84 (total program time was only increased by 3.7 min). The steps added to the program are to insure complete conversion to the PTH-derivative and to insure complete solubility of the PTH-derivative prior to HPLC analysis. There was one software problem encountered during the modification of the Applied Biosystems "O2NVAC" program. The software "bug" is in the cycle editor. If the number of steps "inserted" (approximately 15–20% of the total number of steps in a cycle program) into a cycle program exceeds the space allocated to the program on the cassette tape, the computer will crash. This locks up the keyboard and prevents further access to that program in the editor. The only way to free up the keyboard is to turn the instrument off and then back on again. Rewriting the entire program by "creating" a new cycle circumvents this problem.

Most laboratories possessing an Applied Biosystems sequencer already have a dedicated PTH-amino acid analysis system. The on-line system described here can be easily adapted for use with other HPLC systems and is a viable alternative to the commercially available Applied Biosystems on-line HPLC system. This OL-HPLC system can reproducibly inject up to 90% (100 μl out of 110 μl) of the total sample from the sequencer[6]. The sequencer cycles in Fig. 3 represent an 80% injection. On-line detection has increased the speed with which protein sequence information can be obtained and analyzed from an Applied Biosystems protein sequencer and may actually improve the yield of labile PTH-amino acids. The OL-HPLC system has doubled the productivity of our Applied Biosystems sequencer. The cycle to cycle baseline stability of the HPLC trace provided by on-line analysis make this automation an invaluable tool for subnanomole sequencing.

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